Polarity and Patterns in the Postembryonic Development of Insects

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I. INTRODUCTION

Developmental biology is still a largely uncharted subject: analysis of developing embryos is difficult mostly because so much is happening at any one moment that it becomes impossible to observe

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individual components of the morphogenetic machinery. In all organisms development continues beyond the embryonic stage, where it often takes a simpler form. In insects this postembryonic development usually occurs as growth and metamorphosis, particularly of the epidermis, and here it is a help that any changes in the epidermis are permanently registered in the cuticle. I hope to show in this chapter that basic embryological phenomena do recur at insect metamorphosis, and how work with insects has produced some central information. There are dangers in the assumption that embryonic and later development depend on similar mechanisms, but in spite of these dangers, knowledge in depth should be the first aim.

Developing cells are usually *polarized* in relation to the main axes of symmetry of the organism. During epigenesis, to the continued accompaniment of growth, the possible destinies of individual clones of cells become progressively defined. Once the developmental identity of a cell is established, it is said to be *determined* and the process which orders requisite determinations in space is called *pattern formation.* Often the mechanisms of pattern formation are plastic so that they adapt to interference or loss of parts so as to reconstruct the whole pattern from the available material. This process is termed *regulation.* After a cell reaches its final determined status it constructs specialized organelles and synthesizes specific biochemical products–it *differentiates.* Although these five features of developing systems: polarity, determination, pattern formation, regulation and differentiation, are each components of one process, they can, to a certain extent, be treated independently. In this essay they will be discussed in relation to development of postembryonic insects. I have concentrated only on telling examples and have made no attempt to include all references in any of the areas considered.

II. CELL POLARITY

Individual cells are asymmetric in structure and in function. Insect epidermal cells sit on a basement membrane and secrete a cuticle apically. However, there is another, more subtle orientation: the first layer of the exocuticle contains chitin fibrils that are laid down in a particular direction–usually the antero-posterior axis of the body, or the disto-proximal axis of the appendage (Neville, 1967; Neville and Luke, 1969). This might indicate some extracellular stress which aligns the fibrils, but as we shall see, experiments point to the conclusion that the orientation of these fibrils is a reflection

of an intrinsic polarity in the underlying cells. This polarity in the epidermis also has direct expression in the orientation of asymmetric structures, such as scales or bristles, which commonly grow out antero-posteriorly.

While the basic cue to asymmetry may be in the egg itself, the complex polarities of the highly folded and structured epithelia which make up the mature insect originate as part of the epigenetic process. It is often not possible to analyse these events in the egg, but it is highly probable that the mechanisms which act during the genesis of cell-polarity there, also act to maintain it during growth and regeneration.

A. INSECT SEGMENTAL GRADIENTS

After wounding insect epidermal cells migrate individually from the surrounding area into the damaged parts (Wigglesworth, 1937). It seems unlikely that they retain their polarity during this amoeboid movement, and indeed when bristles are regenerated by the

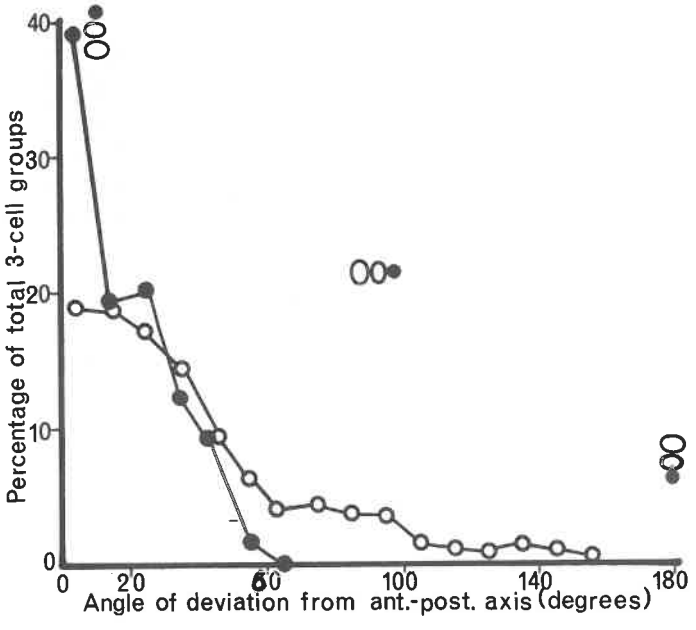

Fig. 1. Two stages in the acquisition of a preferred orientation in the differentiating hair cells in *Oncopeltus*. The alignment of the three-cell groups progresses from the early (open circles) to the late (closed circles) stages. (From Lawrence, 1966a.)

reconstituted epidermis they are often poorly orientated (Wigglesworth, 1940a, 1959). At subsequent moults the bristles are reorientated to their normal alignment, and after large wounds this proper orientation seems to spread in centripetally from moult to moult (Lawrence, 1966a). It would seem that cellular polarity is retrieved from the undisturbed peripheral cells. During the development of scales, hairs and bristles in a moth (Piepho and Marcus, 1957), a bug (Lawrence, 1966a) and in *Drosophila* (Stern and Hannah, 1950; Tokunaga and Stern, 1969) the formative cells acquire an orientation from the cells around them (Fig. 1); an observation which implies that polarity can be communicated from cell to cell.

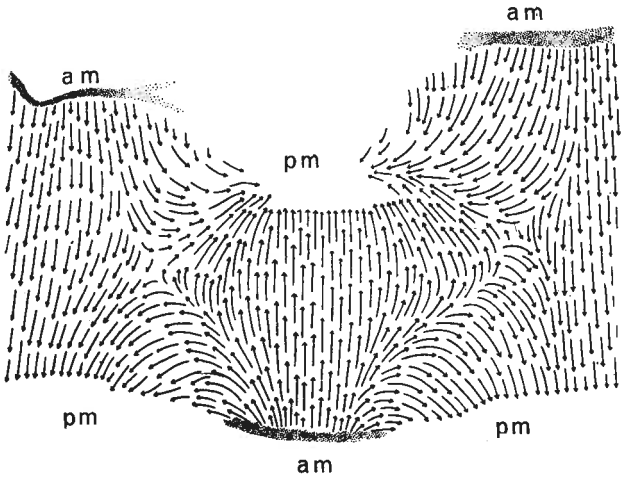

Fig. 2. An abdominal segment of adult *Galleria* after reversal of a square of integument in the last stage larva. Arrows mark the orientation of scales. Note the central area of reversed polarity. am = anterior margin, pm = posterior margin of intersegmented membrane. (From Piepho, 1955a.)

What, therefore, would happen to a cell placed between two masses of cells of mutually opposing polarity? Piepho (1955a, b) investigated this question by rotating a square of *Galleria* larval integument and grafting the piece back (Fig. 2). The scales of the adult were orientated by polarizing influences emanating from both the rotated piece and the area around it; Piepho described these influences in terms of forces, and noted how the scales oriented in the direction taken by the resultant of these opposing forces. Locke (1959, 1967) also found that rotation of pieces of integument in

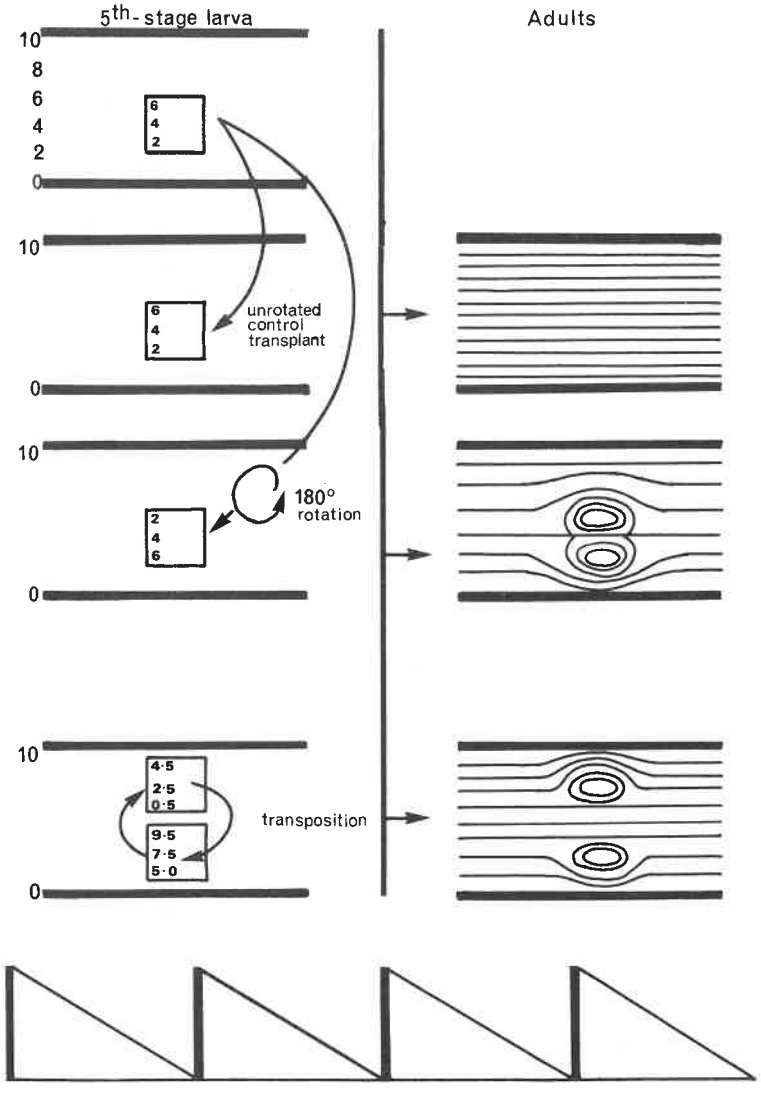

Fig. 3. Locke's experiments on *Rhodnius*. The figures mark the height of the segmental gradient in the host segment and in the transplanted pieces. The adult ripple patterns are shown, in stylized form, on the right. At the bottom of the diagram the serially repeated segmental gradient is depicted; the thick lines represent intersegmental membranes. (After Locke, 1959.)

Rhodnius disturbed the orientation of epidermal structures (in this case folds or "ripples" in the adult cuticle), whereas removal and replacement of squares of unrotated integument were without effect (Fig. 3). Transplantation up and down the segment in the proper orientation also produced alteration of the adult ripple pattern and the degree of pattern disturbance was related to the amount of displacement of the transplant. Since the polarity of the cells in the host and transplant was not altered by this experiment, it was clear that there was a gradient of some property within the segment, and it was interaction between cells of different gradient level which caused the disturbance. As transplantations between equivalent levels of adjacent segments had no effect on the ripple pattern, this gradient must be serially repeated in each segment. Lateral transplantations at any particular level had no effect on the adult pattern; this suggested that the gradient was the same along the segment.

The segmental repetition of the gradient implies that the intersegmental membrane must intervene between the high point of one gradient and the low in the adjacent (Fig. 3), and indeed

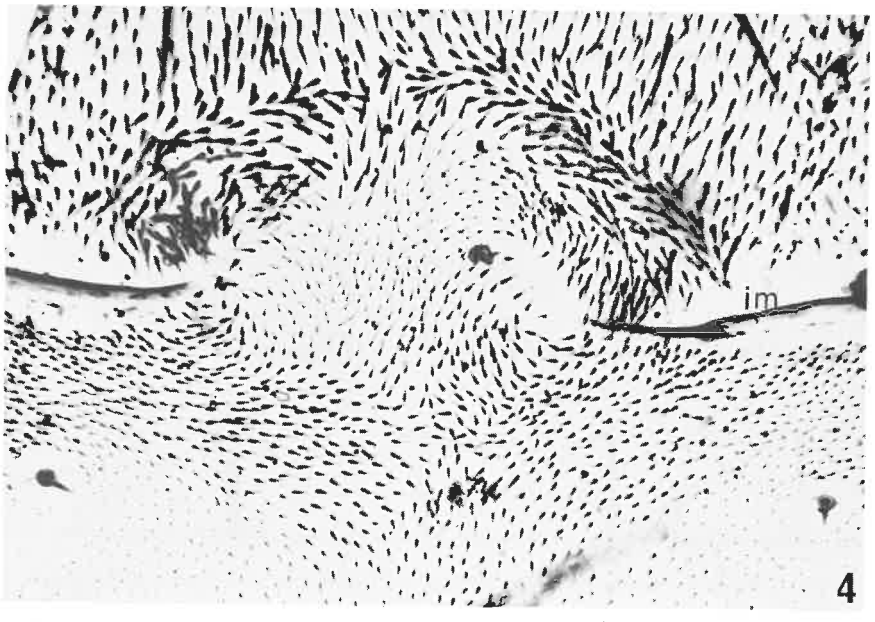

Fig. 4. Hairs near a discontinuity in the intersegmental membrane (im) of an adult *Oncopeltus*. Note the reorientation of the hairs in the centre of the picture. (From Lawrence, 1966a.)

Rhodnius which have an interrupted intersegmental membrane also show a considerably altered orientation of cuticular structures (Locke, 1960). In *Oncopeltus* the hairs are orientated normally in the antero-posterior axis, but in insects bearing a natural discontinuity of the intersegmental membrane there is a complex but precise pattern of hairs, which are oriented in relation to it (Fig. 4). Insects such as these suggested a model of the insect segmental gradient in which I proposed (Lawrence, 1966a) that the gradient shared properties with a sand gradient, in that it has a maximum stable slope resulting from an equilibrium between two forces (gravity and friction between the sand grains). When a steeper slope is created experimentally, flow of sand is initiated and continues until the maximum stable slope is reconstituted. In this view the gap in the intersegmental membrane would result in an unstable situation, the sand then flowing from the high to the low to produce a new stable gradient topography (Figs 5 and 6). This model

Fig. 5. The sand model–1. Glass plates separate two sand gradients. (From Lawrence, 1966a.)

Fig. 6. The sand model–2. The glass plates have been opened and the sand has flowed to set up a new, stable landscape. Note that the orientation of the hairs in Fig. 4 and the direction of the sand gradients are identical. (From Lawrence, 1966a.)

provided a common explanation to Piepho's and Locke's results: their transplantation experiments set up unstable gradient situations ("sand precipices") which resulted in flow of the gradient itself until new gradients were set up at the maximal steepness that was stable. In *Galleria* the scales point down (say) the gradients in the new landscape, and in *Rhodnius* the ripples run along the contours.

A similar model of a concentration gradient of diffusible substance was proposed independently by Stumpf (1965a, b) and Lawrence (1966a). In this model, like the sand model, experiment produces an unstable disposition of the gradient itself, subsequent flow and the formation of a new landscape.

It is not known how the gradient originates, or how it is maintained, but several models have been suggested. The intersegmental membrane of *Galleria* has two margins, one forming the anterior and one the posterior boundary of adjacent segments (Fig. 17). Marcus (1962) implanted small pieces of segment margin into different regions of the segment surface in *Galleria*; and these preparations were studied by Stumpf (1967a). At a distance from the implant the orientation of individual scales was the resultant of two influences: one emanating from the extant segment margins, and the other from the implanted piece. It was possible to deduce the relative contributions of these two influences from the alignment of the scales in relation to the distance from the transplant. There were many sources of experimental error, but Stumpf concluded that the orienting force attributable to the transplanted piece of margin declined more or less linearly with increasing distance from the transplant; whereas that attributable to the undisturbed segment margins was approximately constant all over the segment. This implied a linear gradient, and Stumpf therefore proposed that the anterior and posterior margins of the intersegmental membrane maintain two different concentrations of a substance, which is diffusing constantly from one margin to another. If the passage of material from anterior to posterior (say) were rapid, local disturbances resulting from transplantation would soon be overcome. As this does not happen (disturbed ripple patterns can persist for three moults in *Rhodnius*—several weeks) a substance with a very low rate of diffusion was postulated. An objection to this model arises from an elegant experiment of Piepho's in which he generated a patch of segment surface totally surrounded by just one segment margin. This resulted in a field of centrally pointing scales (Fig. 7) (Piepho, 1955b; Lawrence, 1970) and showed that a gradient of

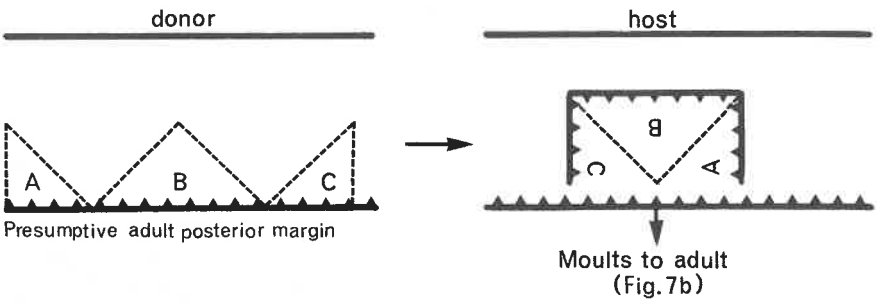

Fig. 7(a). Piepho's method of constructing a piece of *Galleria* segment surface totally surrounded by presumptive adult posterior segment margin (toothed line). The dotted lines mark the cuts made. The three pieces (A, B and C) were transferred to a host as shown. (After Piepho, 1955b.)

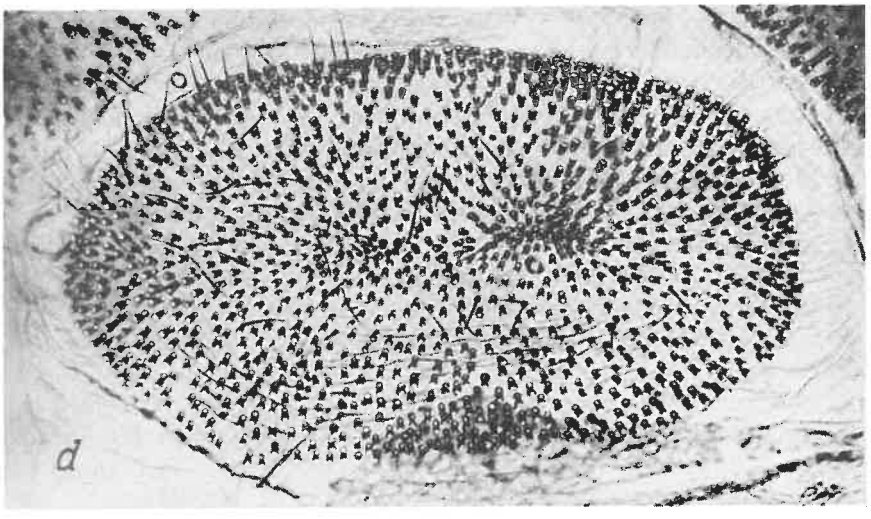

Fig. 7(b). The scale pattern after the operation. Note the even surround of intersegmental membrane, and the centripetal alignment of the scales. (From Piepho, 1955b.)

some sort does not depend on the presence of both margins. However, the additional postulate that the substance spontaneously breaks down can accommodate Piepho's result to Stumpf's model.

I propose that the gradient is generated and maintained by the cells themselves, by active transport of a substance against the gradient. Such pumping by the cells sets up a stable situation, in which the forces of pumping and diffusion are in equilibrium. If the direction of the gradient slope is altered, the cells respond

antagonistically and transport the substance against the new concentration gradient and thereby maintain it in its new orientation. This model suggests that the intersegmental membranes might act simply as passive impermeable barriers. It seems that a combination of both models fits the facts best: on the one hand, transplantation of pieces of anterior or posterior intersegmental margins into the segment surface produces profound alterations in the orientation of scales or ripples which suggests positive participation by two margins of opposite effect (Piepho, 1955b); on the other hand, deformation in the gradient landscape resulting from transplantation within the segment does point to the participation of the epidermal cells themselves.

B. ORIGIN OF THE SEGMENTAL GRADIENT

Natural malformations cast some light on the origin of the segmental gradient system. The abdominal sternites of *Oncopeltus* can conveniently be described in terms of two lateral halves, for although typically these two halves fuse medially to give one entire sternite, occasionally one half fails to appear, possibly because it merges with an adjacent segment half (Fig. 8). Any defect in a sternite is not carried over to the equivalent tergite, and their development can therefore be regarded as independent. Similar malformations occur occasionally in many insects and frequently in stocks of *Drosophila* carrying certain mutations, (e.g. *abnormal abdomen,* Zimmermann, 1954) or can be elicited by heat shocks (Löbbecke, 1958). A detailed study of a strain of *Drosophila* showing a high frequency of segmental defects was undertaken by Sobels (1952) who found, in agreement with Maas (1948) that the adult segment pattern descended directly from the larva with very few alterations. The most interesting type of defect, which was found only rarely, is the genesis of a segment in part bounded by two "anterior" or "posterior" margins. In the latter case the bristles orient towards both margins, with a divide in the middle. A similar defect in *Oncopeltus* is shown in Fig. 9. These malformed segments point to the intersegmental membrane as being vital to the genesis and organization of polarity of the segment surface; they suggest that the proper relations of the two segmental margins are an essential prerequisite for the normal development of each sternite and tergite. Indeed, from study of many kinds of segmental defects, Sobels (1952) concluded "both the anterior and the posterior margin of a segmental border seem to be involved in the determination of the

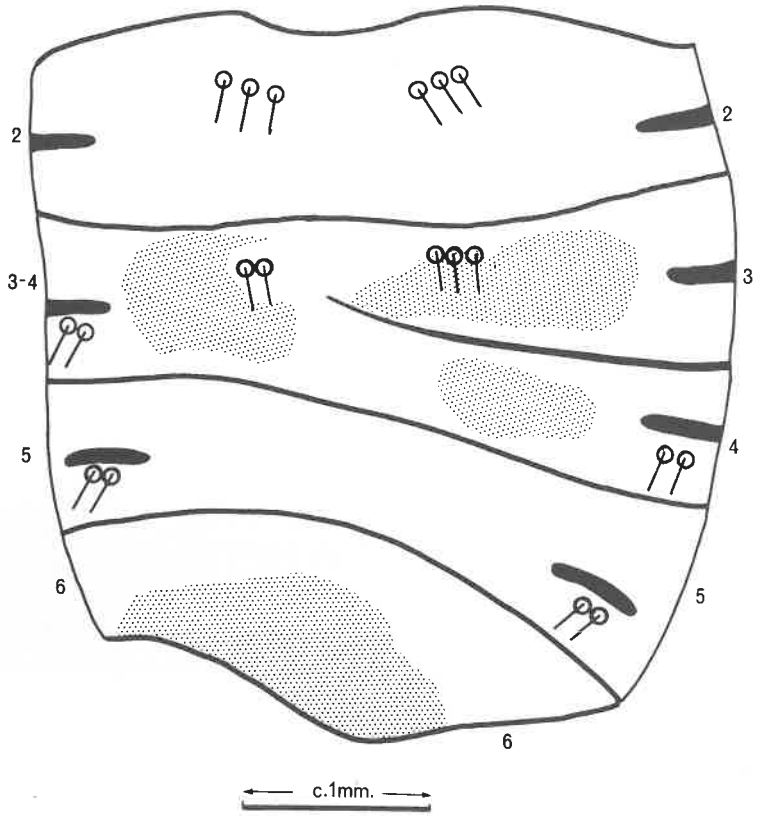

Fig. 8. Diagrammatic sketch of a segmental defect in the sternites of an individual *Oncopeltus* adult. On the right side of the diagram the normal sequence of segments is present. Note that segments 2 and 3 bear paired groups of large bristles centrally, whereas segments 4 and 5 each have two large bristles by the dorso-ventral muscle insertions (black bars). On the left side of the Figure the intersegmental membrane which separates segments 3 and 4 is missing and the combined segment, which is only as wide as one normal sternite, bears large bristles appropriate to both segments 3 and 4. The shading marks pigmentation.

outgrowth of the tergite anlage. The anterior margin mainly governs the differentiation of the anterior part of the tergite, whereas the posterior margin seems to be responsible for the differentiation of the caudal pigment band."

C. POLARIZED TRANSMISSION OF INFORMATION DURING GROWTH AND REGENERATION

The balanced growth of a complex organ is easily taken for granted and yet to achieve it, information exchange must take place

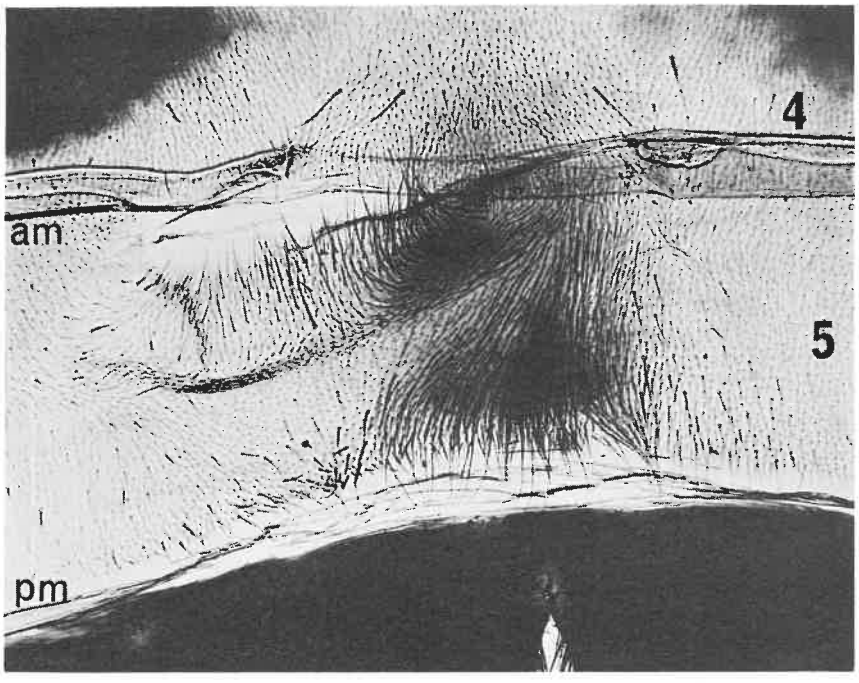

Fig. 9. The fifth (*5*) sternite of an *Oncopeltus* adult. Over a restricted region the orientation of hairs and bristles is outwards from the centre. Note that pigmentation and the length of the bristles suggest that in this region the segment is bounded by two "posterior" margins. am = anterior margin; pm = posterior margin of normal region of segment.

between the various parts. One such organ has been analysed by Locke (1958). In the ramifications of the tracheal system of *Rhodnius* the minimal total area of air tubes at any particular level limits the air-flow in the whole system, and in fact measurements of tracheae showed that the total cross-sectional area of the branches remained constant right through the system, whether measured at the base or at the finely branched tips. When the tracheae branch distally during growth the proximal supply tracheae show a corresponding increase in diameter. If connections between these supply tracheae and the distal branches were cut, they survived normally and the distal system continued to branch. New cuticle was successfully formed by all parts of the system but the supply tracheae did not expand in diameter. This experiment suggested that growth stimuli normally pass down the tracheal epithelium from the tissues to the basal trunks. By means of similar experiments Locke

discovered there were two sources of growth stimuli: the distal branches in the tissues and the nodes (sites of cuticular breaks at ecdysis). Growth information could pass only in *one* direction along the tracheae. Here there was no evidence for a gradient as such, only for polarity. Locke (1964) did however suggest that the nodes might

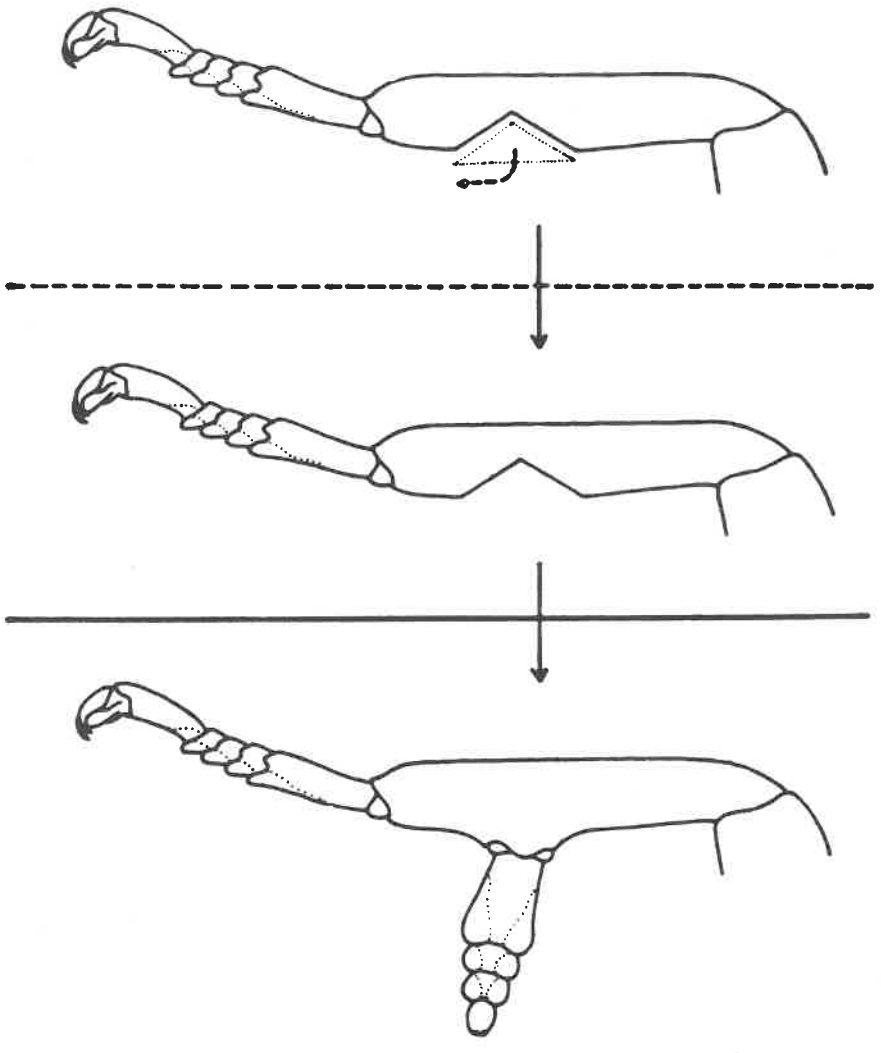

Fig. 10. The effect of cutting a V-shaped piece from the tibia of *Leucophaea*. The next instar bears a lateral regenerate. (From Bohn, 1965b.)

be equivalent to the intersegmental membranes in the part they play in the polarized transmission of growth stimuli (p. 216).

When an insect limb is cut off, regeneration of a new limb from a wound blastema of cells may ensue. A simple cut in the limb, although like amputation in inducing cell multiplication and cellular migration, does not lead to regeneration. It would seem to be the presence of the distal limb itself which effectively inhibits expression of the latent powers of the wounded limb cells to regenerate. If the cut is made in a *Leucophaea* limb as a deep V, the proximal portion of cut epithelium apparently receives no information from the extant limb for it makes a regenerate (Fig. 10) (Bohn, 1965b). This experiment illustrates the inability of these messages to by-pass gaps by making a lateral detour, and suggests, as with the tracheal experiments, that information is transmitted only in a particular direction. Confirmation comes from another experiment by Bohn: amputation, followed by grafting of the distal portion back on to the stump, effectively stops regeneration by the stump blastema (Bohn, 1965a). Thus the epithelium has grown together. If instead of the appropriate limb, a leg from the other side of the animal is grafted on to the stump (Fig. 11) information does not effectively pass to the

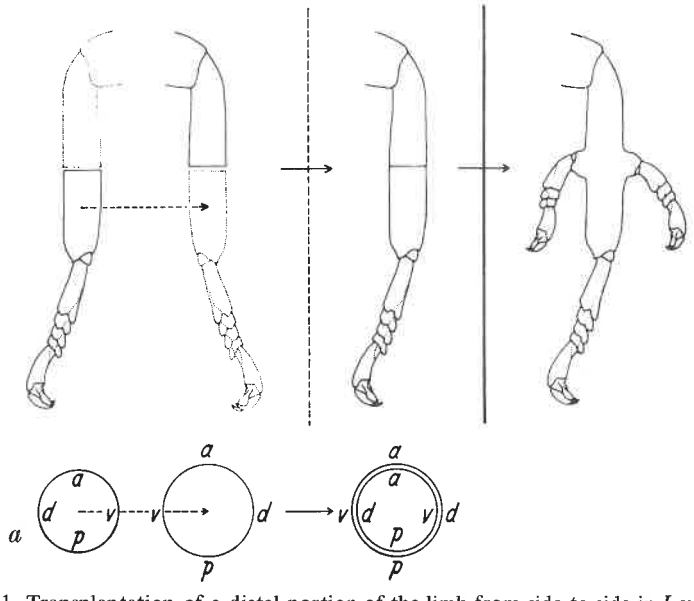

Fig. 11. Transplantation of a distal portion of the limb from side to side in *Leucophaea*. After the operation the antero-posterior axes (*a*, *p*) are compatible in host and transplant, whereas the dorso-ventral axes (*d*, *v*) are not. (From Bohn, 1965b.)

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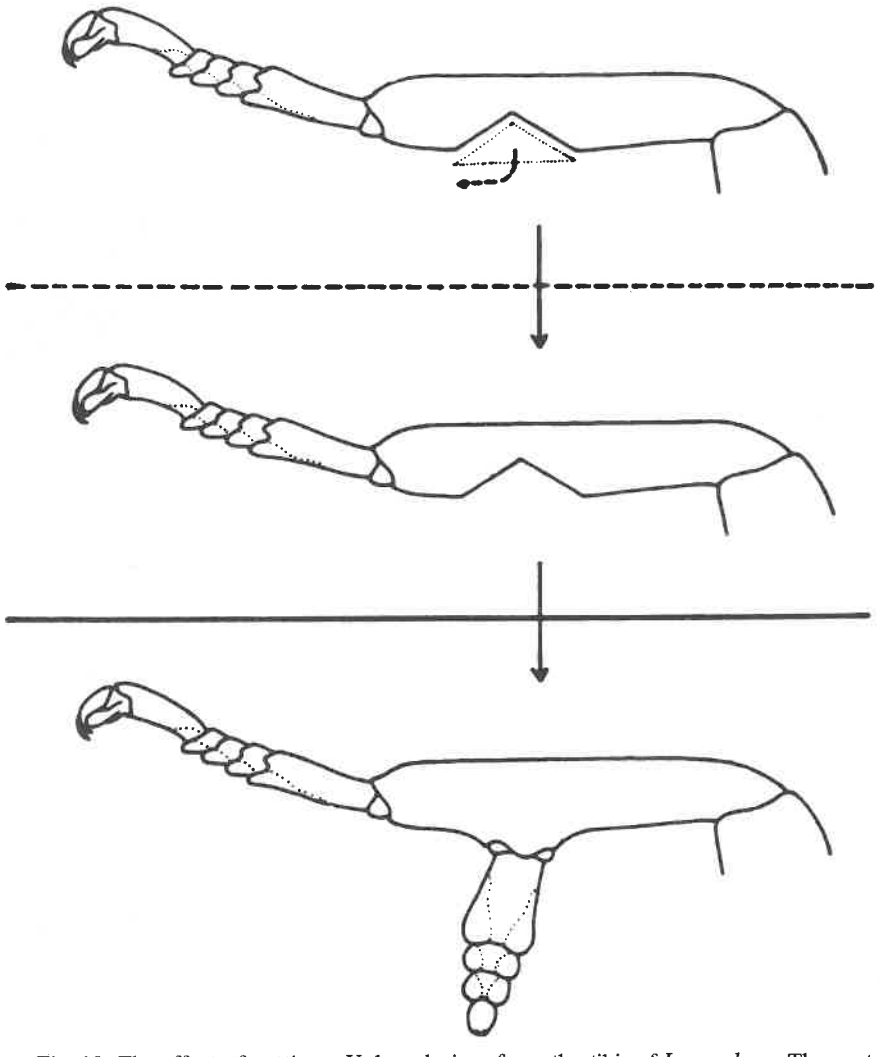

Fig. 10. The effect of cutting a V-shaped piece from the tibia of *Leucophaea*. The next instar bears a lateral regenerate. (From Bohn, 1965b.)

stump, and at the two sites of axial incompatibility two supernumerary limbs regenerate from the proximal wound tissue (Bodenstein, 1937; Bart, 1965a, b; Bohn, 1965b). Transplantation from left to right with rotation through 180° (Fig. 12) results in axial incompatability in the other axis of the limb and here also two supernumerary limbs grow out (Bohn, 1965b). One may conclude

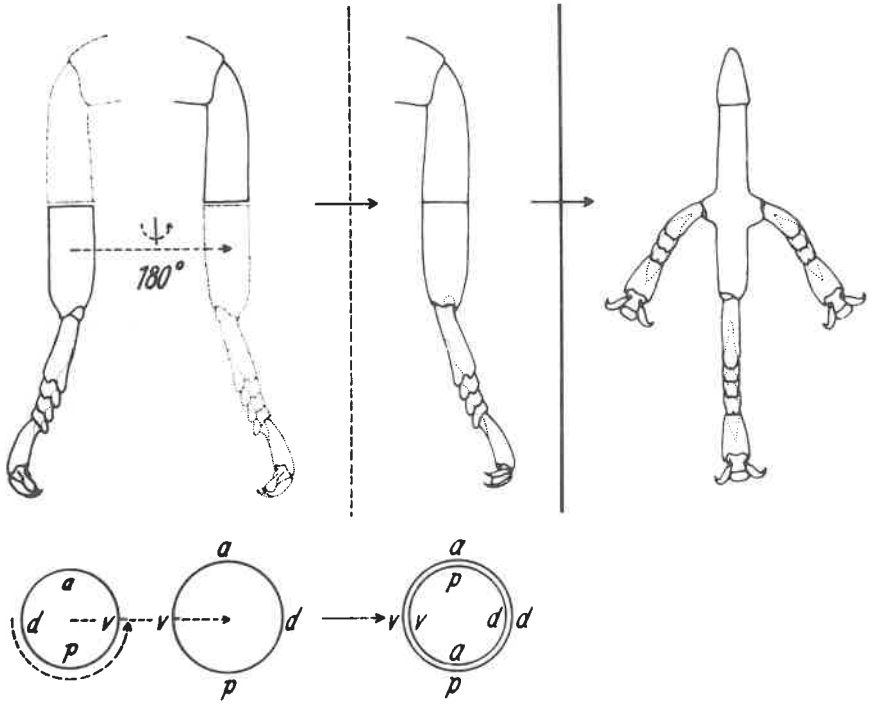

Fig. 12. Transplantation of a distal portion of the limb from side to side, with 180° rotation in *Leucophaea*. This experiment creates incompatibility in the antero-posterior axis, but not in the dorso-ventral. Note that the regenerated limbs are in a different plane than in Fig. 11. (From Bohn, 1965b.)

that information which inhibits regeneration can only move between cells whose symmetry is equivalent.

This conclusion is complicated by some other experiments by Bohn in which he rotated the amputated portion of the limb through 90° or 180° and then grafted it back. This altered the relative situation of both axes and one might predict the regeneration of four extra limbs. However, the topological quandary was resolved by the gradual rotation of the distal portion of the limb until the proper

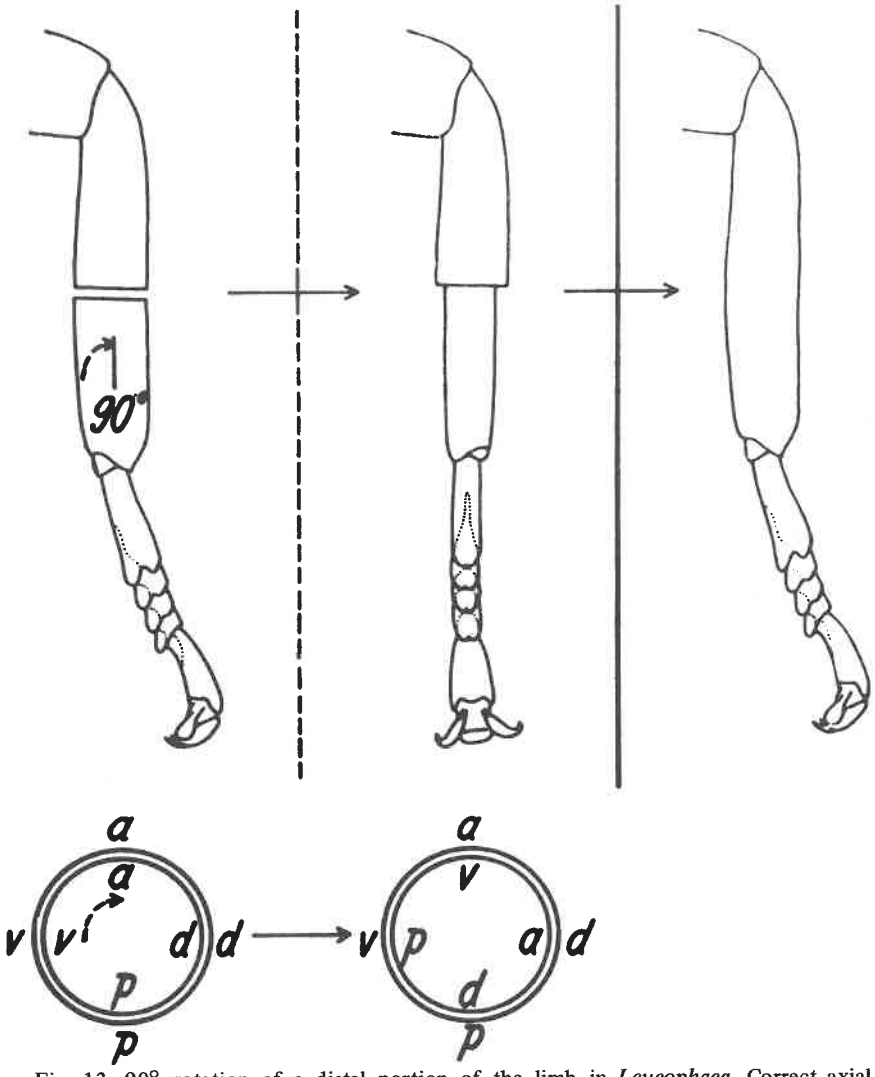

Fig. 13. 90° rotation of a distal portion of the limb in *Leucophaea*. Correct axial alignment is regained by compensatory rotation of the transplanted portion. No regeneration occurs. (From Bohn, 1965b.)

relationship between stump and transplant was re-established (Fig. 13). There is no explanation for this simple solution by the insect.

D. GROWTH AND GRADIENTS

There is other evidence that segmental gradients are connected with the organization of growth; it would seem that the tissue has an

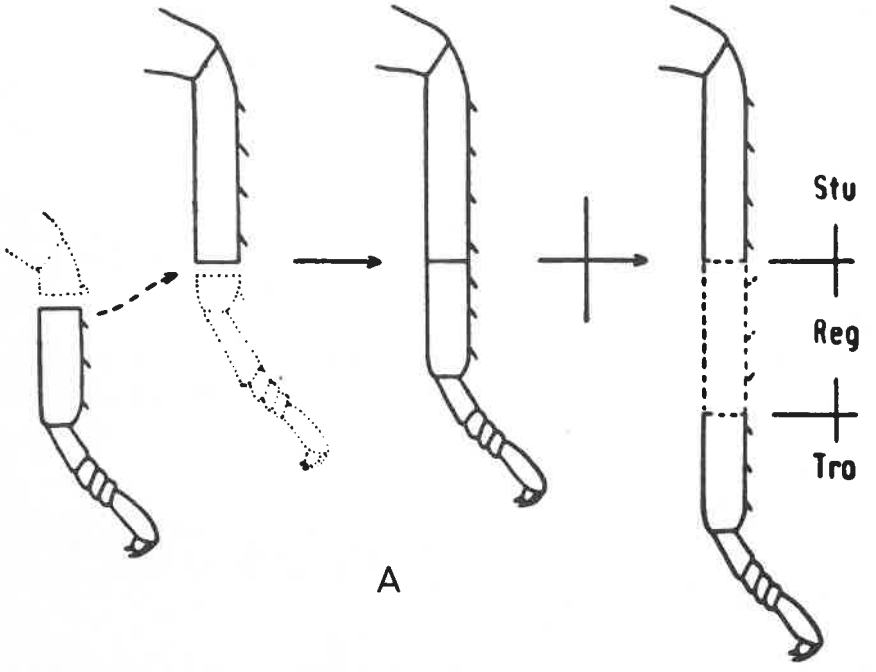

Fig. 14. Transplantation between two tibia in *Leucophaea.* (a). Note after transplantation there is a gradient discrepancy at the junction. Intercalary regeneration occurs, and the spines on the regenerated portion *Reg,* unlike those on the rest of the limb, grow out disto-proximally. The final tibia is twice the length of a normal tibia.

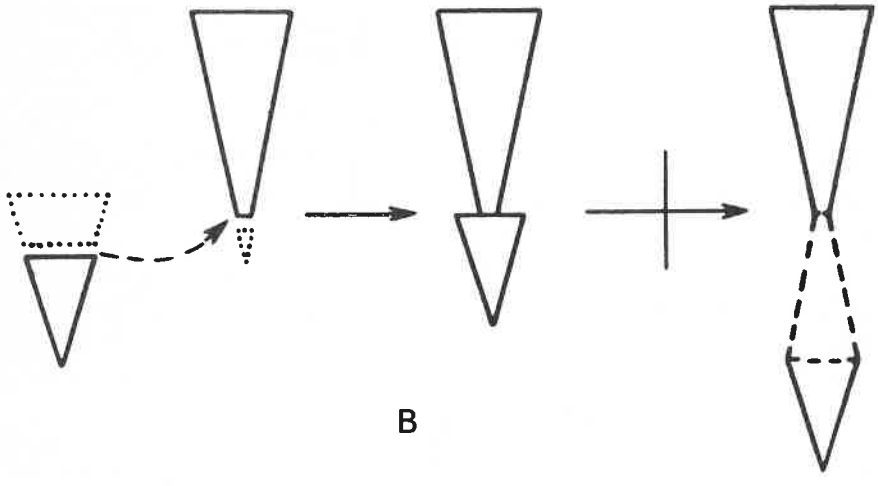

Fig. 14(b). Diagram of the gradient situation. (From Bohn, 1967.)

inherent steepness of gradient and growth continues until that steepness is reached—the gradient being determinant therefore of the size of the structure. For the clearest example of this we must turn again to the insect leg. In experiments homologous to his studies on the abdomen, Locke (1966) showed that the insect leg segments bear serially repeating gradients which are concerned in the orientation of bristles. Bohn (1967) found that individual segments of *Leucophaea* could regenerate excised tissue. Regeneration proceeded only until the lost tissue had been reconstituted, and he showed, moreover, that it was not the length of the tibia *per se* that the system was reconstructing but the steepness of the gradient (Fig. 14). By transplantation between the short mid tibia and the long hind tibia Bohn also demonstrated that the differences between the two

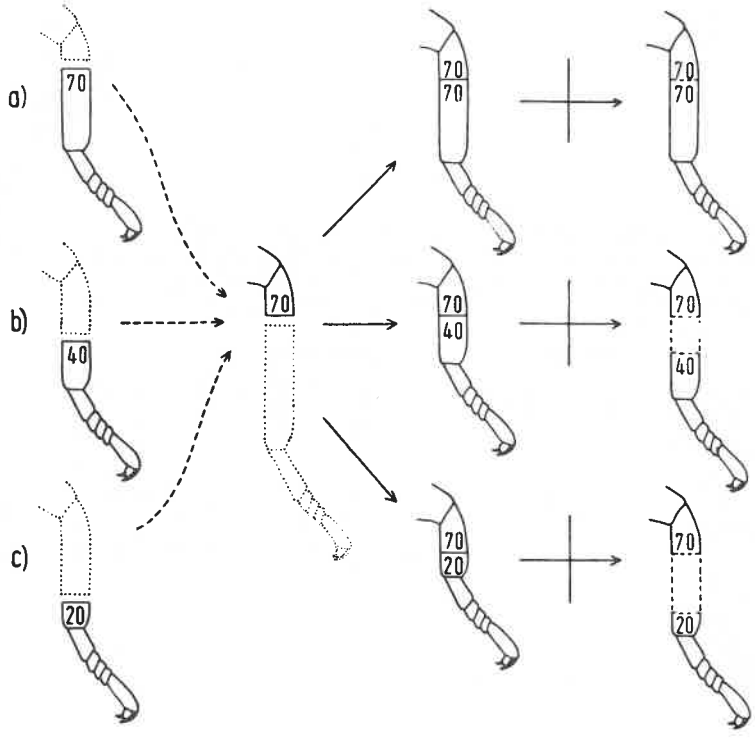

Fig. 15. Transplantation between the short mid-tibia and the longer hind tibia. The numbers represent the percentage of the total length of the tibiae. These experiments show that regeneration only occurs when there is a gradient discrepancy, and that the gradients are of different steepness, but not extent, in the tibiae of the two legs. (From Bohn, 1967.)

tibia reside not in the total extent of the gradient, but also in the declivity (Fig. 15). The gradients are homologous in the two limb segments for they will fuse into one, but nevertheless are of different steepness. Needless to say these observations raise further problems: How is the steepness of the gradient system varied in the different limbs? How is growth controlled quantitatively from moult to moult? This latter problem is highlighted by a segmental defect found occasionally in cultures of *Oncopeltus* (Lawrence, 1966a) (Fig. 16).

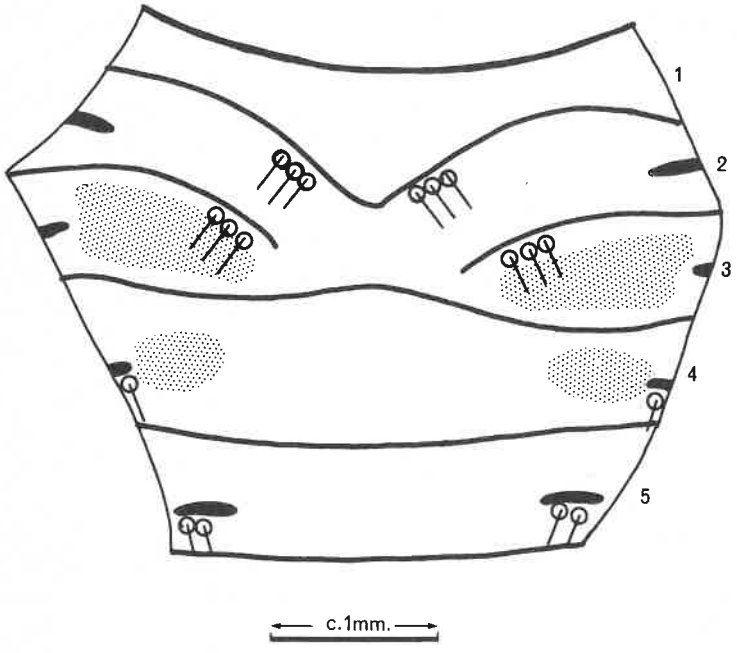

Fig. 16. Sketch of *Oncopeltus* sternum, showing central fusion of sternites 2 and 3. Note that sternites in the fused area are only together as wide as one normal segment.

In such insects the sternites 2 and 3 are fused medially, and together are only about the width of one normal sternite. Consequently the ventral abdominal cuticle is considerably shorter than the dorsal, and the whole abdomen is uncomfortably hunched. Earlier studies (Wigglesworth, 1942, 1964) had demonstrated that growth of the epidermis is a homeostatic response to cuticular expansion due to feeding. In the defective insects the lateral parts of sternites 2 and 3 and the tergites are the proper width, and witness that normal distension has occurred during feeding. If growth were

completely dependent on stretching one would have expected the medial parts of segments 2 and 3 to grow as wide as two segments. They do not do so, showing that in addition to the effects of distension on epidermal cell division, there is also some kind of growth control stemming from the segment margins. Locke (1959) reported that integumental grafts only grew properly when they were oriented correctly, in relation to the intersegmental membranes. I found (Lawrence, 1965) that such grafts did grow regardless of their orientation, providing that the graft included a piece of intersegmental membrane, which again points to the segment margins as sources of growth control—as suggested by Locke (p. 209).

E. GRADIENTS AND PATTERNS

The insect segment is an excellent model system of pattern formation. When, in the egg, a presumptive segment is generated, it may consist of fewer cells than the number of qualitatively different regions that will be present in the mature segment. Thus during growth, there is diversification into different regions. Formally this could either occur by means of unequal cell divisions, by which the different parts of a single cell's cytoplasm are partitioned amongst the daughters (mosaic development) or alternatively by some supracellular determinative process which occurs much later (regulative development). An experimental investigation of the developing segment can allow a choice between these two hypotheses.

The adult segment of *Galleria* bears strips of qualitatively different integument, but the larval cuticle is more or less homogeneous. Marcus (1962, 1963) showed that if small pieces of larval segment from different parts are cultured through metamorphosis in the abdominal cavity of a mature caterpillar, they develop into cuticle according to their prospective fate. This experiment indicates that the different regions of the segment are already determined prior to metamorphosis. However, transplantation of small pieces of cuticle in the larva, from presumptive region 0 into presumptive region 2 (Fig. 17) resulted in the reorientation of scales in accordance with the segmental gradient system, and moreover the two tissues interacted to produce a region of integument type 1 between them (Fig. 18). I have argued that region 0 was the posterior margin of the intersegmental membrane (Lawrence, 1970), which maintained the lower gradient position; this resulted in flow and the lowering of the surrounding level 2 cells to level 1. Marcus (1962) showed that none

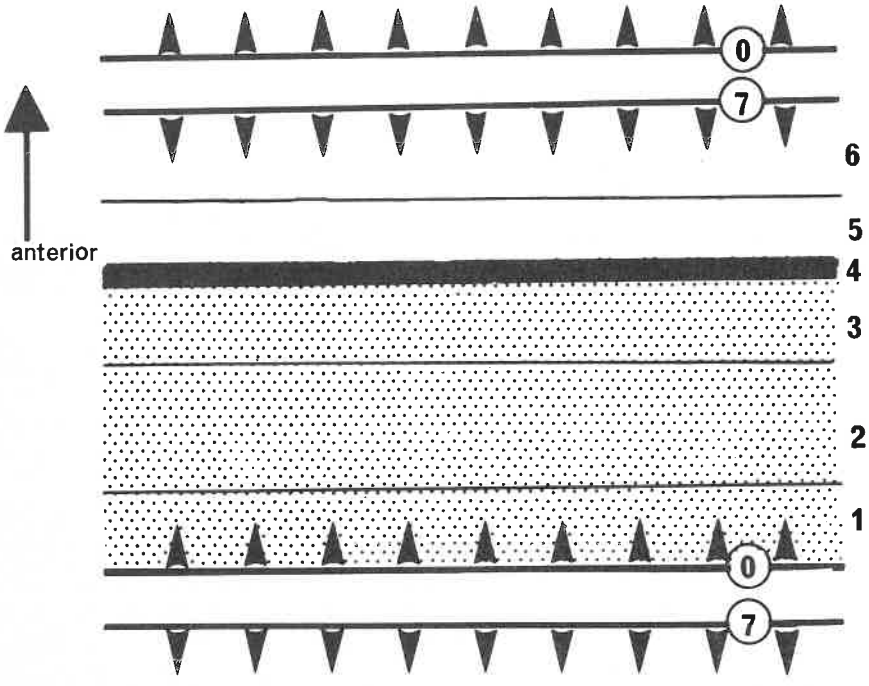

Fig. 17. Diagram of the adult *Galleria* abdominal segment. The thick bar is a ridge of hard cuticle (region 4). The dotted areas (3, 2 and 1) represent regions bearing scales of three different sizes. The intersegmental membrane has a posterior margin (0) and an anterior margin (7). The arrow points anteriorally. (After Marcus, 1962; interpretation from Lawrence, 1970.)

of the type 1 cells originate from cells of the transplant (Fig. 18); they were all presumptively type 2 cells from the segmental surface and effectively therefore the posterior margin imposed its gradient position on the surrounding cells. This result confirms that the intersegmental membranes do have a special part to play in the maintenance of the gradient, for if their gradient position were as labile as the cells of the segment surface after transplantation, some of their cells would also have made type 1 structures. The actual formation of the adult integument was elicited by general metamorphosis of the host, but the nature of the product depended on interaction between the cells within the segment. Thus the determination of cells in the larval segment was only "provisional". These elegant experiments (Marcus, 1962, 1963) showed that orientation of scales and the sequence of cuticular types in the segment had a common gradient basis, the level of the segmental

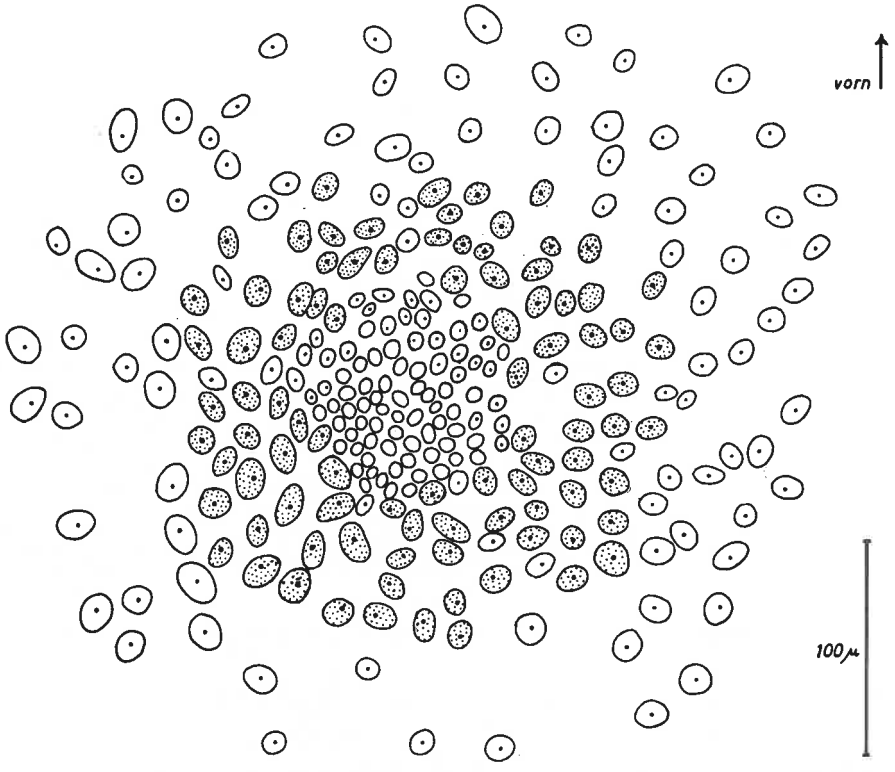

Fig. 18. Transplantation of presumptive region 0 into presumptive region 2 in *Galleria* (see Fig. 17). The large peripheral nuclei mark scales of type 2, the shaded nuclei mark scales of type 1. The host nuclei (presumptively type 2) are each marked with a central dot, the donor nuclei are undotted. Note that some host nuclei have become cells of type 1, and others have become nuclei of type 0 (small nuclei in centre). (From Marcus, 1962.)

gradient being determinant of the type of integument formed. The proper development of isolated pieces of segment suggest that the gradient level can be autonomously maintained for some time, again pointing to active participation by the epidermal cells in the maintenance of the segmental gradient, rather than only the intersegmental membranes. Such experiments argue against mosaic development and demonstrate that the interaction of growing cells is responsible for the increasing complexity of the epidermis during growth. They further indicate two central points: one, that cellular polarity and pattern formation are causally interrelated and two, that pattern formation, at least in this example, can be analysed as two systems—a basic underlying gradient system and the response of the

cells to it. Both could vary independently. These two points will be illustrated further in the next example, and in the section on pattern formation.

In *Galleria* pupae and adults there is a ridge of cuticle (region 4 in Fig. 17) which is present on segment 6 but not on segment 4. In a series of transplantation experiments Stumpf (1966, 1967b) showed that ridge cuticle developed wherever the appropriate level was generated by the process of gradient flow. Rotation of a square of cuticle with a slightly eccentric presumptive ridge gave the predicted pattern of an isolated ellipse and deflected line of ridge cuticle (Fig. 19). Here many cells which were not normally destined to make

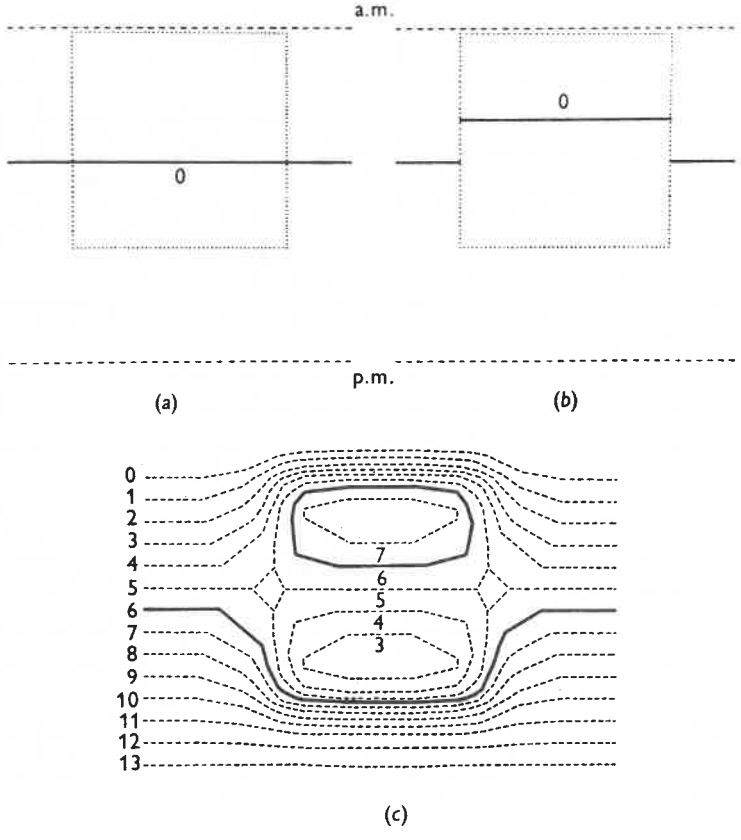

Fig. 19. (a) Rotation of a square piece of *Galleria* larval cuticle containing eccentrically placed presumptive ridge cuticle (thick line). (b) Situation immediately after rotation. (c) Predicted pattern after flow; the contour for level 6 is marked with a thick line. a.m. = anterior margin; p.m. = posterior margin. (From Stumpf, 1968.)

ridge cuticle now did so. Stumpf (1968) then turned her attention to the difference between segment 6 and segment 4. She transplanted pieces of tissue between these two segments and the shape of the ridge, which only developed from sixth segment cells, showed that the segmental gradients were equivalent and that they interacted as before. The difference between the segments was totally due to varied cellular responses to identical gradient systems.

F. INSECT SEGMENTAL GRADIENT: A SUMMARY OF THE WORKING HYPOTHESIS

It has been established that the scales and bristles of *Galleria* and *Oncopeltus* mark the direction of slope of a linear gradient, which is set up between the anterior and posterior margins of the two bordering intersegmental membranes and behaves as the sand gradient model. The direction of slope can be altered predictably by experiment. The maintenance of the gradient seems to depend on some active participation by the cells themselves between the limits set by the organizing margins of the intersegmental membrane. A chemical model which would fit the facts would consist of the following points:

(i) that the gradient is of a diffusible substance;
(ii) that one margin produce, and the other absorb this substance;
(iii) that the substance is actively transported by the cells against the concentration gradient in which they find themselves;
(iv) that the substance is labile or broken down by the epidermal cells.

Some kind of gradient would be set up by points (i) and (ii) alone or point (iii) alone. However, the experiments of Piepho (1955b) and Marcus (1962) which show that both membranes are opposite in effect and that they act on the adjacent epidermal cells, and those of Locke (p. 201) which demonstrate that the orientation of the cells themselves is not totally dependent on the intersegmental membranes, together demand the bipartite hypothesis.

Points (i), (ii) and (iii) still do not predict the result when a segment is bordered by two margins of identical effect, which occurs naturally (Fig. 8) and has been created experimentally (Fig. 7): a further premise (iv) is required.

This working hypothesis is little more than a description of the experimental results, and must be regarded as very provisional. Indeed, Goodwin and Cohen (1969) have shown one way in which a

gradient with the required properties could be generated without a unique substance.

G. GRADIENT PHENOMENA IN OTHER ORGANISMS: A COMPARISON

The insect offers a very clear advantage over other systems in the elucidation of the morphogenetic gradient: there are markers such as bristles, which point to the direction of the gradient in any small locality, and provide an accurate register of gradient changes after experiment. No other system possesses such local polarity indicators. Moreover, as we have seen, there is convincing evidence that at least in the insect segment, the gradient itself is the basis for qualitative pattern development, particular levels in the gradient being determinate of particular integumental types. While there is no compelling reason to believe that morphogenetic phenomena in different groups have a common basis, it is often instructive to see how far information on one group is in accord with evidence from other groups. In this case the experiments on the insect segment have produced an incomplete, but moderately demanding hypothesis; how far can this hypothesis be applied to these other groups where morphogenetic gradients have been mooted?

We shall restrict ourselves chiefly to studies of *Hydra* and *Tubularia* although most gradient phenomena have also been found in planarians (Wolff, 1962; Lender, 1965; Hay, 1966). These hydroids possess the ability to regenerate new distal and proximal ends, and to reorganize the tissues between, after section. There is a suggestion that all cells are normally restrained from developing into distal structures by inhibitory influences emanating from extant distal structures (Rand *et al.*, 1926; Webster, 1966). Webster and Wolpert (1966) have demonstrated that in addition to this inhibitory influence there is a gradient of hypostome forming propensity, and that the most anterior portion of the cut piece, now released from inhibition, will develop fastest to the state where it can induce a secondary axis when grafted to an intact hydra.

A similar gradient of head forming propensity has been postulated for planarians (Brøndsted, 1955).

Rose (1957a) has argued the case for specific inhibitors; he believes that each organ of the regenerating hydroid produces an inhibitor which prevents other parts from developing into that structure. Under any circumstances the cells of *Tubularia* will transform into the most "efficient" state not already occupied. This

hypothesis proposes that the sequence of structures along the disto-proximal axis is stored in the cells' developmental repertoire as a series of states of different "efficiency" and that each of these differentiated structures, when formed, will inhibit the development of like structures. After removal of the hypostome, the most distal remnant tissue will transform into new hypostome, and the specific sequence of structures will spread proximally from the new hypostome until it includes the whole reconstituted animal.

Rose (1963) has moreover gathered an impressive body of evidence which speaks for the existence of specific inhibitory substances. Pieces of *Tubularia* will normally regenerate new distal tentacles, but homogenates of distal structures incorporated into agar could be made to inhibit this regeneration. The agar was inserted into a piece of hydranth stem and the preparation subjected to an electrophoretic current. In this situation distal regeneration was always inhibited if the distal end of the test piece faced the cathode, but not if it faced the anode. If clean agar, or agar impregnated with a homogenate of proximal material, was inserted into the hydranth stem, the current was without effect on regeneration. Quantitatively the results from these experiments are impressive. Further experiments (Rose, 1963) suggested that it was possible to influence the movement of inhibitory substances *in situ*. These observations justify the notion that an electric field can interfere with the disto-proximal passage of something which blocks regeneration of distal structures behind pre-existing distal structures.

Specific inhibitory effects have also been demonstrated in nemerteans (Tucker, 1959), polychaetes (Smith, 1963) and planarians (Lender, 1956).

So far the insect segmental gradient and the morphogenetic gradient of hydroids seem to have little in common. One phenomenon, which brings the two systems together, is that of the direction of transmission of inhibitory information. It appears that inhibitory information from an extant or nascent hypostome can only pass disto-proximally. If two distal portions of *Tubularia* were grafted together in the same polarity one was overcome by the other and they merged (Rose, 1957b) (Fig. 20(b)). But when two distal portions were grafted together in opposite polarity they did not inhibit each other and maintained their independent status (Fig. 20(c)). Rose argued that the inhibitory information could not pass up the stem proximodistally, so that the two distal ends were effectively insulated from each other.

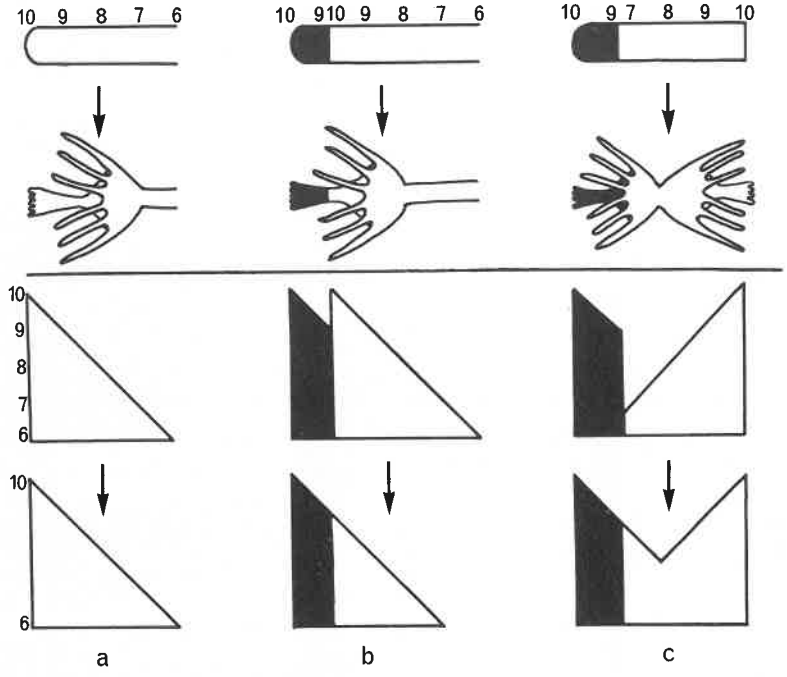

Fig. 20. Experiments on *Tubularia.* Above: appearance of hydroid. Below: gradient situation. Transplanted tissue is shown in black, host tissue in white. (a) Control. (b) Grafted distal portion on to complete presumptive hydranth in same orientation. (c) Distal portion grafted in opposite orientation to host, which leads to development of twin-headed hydranth. Note how part of the host becomes changed in polarity. This change can be explained in terms of the kind of flow which happens in the sand model. (After Rose, 1957b.)

The hydroid hypothesis now stands as follows:

(i) there is a gradient of propensity to transform into distal structures;

(ii) each organ makes a specific inhibitory product which inhibits the development of the same organ elsewhere;

(iii) there is polarized transmission of these inhibitory agents.

This hypothesis offers no explanation for polarity reversal. When two pieces of hydroid of different length are grafted together some of the stem of one becomes reversed in polarity (Rose, 1957a, b) (Fig. 20(c)). The Rose hypothesis does in no way predict this reversal but the sand-model type of gradient does (Fig. 20(c)). Is it possible that a gradient of the sand-model type is set up below the hypostome in hydroids? Are there any advantages to such a hypothesis?

The neogenesis of a hypostome after section is not predicted by

the sand-model hypothesis, but once one is formed, because of the plastic nature of the gradient, depending on active transport of a diffusible substance between the two new limits (basal disc and hypostome in hydra) a new gradient would be reconstituted. If two pieces of different gradient level are grafted together there should be "flow" from high to low, and over the area affected by flow a newly oriented gradient covering intermediate gradient levels would be established. After these gradient alterations the sequence of structures would develop because each cell responds appropriate to its level in the gradient. This hypothesis requires no specific inhibitory substances, but demands that the cells respond to the concentration of one gradient substance.

In this respect it is simpler than Rose's, but if one were to adopt the sand-model hypothesis for hydroids, and perhaps planarians, one needs to explain away the demonstrations of specific inhibitory substances (p. 222, Tardent, 1963). Of course, one can find difficulties: the substance ought to interfere with the growth of the producing organ itself, particularly as it can allegedly cause the regression of extant structures of the same type as the producing organ (Rose 1957b). One might argue that the substances discovered by Rose are internal growth regulators rather than morphogenetic messengers.

One must conclude, unsatisfactorily, that a choice between these two incomplete hypotheses is premature now and probably will be until the basic mechanisms of gradient phenomena are uncovered.

III. PATTERN FORMATION

Embryonic development depends on the spatial organization of cellular differentiation. One apparently simple, and two-dimensional, example of this phenomenon is the generation of spaced bristle patterns in insects: there have been two main approaches to analysis of this system. One is a cytological study of the development of bristles in two genera of bugs, and the other utilizes *Drosophila* which are mosaic for tissues of two genotypes that have different effects on bristle pattern. These are both reviewed below.

A. THE DEVELOPMENT OF SPACED BRISTLES AND HAIRS IN *RHODNIUS* AND *ONCOPELTUS*

The abdominal cuticles of larval *Rhodnius* and *Oncopeltus* are covered with evenly-spaced bristles. Bristles are tactile sensilla, and

their constituent cells develop from single epidermal cells which transform into bristle mother cells and then undergo special differentiative divisions (Wigglesworth, 1953; Lawrence, 1966b).

As a result of cell divisions during each moult cycle the number of epidermal cells intervening between extant bristles increases, and in the subsequent moult cycle some of these cells are transformed into bristle mother cells. In this way the bristle density is kept more or less constant from instar to instar. It has been experimentally demonstrated that the number of new bristles is related to the number of cells rather than to the distance intervening between extant bristles (Wigglesworth, 1940a; Lawrence, 1966b). Wigglesworth noted that new bristles were likely to appear in any large space which existed in the old bristle pattern, and that they never formed very close to existing bristles; it was clear that particularly situated cells were being selected as bristle mother cells. To explain his observations Wigglesworth (1940a) postulated that extant bristles effectively inhibited the appearance of new bristles nearby.

During the last moult cycle in *Oncopeltus,* but not in *Rhodnius,* new structures develop, so that in addition to the bristles the adult abdominal sternites are covered with dense *hairs.* These hairs also develop as a result of unusual divisions by transformed epidermal cells, but unlike the bristles, lack innervation (Lawrence, 1966b, 1968). They form a completely integrated pattern with the bristles (Fig. 21). As these hairs are added to the pattern the density rises from about 150/sq mm for the larval bristles alone to 2000/sq mm for the bristles and hairs; these hairs are so precisely spaced that the uniformity of distribution* of the pattern rises from $R = 1.44 \pm 0.03$ in fifth-stage larvae to $R = 1.70 \pm 0.02$ in adults.

Analysis and understanding of this process requires detailed knowledge of cellular events during metamorphosis. We shall consider some of the relevant information:

(i) Cellular metamorphosis: Juvenile hormone is present in the earlier instars and maintains the larval state; in its absence in the fifth-stage larva the moult cycle is prolonged and metamorphosis occurs. During metamorphosis the epidermal cells change in many ways and synthesize a qualitatively different cuticle (Lawrence, 1969). If juvenile hormone is injected at the beginning of the fifth

* The uniformity of distribution is defined as a scalar quantity, R, which is equal to the product of twice the square root of the density and the average distance from each unit in the pattern to its nearest neighbour (Clark and Evans, 1954; Claxton, 1964). For a random distribution $R = 1.00$, and for perfect hexagonal packing $R = 2.15$.

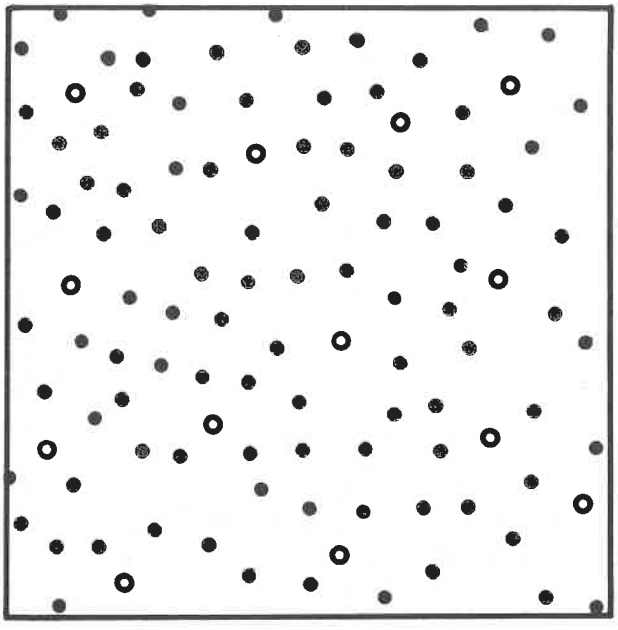

Fig. 21. Distribution of hairs (closed circles) and bristles (open circles) on the sternite of adult *Oncopeltus.* Note hairs and bristles are distributed in the same way. (x360.)

larval stage, metamorphosis is inhibited and no hairs develop (Lawrence, 1969). Two experimental results show that development of hairs does not result from the altered moult cycle but is a local phenomenon dependent on the state of the epidermal cells themselves; firstly if the hormone is applied topically to the insect, local patches of hairless larval cuticle are formed—even though the length of the moult cycle is not affected and the remainder of the insect is a perfect adult (Lawrence, 1969) and secondly, adult tissue transplanted back on to a larva will develop new hairs even during a fourth- to fifth-stage moult of the host, in the continuous presence of the juvenile hormone (Lawrence, 1966c).

(ii) The timing of determination: Uniformly sized fifth-stage larvae, which normally develop into adults of a certain size, will moult into very small adults if they are partially starved (Lawrence, 1966b). These small adults have approximately the normal epidermal cell and hair density and accordingly the number of hairs in the particular region studied may be as low as half the normal. As was earlier shown by Wigglesworth (1940a) for *Rhodnius,* the density of epidermal cells is homeostatically controlled and their number therefore related

to the amount the cuticle expands during each moult cycle. In the starved *Oncopeltus* the number of hairs has responded to the lower number of cells, and consequently hair determination must have occurred after at least the majority of cell divisions. We may note that in contrast bristle determination in larvae does not respond to starvation in any one moult cycle, and by the same argument must therefore have occurred *prior* to the majority of cell divisions in that moult (Lawrence, 1966b) (Fig. 22).

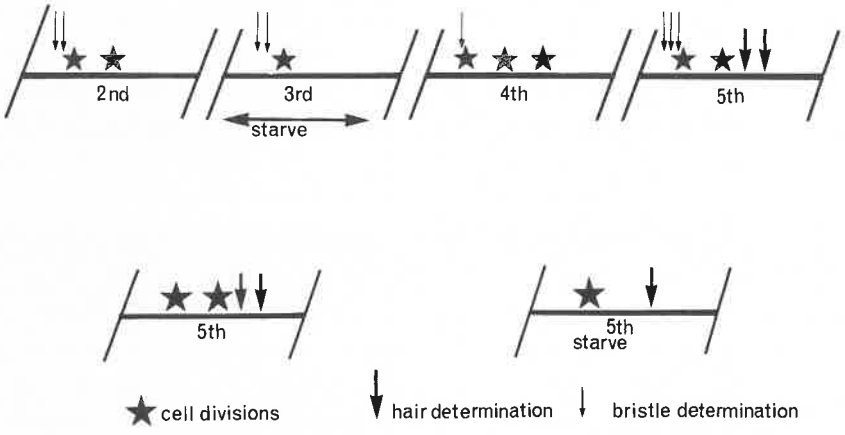

Fig. 22. The timing of bristle and hair determination. Starvation in the third larval stage reduces cell divisions. Bristle determination responds to this lower cell number in the fourth larval stage, so that the reduced bristle number can be seen on the fifth instar. By contrast, starvation in the fifth larval stage, because hair determination follows cell divisions, directly reduces hair determination in that moult.

(iii) The process of hair spacing: Cell divisions in the fourth-stage larva effectively separate the bristles and uncover competent areas, which are now outside the bristles' "inhibitory influence". These areas are then populated with some new bristles during the determinative phase which begins before the majority of cell divisions in the fifth-stage larva. Cell divisions in the fifth-stage larva will again separate old and nascent bristles, and uncover areas free from inhibition. These divisions are followed by hair determination. So many hairs are formed that in addition to those sponsored by divisions in the fifth-stage larva, there must be a considerable reduction in the extent of inhibition by the bristles so as to increase massively the total competent area. This drop in the diameter of the inhibitory circle which surrounds each bristle could either occur suddenly prior to the initiation of hairs, or could be continuous with

the process. If one knows the spatial order in which hairs are added one can distinguish between these possibilities; in the first instance large competent areas are defined and within them hairs with their inhibitory circles are introduced at random until the area is saturated, and in the second instance the initial hairs are confined to areas relatively distant from extant bristles, and as the inhibitory

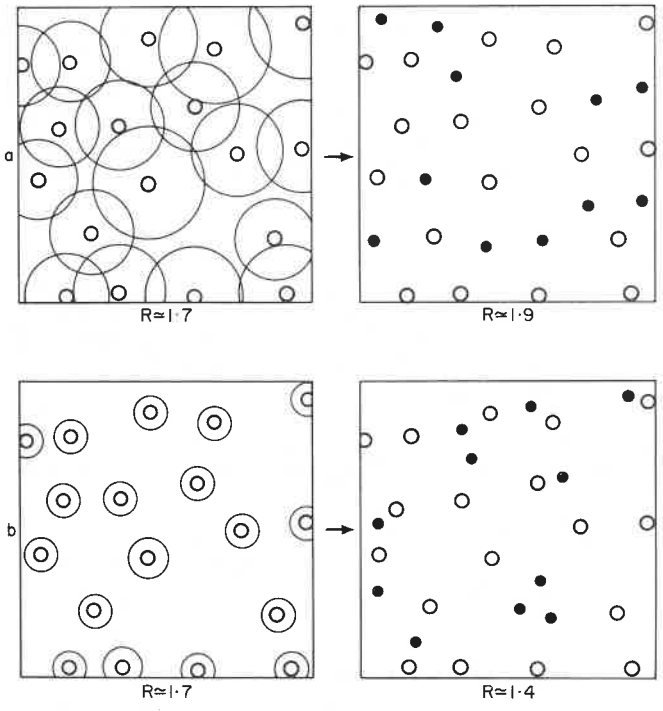

Fig. 23. Identical patterns of bristles with (a) large and (b) small inhibitory regions. The first new hairs in (a) improve the uniformity of distribution, and in (b) spoil it.

circles shrink, the hairs are sited closer and closer to each other and to the bristles. The uniformity of distribution will be very different during the early phases of pattern development in the two cases (Fig. 23).

With the aid of a computer we (Lawrence and Hayward, 1970) have been simulating hair pattern development of various models of this type. We have found that patterns which are almost identical to the natural hair pattern can be generated using the simple rules developed by Claxton (1964). The starting point is a typical larval bristle pattern, and each bristle is surrounded by an inhibitory field

within which new structures are forbidden. Points are generated at random, and added (with an appropriate inhibitory circle) if they fall within competent regions. During the process the computer periodically pauses and measures parameters of the pattern under construction. The process continues until no competent areas remain. We found that the adult pattern cannot be generated if at any one time the inhibitory circles are all exactly the same size (this gives a probability surface around the average hair or bristle shaped

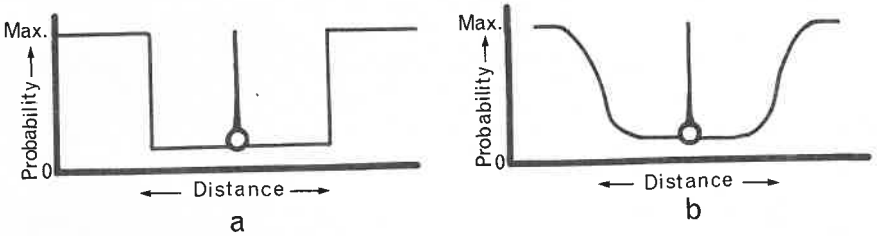

Fig. 24. The probability of a new hair developing in relation to distance from an extant bristle. (a) With all inhibitory circles the same size and (b) with normal variation in the size of the inhibitory circles.

as in Fig 4(a) and a final distribution that is too uniform) and that some normally distributed variation of these circles is required (Fig. 24(b)).

The simplest model supposes that the reduction in circle size, which is a prerequisite for adding a large population of hairs, occurs at the very beginning; the first and all subsequent hairs are therefore added in random order. When this is done the first hairs lower the uniformity of distribution, and it only recovers later (Lawrence, 1969). If however the reduction in circle size occurs later, or gradually during the process, then the first hairs formed will be a highly dispersed sample. There is direct evidence that this latter system obtains: when accurate drawings were made of the differentiating epidermis in the late fifth-stage larva the most advanced stages, that is the earliest formed hairs, were so precisely dispersed that the uniformity of distribution for the fifth-stage bristles and these stages together, was higher than for the bristles alone.

Further evidence that shrinkage of the circle size is occurring while the hairs are being added comes from insects in which the process has been curtailed, either by an inhibitor of DNA synthesis, hydroxyurea, or by means of the insect moulting hormone, ecdysone

[which causes premature deposition of the cuticle (Williams, 1968)]. When injected late in the moult both these agents specifically curtail hair development, and do not inhibit cuticle formation or cellular metamorphosis. Measurements of the partially completed hair patterns of such insects are completely at variance with computer simulations in which the diameters of inhibitory circles are fixed at the outset, but much closer to simulations incorporating a gradual shrinkage of the inhibitory circle size (Lawrence and Hayward, 1970).

(iv) Models to explain spatial inhibition: In order to simulate the development of hair and bristle patterns, we require only that extant and nascent bristles should effectively inhibit the transformation of nearby epidermal cells into new structures. The extent of this inhibitory effect is decreased during hair determination. We need to know how this inhibition is achieved. As a model Wigglesworth (1940a) proposed that extant bristles continuously absorb some diffusible substance which is made by the epidermal cells. Only epidermal cells in an area of sufficiently high concentration of this substance can transform into bristle mother cells and as soon as the transformation has occurred in an individual cell, this cell begins to absorb the substance, reduces the concentration around it, and thereby precludes nearby determinations. The longer this process takes the greater would be the chance of two simultaneous determinations occurring in neighbouring cells. Such determinations almost never occur, and this makes literal application of the model a bit difficult. Another problem is that the inhibitory influence which is presumed to emanate from a hair or bristle must be maintained independent of the cytological state of the organ itself: old bristles, whose cells have secreted several previous cuticular structures; nascent bristles whose cells have not yet differentiated; new hairs in the various phases of cell division and hair mother cells only barely distinguishable from epidermal cells. All clearly have equivalent inhibitory effects which result in an even, integrated pattern. Each of these organs, in their divers states has to, according to the Wigglesworth model, absorb a substance at the same rate, and it is difficult to imagine how this could be done. The essential factor that they all have in common is the surrounding epidermal cells and this suggests to me that it is the epidermal cells rather than the bristle cells which are the prime mover. This hypothesis proposes that a small group of epidermal cells sponsor the appearance of a new organ in their centre and in this view the developmental state of the bristle

itself becomes irrelevant to the pattern forming process. This hypothesis is not without disadvantages, is more complex than the Wigglesworth model, but does bring the bristle patterns more obviously into line with other field systems in embryology, where a population of cells is clearly capable of subdividing itself into two or more equivalent independent field systems (e.g. "Homonomous arealization", p. 244). This means also that they adopt the same mantle of mystery which surrounds embryological fields generally.

There is no doubt that the epidermal cells surrounding bristles are sometimes different from other cells. In *Rhodnius* (Wigglesworth, 1933) these cells secrete a plaque of smooth cuticle; and as a result of delicate manipulation of hormone timing (Wigglesworth, 1940b) can be made to do so even when the bristle itself has degenerated. In *Drosophila* some bristles are always accompanied by a small cuticular process from a neighbouring cell termed a bract (Peyer and Hadorn, 1966). After dissociation and reaggregation of leg disc cells the orientation and site of this bract is related to the orientation of the bristle itself (García-Bellido, 1966a). These examples have been considered to result from induction by the bristle cells, but there is no evidence against their determination occurring synchronously with the bristle.

From this viewpoint it becomes likely that in some mutants the epidermal cells could become organized into a local bristle-supporting field, but because of some genetic alteration their central cells might not be able to form a bristle. This realization separates the inducing from the responding systems, ideas that have been developed by Stern and his school, reviewed in the next section.

B. GENETIC MOSAICS

Drosophila, homozygous for the recessive gene achaete (*ac*), lack two large bristles on the thorax. Flies were bred which carried *ac* and the gene yellow (*y*) [which alters the colour of the cuticle of each cell bearing it (Hannah, 1953)] on a normal X chromosome, and *ac* + *y*+, on a ring X chromosome.

The ring X chromosome is eliminated with fairly high frequency during development to give clones of male cells which are hemizygous for *ac y,* and consequently the extent of the *ac* tissue is independently marked by yellow cuticle (Hannah, 1953). Analysis of such mosaics (Stern, 1954, 1968) showed that regardless of the amount of *ac* and *ac*/*ac*+ tissue the expression of *ac* was almost always autonomous to the yellow patch of tissue. When yellow

cuticle covered the bristle site the bristle was missing; when wild-type cuticle covered it, the bristle was present (Fig. 25). There was no gross interaction between the two tissues; it was as if the only difference between the *ac* and the *ac*+ epidermis was the presence or absence of the bristles themselves, rather than in the pattern forming process. Since bristles are, as we have seen, particular epidermal cells selected because of their position in the whole, this lack of interaction was surprising until the dual nature of the pattern

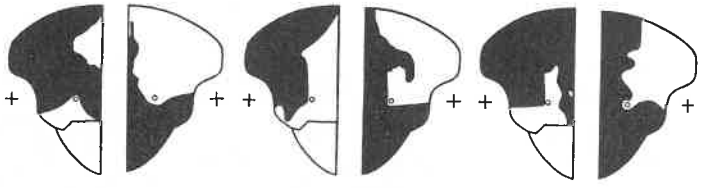

Fig. 25. Some mosaic half-thoraces of *Drosophila.* Black = *ac* tissue, white = ac^+ tissue. Wherever the ac^+ tissue covers the site of the bristle, the bristle develops normally. (From Stern, 1954.)

forming process was perceived. In both *ac* and *ac*+ tissue, Stern argued, there are identical co-operative pattern forming systems (prepatterns) but in *ac* tissue the cell selected by the prepattern is unable to respond. The genes involved in the pattern forming process which locates the bristle, and those concerned in the development of the bristle itself, were different. These lines of thought converge with those expressed above when I suggested that it was the epidermal cells not the bristle cells which are the prime mover in pattern formation; and it should be possible, as apparently in achaete tissue, to have a bristle supporting field without a bristle.

However the situation is more complicated, because occasionally when *ac* tissue only just covers a bristle site, a slightly displaced *y*+ bristle will sometimes form in wild-type tissue near by. Here we have evidence that the prepattern does not define a bristle site absolutely, and that some regulation is possible (compare Schubiger's experiments with Edgebristle, p. 254). How then in wild-type tissue is it ensured that only one bristle is formed? There is no answer as yet.

Some mutants of *Drosophila* bear adventitious structures: male flies homozygous for the gene engrailed (*en*) have a secondary sex-comb. Somatic cross-overs produced, in stocks of suitable genotype, mosaics of *en*/*en* and *en*+/*en* tissue (Tokunaga, 1961). The homozygous tissue was marked with yellow. It was found that regardless of the relative amount of *en*/*en* and *en*+/*en* epidermis that

were present, the development of a secondary sex-comb was always autonomous to the patch of yellow tissue. In some cases a little yellow patch behaved always as if it were part of a limb which was all mutant; the large areas of wild-type tissue likewise developed as if the limb were all wild-type. In other cases the limb would be mostly of *en/en* tissue and again the expression of pattern was quite autonomous to the patches of different genotype. Tokunaga concluded that in the wild-type leg there is normally an underlying facility to make a secondary sex-comb, and that *en*+ cells have lost the ability to respond to it.

In fact nearly all the pattern genes tested by means of mosaics turned out to be alterations in the mutant cells' reaction to an invariant prepattern (Arnheim, 1967). However, an example of a prepattern mutant has been recently described (Stern and Tokunaga, 1967). Male insects heterozygous for the mutant eyeless-dominant bear extra rows of sex-comb bristles. Mosaics of eyeless-dominant and wild-type tissue behaved non-autonomously: patches of wild-type tissue in a primarily eyeless-dominant background conformed with the general pattern of the eyeless-dominant tissue and formed sex-comb teeth in the extra rows not found in exclusively wild-type insects. It was noticed that the eyeless-dominant gene interferes with the proper segmentation of the leg, which confirms the importance of the segmental gradient, and the intersegmental membranes in the development of prepattern.

We have seen how the orientation of hairs is an indicator of the underlying cellular polarity (p. 203). Mosaics of two alleles of aristaless (*al*), which have differing effects on bristle orientation of the scutellum, have been studied by Tokunaga and Stern (1969). They found that bristle orientation was not expressed autonomously, but was related to the proportion of mutant and wild-type tissue on that side of the scutellum. The posterior part of the scutellum grew less in mutant than in wild-type tissue, as did patches of mutant tissue in a mosaic. This growth, autonomously expressed by mutant tissue, controlled the orientation of the bristles near by, regardless of the genotype of the bristles themselves. These observations closely related the fundamental pattern forming system of the organ to the orientation of the bristles.

Homeotic mutants cause the development of an inappropriately situated appendage; one example is aristapedia where tarsal segments develop in place of the antennal arista. A suitable genotype will give flies mosaic for aristapedia (marked with yellow) and wild-type

tissues, after somatic crossing over. Roberts (1964) found that patches of mutant tissue were autonomously expressed as tarsal tissue in amongst wild-type aristal filaments. These observations imply that the underlying prepattern is equivalent in the two appendages and the essential variable, which has been so strikingly altered in the mutant tissue, is the competence to respond to it.

A similar point was beautifully illustrated much earlier by Bodenstein (1935) who took advantage of the very different structure of the fore- and hind-legs of adult *Vanessa urticae*; the fore-leg being exceptionally short and covered with long hair-like scales. The adult leg develops initially from a region in the second segment of the caterpillar limb and can first be cytologically distinguished at the beginning of the fifth and last larval stage. When different amounts of forelimb were transplanted on to part of the hindlimb in the third-stage larva, harmoniously formed limbs resulted, each with only one femur, tibia and the normal number of tarsi. The limbs were chimaeric and made up of different amounts of tissue of the two limb types: when second, third and fourth segments of the forelimb were transplanted on to the first segment of the hindlimb the adult leg was almost pure donor, but when only segments 3 and 4 were transplanted the adult limb was about half and half fore- and hindlimb. The contribution of the transplanted limb to the adult structure depended also on the amount of material left as the stump on the donor. If segments 4 and 3 of the forelimb were transplanted on to segments 1 and 2 of the hindlimb the grafted tissue only formed tarsal material, but if the same tissue was transplanted on to segment 1 of the hindlimb, it formed both tarsi and some of the tibia. Clearly segmentation of the limbs was not established at the time of the operation. But the chimaeric nature of the limb showed that determination to limb type had been completed, although nevertheless these differently determined structures were responsive to one organizing system.

Again, Kroeger (1959, 1960) tied fore- and hind-wing discs *Ephestia* together and implanted the combinate into a mature caterpillar. These two alien discs combined to form single integrated wing hinges in which, although the characteristic type of structure was formed by each tissue, the components fitted together neatly. Both different discs clearly conformed to a single combining pattern influence, but the precise way they reacted depended on the established identity of the disc cells themselves.

These studies build up a picture of the pattern forming system as a

series of independent gradient systems which determine the shape and size of an organ. In different organs such systems are much more similar in construction than are the final organs themselves—the difference being primarily due to myriad variety in the competence of the cells. It is possible that these cells respond to a level in the gradient system, as occurs in the segmental gradient of *Galleria* (p. 218), and that gradient systems are based on the same mechanisms, wherever morphogenesis is to be found. Similar ideas are also being developed by Wolpert (1970). Differences between competences of cells are, therefore, mostly responsible for the structural heterogeneity. This pattern of responses by the cells to a gradient system depends on the earlier acquisition by those cells of an identity (that they are determined as fore-wing cells, hind-wing cells and so on); this identity—the determined state—will be considered in the next section.

IV. DETERMINATION AND REGULATION

A. INTRODUCTION

Although "Determination is among those indispensable terms in the vocabulary of embryologists and geneticists which are most difficult to define" (Hadorn, 1965), it is obviously important to know when, in a growing and diversifying population of cells, the developmental identity of an individual cell becomes established. Determination is this choice of a developmental route, and Waddington (1956) argues that when "determined" the cell is irrevocably committed to develop along that pathway. However, as he points out, what is irrevocable may depend on experimental circumstances: Marcus has shown (p. 216) that isolated pieces of the *Galleria* larval segment will develop according to their prospective fate, and most would argue that this shows that the cells are determined; and yet if placed adjacent to cells of different gradient position, they develop differently. In that case, moreover, determination to form a particular type of scale is provisionally a property of a local population of cells. Still later individual cells from that population are further determined as scale mother cells, leaving the others to make cuticle (Lawrence, 1970). In this case determination is progressive; as growth proceeds diversification continues, to result in a narrowing developmental repertoire of the cells.

If the development of an embryo is completely and irrevocably

mapped out, parts dissected from it would always develop, as far as conditions allow, towards their prospective fate. If small sections of an embryo were extirpated the embryo would mature with these parts wanting. Often, however, there is plasticity in the developmental process and isolated pieces, or incomplete embryos, overreach their prospective fate so that a whole embryo may be constructed from some of its parts. This process is termed regulation.

In theory, regulation could only occur in the absence of determination, but in fact these two cellular properties are not as mutually exclusive as abstract definitions demand. We shall discuss these matters in connection with experiments on imaginal discs.

Many of the properties of the determined state have been elucidated by a brilliant series of experiments on imaginal discs of *Drosophila*: "one of the great stories in modern biology" (Williams, see Hadorn, 1965). Diptera are almost completely remade in the pupal stage: this is not done by "demolition and reconstruction" but by "progressive substitution" (Wigglesworth, 1965) as many of the larval tissues break down and the adult develops from small clusters of cells which have been sequestered during larval growth. These clusters of cells, termed imaginal discs, appear in the egg and grow during larval life. At metamorphosis the cells of the disc differentiate and form adult organs, including much of the integument. Each disc has independent generative powers, for if removed from a fully-grown larva and implanted into the abdomen of another, it will develop almost completely normally, although many structures fail to evaginate (Bodenstein, 1941; Vogt, 1946). Younger and smaller discs will attempt to make their own structures if forced to metamorphose prematurely by implanting them into a fully grown larva (Bodenstein, 1939). These experiments show that the discs are determined as a whole to make their appropriate organ but they do not tell how precise determination is at the cell level.

Vogt (1944, 1946) investigated the determined state of the eye-antennal disc of *Drosophila.* She transplanted bisected discs into late third-stage larvae, and found, unlike Bodenstein (1941), that the development of half discs quantitatively overreached their prospective fate and, taken together, the two half discs made more than the normal number of ocelli and aristae.

Hadorn and Gloor (1946) investigated the determined state of parts of the female genital disc of *Drosophila.* By cutting the mature larval disc into several pieces and transplanting those pieces directly into another full grown larva, they found that different parts of the

disc developed independently, into organs of the genital system and associated structures, according to a pattern. They concluded that the disc was divided into areas which were committed to form the different organs of the genital system and constructed a determination map of the disc. In addition they noted that the disc pieces often made complete supernumerary structures. As each portion of the cut region reacted to the interference by regenerating a complete structure, this process was termed regulation. It seemed that the determined regions of the disc possessed independent powers of regeneration, and Hadorn and Gloor (1946) therefore proposed that the disc was a mosaic of separate field systems or anlagen. In an examination of this disc, Hadorn and Chen (1956) studied regulation of the spermatheca and found that regulation occurred most often when the presumptive spermathecal region was bisected. But if only small portions of this area were isolated by the cut they had a reduced chance of forming a spermatheca. These studies raised two interrelated questions: first, what is the mechanism of regulation? Second, how precisely determined are the disc cells prior to metamorphosis? The first question has been investigated primarily by studies of the discs' performance after section and different periods of time in culture, and the second by dissociation and reaggregation of disc cells. I shall review these approaches separately.

B. EXPERIMENTS WITH CUT DISCS–REGULATION

The male genital disc was examined in great detail by Hadorn, Bertani and Gallera (1949) and Ursprung (1957, 1959). This disc will develop into structures illustrated in Fig. 26 if implanted into a mature larva. Some structures are paired (anal and lateral plates, claspers, paragonia, vasa) others unpaired (ductus, sperm pump and penis plate). If the male disc was cut into two sagittal halves (Fig. 27) and both pieces implanted into a full-grown larva, regulation usually occurred only of the unpaired parts (Fig. 28); so that each half disc developed into a genital system with complete ductus, sperm pump and penis plate, but only one of each of the paired structures. Sometimes, however, the paired structures regulated so that each part of the disc made two of these organs. Hadorn *et al.* (1949) noted that regulation was more likely in younger hosts, and Ursprung (1959) refined these delicate methods still further and found that variability in the results could be reduced if the age of the host was carefully controlled. He moreover found that a host of only 55 hr

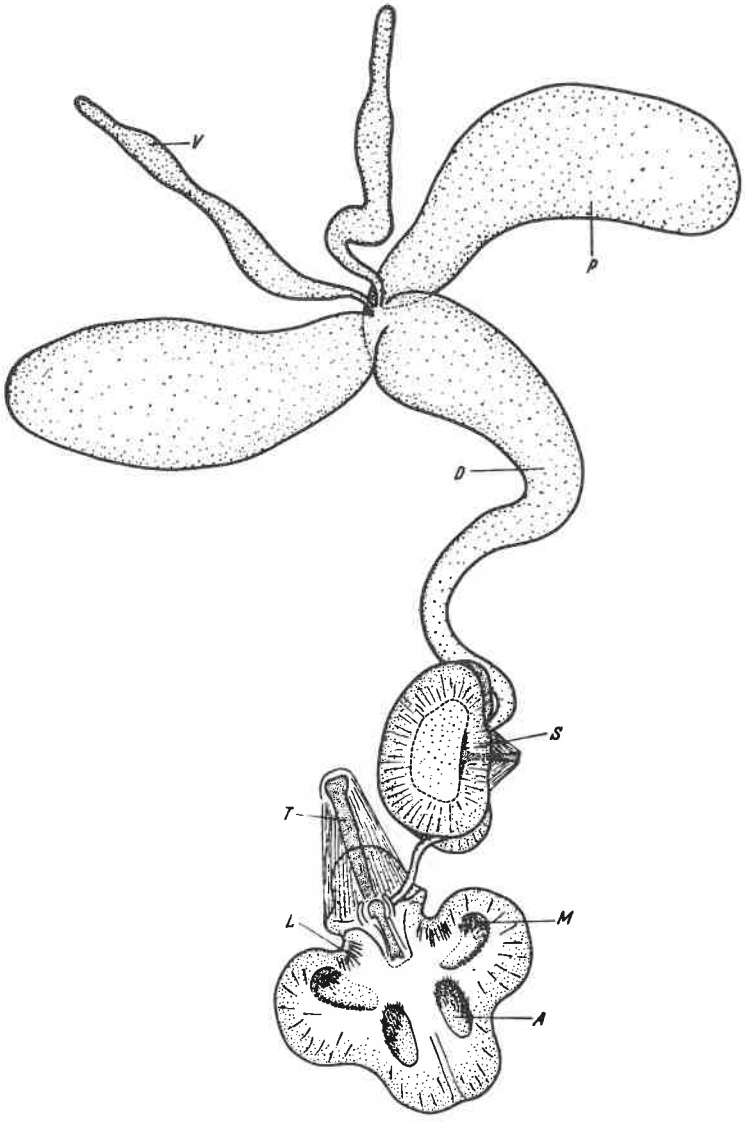

Fig. 25. The male genital system of *Drosophila.* P = paragonia, V = vasa, D = ductus, L = lateral plate, A = anal plate, M = claspers, S = sperm pump, T = penis plate. (From Hadorn *et al.,* 1949.)

old was capable of supporting complete regulation by both halves of the disc. Even half a disc of a mature (96 hr) larva was capable of regulation to a complete male genital system when implanted into a 55-hr-old larva.

It seemed that regulation was dependent not on the age of the disc itself but on the time available prior to metamorphosis, and this was confirmed later (Ursprung, 1962). Hadorn *et al.* (1949) noted also that when the disc was cut paramedially (Fig. 27) the unpaired

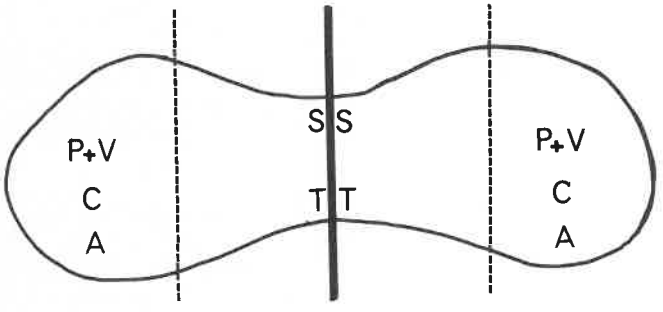

Fig. 27. The genital disc showing approximate position of the anlagen in relation to a sagittal cut (thick bar) and paramedial cuts (dotted lines). P = paragonia, V = vasa, C = claspers, A = anal plates, S = sperm pump, T = penis plate. (From Hadorn *et al.*, 1949.)

central structures usually did not duplicate while the paired ones did. This drew further attention to the observation of Hadorn and Chen (1956) that cut areas regulate particularly well. UV damage does not act in the same way as a cut, for Ursprung (1957, 1959) found that local irradiation often deleted parts of the genital system without inducing regulation. Hadorn *et al.* (1949) had noted that cutting induced growth and proposed that "cell proliferation and regulation go hand in hand".

The size of one of the paired plates can be gauged by the number of bristles it bears, and because Hadorn *et al.* (1949) found no difference in the mean bristle number of anal plates or claspers, whether two were formed by each of the three pieces resulting from paramedial cuts, or whether there were only two plates in all, they argued that regulation is an all-or-none phenomenon.

However, soon Hadorn (1953) reported cases of partial regulation in *D. séguyi.* The anal plates of this species bear four different kinds of bristles, and if disc transplantation was performed some time before pupation, two anal plates of different size were found in the

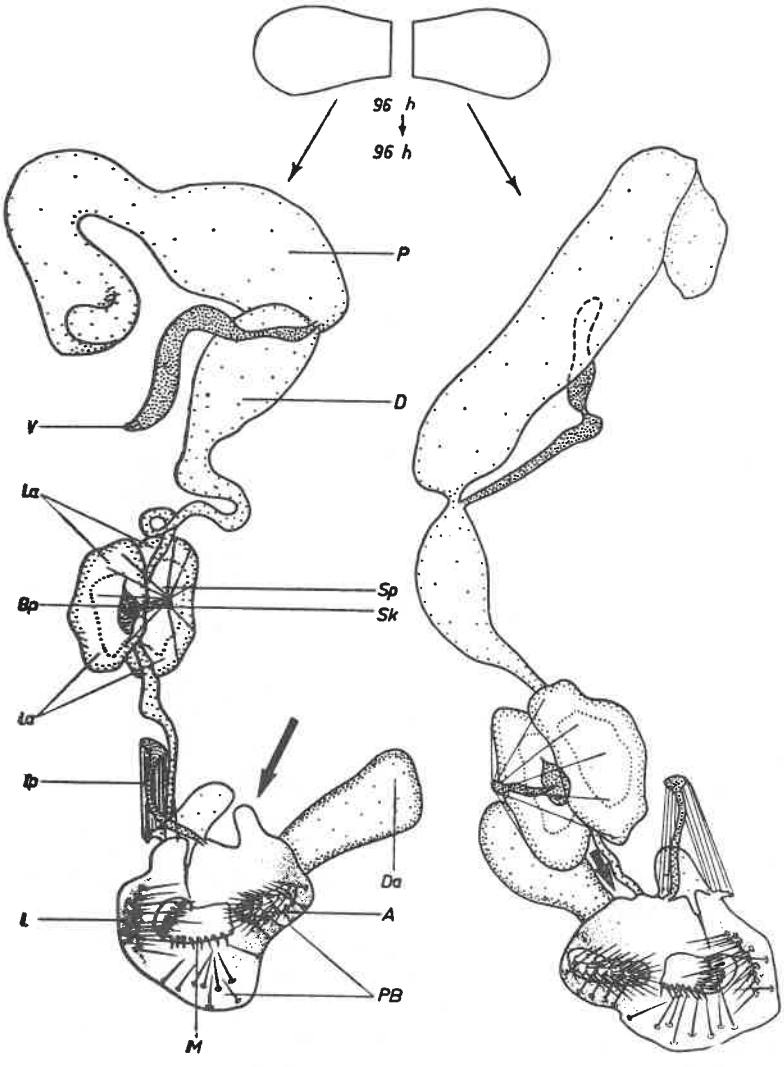

Fig. 28. The genital disc of *Drosophila* from a larva of 96 hr is bisected and the half discs implanted into another larva of 96 hr. After metamorphosis of the host each half has a complete set of unpaired structures, but no duplication of paired structures. *A* = anal plate, *D* = ductus, *Da* = hindgut, *L* = lateral plate, *La* = lappetts of sperm pump, *M* = claspers, *P* = paragonia, *PB* = peripheral bristles, *Sk* = sperm canal, *Sp* = sperm pump, *V* = vasa. (From Ursprung, 1959.)

adult, one was normal in size and shape and the other was very much smaller (Fig. 29). Hadorn argued that the small disc was regenerating at the time when metamorphosis of the host commanded the cells to express their current status. Other very carefully timed experiments caught "regulation actually under way" (Ursprung, 1959). If a sagittal half of a disc were implanted into a third-stage larva of 72 hr,

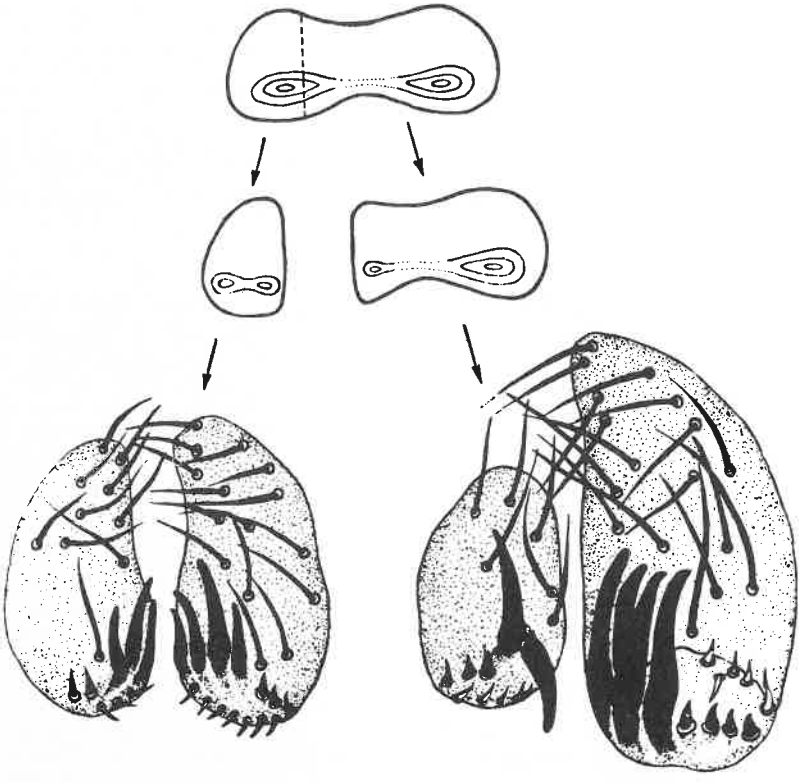

Fig. 29. Section and implantation of the genital disc of *D. séguyi.* After metamorphosis the anal plates show incomplete duplication. Note that all four types of bristles are present on each plate, although their numbers may be much reduced. (From Hadorn, 1953.)

each half gave rise to a complete set of unpaired structures. However, the anal plates and claspers were asymmetric; one of each pair on the same side bore the normal number of bristles, whereas on the other side (the cut side) the anal plate and clasper bore a reduced number of bristles (Fig. 30).

This allows an insight into the mechanism of regulation: as

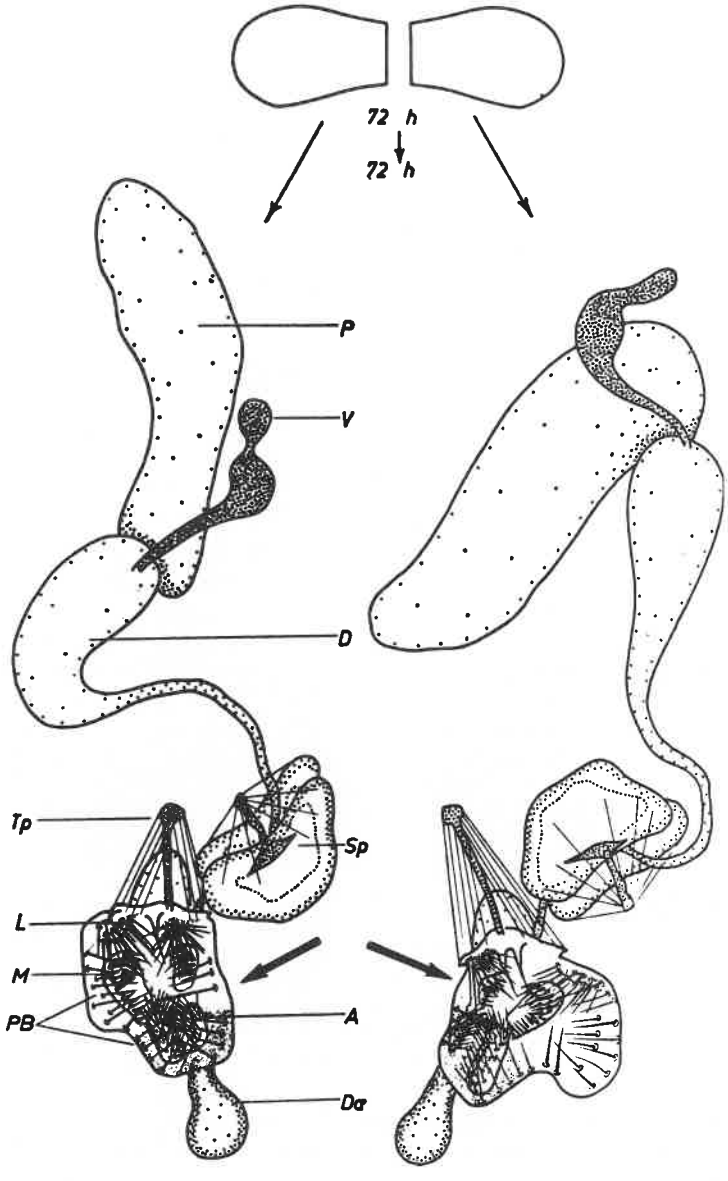

Fig. 30. Compare with Fig. 28. Transplantation of bisected male genital disc of *Drosophila* performed at 72 hr with host also aged 72 hr. Note partial duplication of the lateral plates, anal plates and claspers on the cut side (arrow). The paragonia, vasa and ductus remain unduplicated. For abbreviations see Fig. 28. (From Ursprung, 1959.)

pointed out by Ursprung, regulation could either result from growth of the field of determined cells, followed by its subdivision into two equal parts at metamorphosis (this would give rise always to two equally sized organs, even if they might be abnormally small), or by growth of an independent field system from the wound blastema caused by the cut. The original half of the field would develop normally. Ursprung's observations support the second hypothesis. In a further analysis of this question Ursprung (1962) made use of Bodenstein's discovery (Bodenstein, 1943) that discs could be cultured *in vivo* in the abdominal cavity of adult flies. He removed the implanted half of the disc after various periods of time in culture, and at seven days illustrate a small blastema which could be clearly seen growing out from the half disc. Similarly Kroeger (1958) had observed that during duplication of an implanted wing disc of *Ephestia,* mitosis was mainly restricted to the outgrowing blastema.

Lüönd (1961) transplanted male genital discs of *Drosophila séguyi* taken from fully mature larvae into hosts of different age. In mature hosts, as before, only the unpaired structures regulated, each half disc only formed one anal plate and one clasper although occasionally (6%) two such structures were formed by regulation. The percentage of regulation, as in earlier studies, was increased when younger hosts were employed. Lüönd demonstrated that there is hierarchy in regulative ability; certain structures invariably regulated before others, and this suggested that the regulative process proceeded continuously in the cut disc, at differential rates, until metamorphosis. During initial regulation of the anal plate the proportion of bristles of the various types is different from the final proportion (a feature also noted by Ursprung, 1959) but the proper proportions are re-established by the time the bristle number is half the normal. In these very small anal plates it is the medial teeth-shaped bristles which are relatively in excess, and Lüönd argues that this implies a mediolateral outgrowth of the anal plate primordium. Moreover, intermediate bristles, with sockets like the teeth-shaped bristles and more typical shafts, were found between regions bearing the pure bristle types. Since the cuticular parts of bristles are formed by only two cells, which have certainly descended from one mother cell (Lees and Waddington, 1942; Lawrence, 1966b) this points to the plastic nature of this morphogenesis. Lüönd postulated a mediolateral gradient of something whose concentration in any one place determines the type of bristle that will be formed there.

Unlike the genital disc the eye-antenna disc is mapped out asymmetrically so that section separates two qualitatively different pieces (Gehring, 1966). For instance, when the cut is made at a particular level and the pieces implanted into a mature larva the ability to make palpus is almost exclusively restricted to one half. We know that half of a genital disc, given time, can regulate to form a complete genital system, but since the disc and the genital system itself is symmetrical this only implies duplication of parts already present. Gehring asked if during regulative growth the half discs could surpass their prospective fate and make *qualitatively* different structures.

The anterior half of the antennal part of the eye disc invariably made palpus when implanted into a mature larva, and the posterior half never made palpus. Gehring therefore implanted these halves into young (72-80 hr) hosts to allow time for growth and regulation. After metamorphosis of all 30 implants of posterior half, the palpus was lacking. In the anterior halves palpi were formed and often duplication occurred. After more prolonged culture in adult hosts, followed by retransplantation into larvae, duplication of the palpus in the anterior half was more frequent and in 3/23 cases palpus was formed by the posterior half. Gehring then isolated the most posterior third of four discs, fused them together and implanted them into adults as before. Of 12 combined implants of this type, six formed palps, the other six were completely free of palp. Gehring argued that since even after extensive growth of these combined implants half were without palp, this must imply that basically the posterior third of the disc contains no prospective palp cells, and that its appearance in the remainder must have been spontaneous (*regenerative Neubildung*). Such a change of determined state from one structure to another normally found in the same disc is called regional specific transdetermination (p. 255). Gehring then turned his attention to the duplication of the palpus. Because the bristle numbers are always equal on each side, the duplication of palpus seemed to occur by growth of the palpus anlage and its subsequent subdivision into two equal parts: he called this "homonomous arealization". This is a different method to that described for the genital disc by Ursprung (1962) and Lüönd (1961).

The leg disc is also asymmetric and the results of similar studies to Gehring's allowed Nöthiger and Schubiger (1966) to conclude that regulation of discs depended on proliferation of existing cells, which handed down the determined state to their daughters. It did not

seem possible for cells belonging to one anlage to reliably replace missing parts normally made by cells from another. In order to test this hypothesis further Nöthiger and Schubiger made the genital disc asymmetric by irradiating one half of the disc in approximately the region of the claspers. The irradiated disc was then cut into an irradiated and a non-irradiated half and these were implanted into young larvae (55 hr), or for a time in an adult, to allow regulation to proceed.

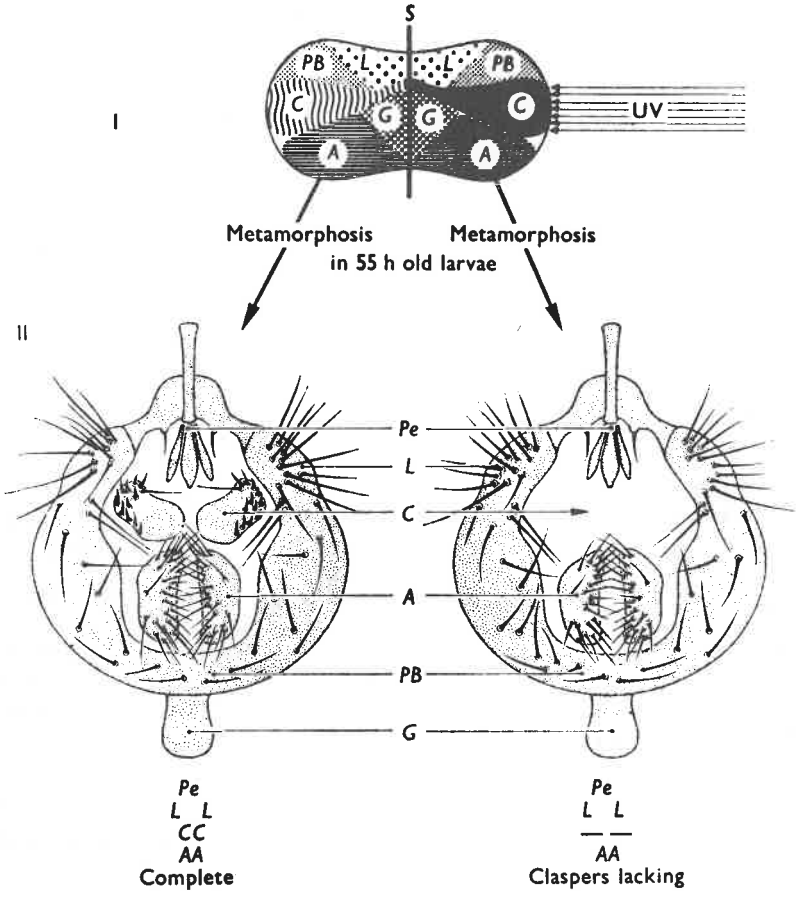

Fig. 31. Diagram to illustrate proliferative regulation. UV irradiation destroys the clasper anlage (*C*) on the right side of the disc. On this implanted half, even though other organs duplicate, the claspers are not replaced. On the left half all organs duplicate. *G* = hindgut, *Pe* = penis. For other abbreviations see Fig. 28. (From Nöthiger and Schubiger, 1966.)

The non-irradiated half often regulated completely and almost invariably contained at least one of the paired structures; however, about 60% of the irradiated halves developed into genital systems which completely lacked one structure, even though other structures were duplicated. Here even after extensive growth, a particular anlage could not be replaced by other parts (Fig. 31). At first sight this study contradicts Gehring's observations on transdetermination to palpus by cells of the posterior third of the eye-antennal disc. This occurred in only one half of Gehring's compound implants, however, and such changes if they occurred only rarely in the irradiated disc, could not be individually detected. These two studies allow the conclusion that reliable replacement of completely lost parts does not occur and further illustrates the mosaic nature of the disc. Regulation of this kind, which is not a complete restructuring of the available material into an entire system, but involves cell division and results only in a duplication of parts already present, was termed *proliferative regulation* by Nöthiger and Schubiger.

Proliferative regulation could be achieved by:

(i) cell division in the different determined anlagen of the disc followed by migration to the new sites;

(ii) migration of determined cells from the different anlagen to the cut and their subsequent division and spatial reorganization;

(iii) a combination of (i) and (ii).

The experiments of Nöthiger and Schubiger do seem to rule out the formation of a "dedifferentiated" blastema, which develops into a new half-genital system autonomously.

Some further observations relevant to this problem have come from studies on the proboscis disc. The proboscis of *Drosophila* is constructed from two equal halves, each made by one separate labial disc. When a single disc is implanted into a 72-hr-old larva it may produce an entire proboscis (Wildermuth and Hadorn, 1965) and the frequency of such duplication can be increased by culturing the disc for several days in the adult (Wildermuth, 1968a). It would seem that the disc *in situ* is normally under some developmental control, and indeed some supernumerary bristles are formed by a single disc even when it is implanted into a larva of the same age as the donor. The development of these extra bristles could result from the shock of the operation itself [Loosli (1969) has suggested that the formation of similar adventitious bristles in the implanted haltere disc is due to such a shock], or be due to the absence of contact with other tissues

(Gehring has demonstrated that palpus duplication in the eye-antenna disc is inhibited when the eye part of the disc is in contact with the palpus area: in this case, however, there may be less wounding as the cut is located far from the palpus).

It is noteworthy that duplication of the proboscis disc can occur without section [although the operation itself would provide some wounding stimulus (Nöthiger, personal communication)], and from a study of partially duplicated discs Wildermuth concluded that duplication resulted from growth and the subsequent reorganization of the cellular material into two parts. He regarded the process as an example of proliferative regulation. This hypothesis demands that the cells of the new structure should descend from the different determined regions of the regulating disc and not from a local blastema. Kroeger (1958) reported that in duplication of the wing disc of *Ephestia,* mitoses were concentrated along the outgrowing new wing. However an autoradiographic study of the duplicating labial disc (Wildermuth, 1968b) did not show such a concentration. Because of the short life of the thymidine in the fly, a pulse of label was taken up by all of the cells undergoing DNA synthesis within only about 1 hr of injection. Labelled cells were found evenly distributed over the disc when uptake of thymidine was assayed immediately after implantation into an adult. In a disc labelled at the beginning of regulation and left to go through metamorphosis prior to fixation, the label was localized only on the old side of the now complete proboscis. This result could well be due to dilution of the label on the new side by repeated divisions, as has been shown to occur during the larval-pupal moult of silkmoths (Krishnakumaran *et al.* 1967), but there are other possible explanations. Even in the case of the genital disc, where regulation of a half occurs as a result of growth of a new blastema from the cut, the source of new cells is not established.

Choice between the basic models for proliferative regulation is not yet possible, but the autoradiographic method pioneered by Wildermuth should, if the whole process of regulation is followed in detail, give the answer.

C. DISSOCIATION EXPERIMENTS

The studies on regulation have suggested that the imaginal disc is a mosaic of independent populations of cells determined to make particular structures. During superfluous or regulatory growth, the cells pass on their determined state to their offspring. We do not yet

know the precision of cellular commitment within each anlage of the mature larval disc. It is conceivable that each cell is rigidly determined to make each component, say a particular bristle, of the pattern, and that pattern reconstruction after section and regulative growth, or after excessive growth leading to duplication, occurs by the ordered assortment of daughter cells. Alternatively, the determination of cells could only be a commitment to form a particular region of the disc such as clasper, and deployment of the available clasper material could depend on a supercellular organization process occurring at the onset of metamorphosis itself.

Hadorn *et al.* (1959) began to investigate this matter by means of genetically marked discs that were partially dissociated and then mixed up together. They mixed wing discs marked with yellow (*y*) with others marked with ebony (*e*). These chimaeric discs were then implanted, allowed to pass through metamorphosis, and the cuticle formed by the combinates examined. Many areas were genetically mosaic, and they found that the cells from both discs combined to make integrated patterns (Fig. 32). The authors argued that at the

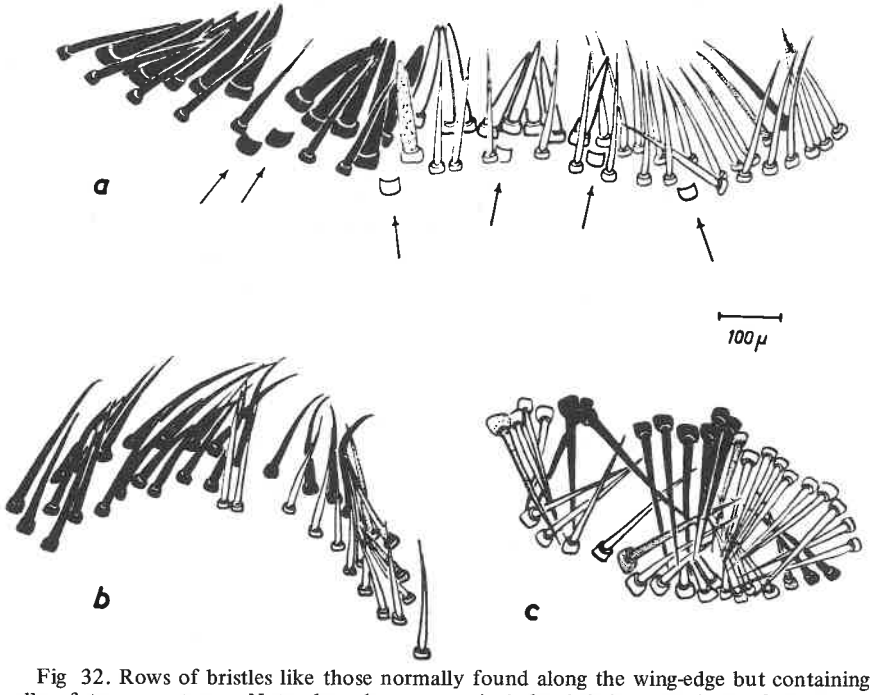

Fig 32. Rows of bristles like those normally found along the wing-edge but containing cells of two genotypes. Note that the pattern includes bristles regardless of genotype. Arrows mark sockets without shafts. (From Hadorn *et al.*, 1959.)

time of dissociation the pattern was "not anchored in single cells" and that some pattern forming process integrated their differentiation. Sometimes the patterns were imperfect; Ursprung and Hadorn (1962) found lines of partially ordered bristles which continued across genetically different areas. These authors argued that such a pattern, which did not have all the components of the pattern found *in situ,* and yet clearly indicated an integrating influence that extended across the genetic boundaries, could only have come from epigenetic development of pattern, which had not been completed in time for metamorphosis.

Nöthiger (1964) extended these studies and mixed pieces of genetically marked discs from both sexes, two species and from different discs. He found that partially dissociated discs from genetically marked males readily formed chimaeric but mostly normal structures, and that sometimes even single bristles from one donor could be found isolated in a homologous structure: such bristles were integrated into the entire pattern (Fig. 33). Male

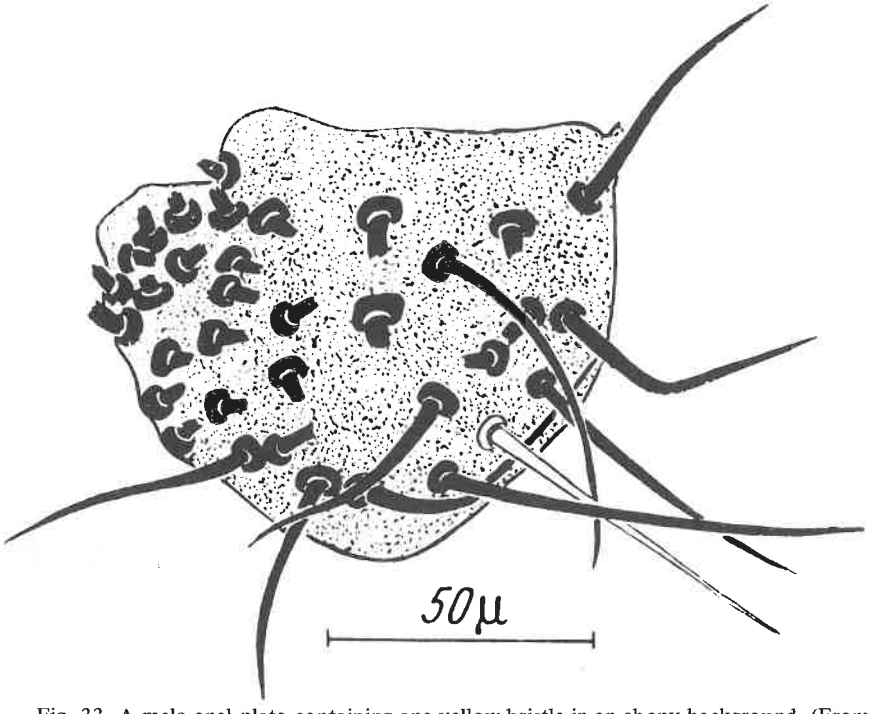

Fig. 33. A male anal plate containing one yellow bristle in an ebony background. (From Nöthiger, 1964.)

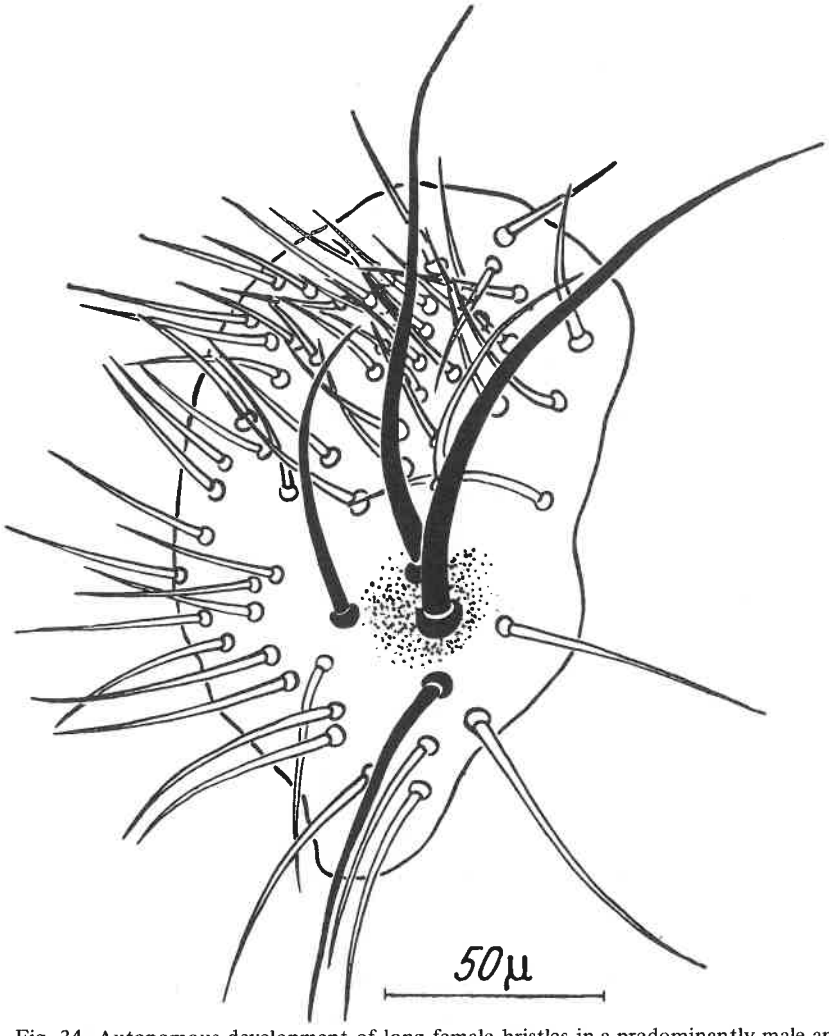

Fig. 34. Autonomous development of long female bristles in a predominantly male anal plate. (From Nöthiger, 1964.)

genetical discs from two species of *Drosophila (D. melanogaster* and *D. séguyi)* also formed chimaeric structures. Here the cells from each donor could only form organs characteristic for that species, although cells of both species combined to form integrated patterns. Likewise, male and female cells also combined to form integrated structures, but only in the anal plates which occur in both sexes (Fig. 34). That mosaics of claspers or vaginal plates were not formed

suggested that these cells separated from each other. Indeed Nöthiger discovered that wing and genital disc cells segregated completely. The sorting of disc cells has moreover been observed *in vitro* (Lesseps, 1965) and can be accomplished without growth (García-Bellido, 1967). These experiments illustrated that, at the time of the operation, the disc cells were already determined to form particular structures, and while cells which were determined identically associated, those determined differently segregated.

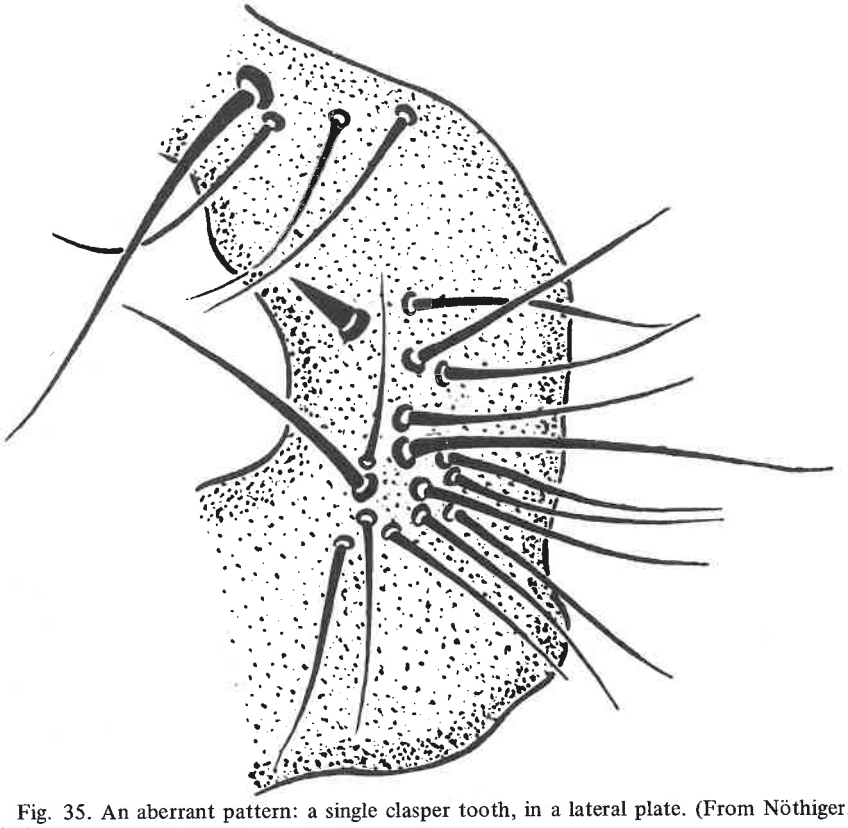

Fig. 35. An aberrant pattern: a single clasper tooth, in a lateral plate. (From Nöthiger, 1964.)

Occasionally some isolated cells belonging to, say, clasper, were found inappropriately placed, in a lateral plate (Fig. 35): this result is of importance because it shows that such errant cells are not sensitive to the nature of the surrounding cells and develop autonomously.

Tobler (1966) did some rather similar experiments on the leg disc and in particular partially dissociated and combined the central part

of one leg disc (corresponding to tarsus and tibia) with the periphery (corresponding to coxa, femur and tarsus) of another differently marked disc. If alterations of prospective fate were to occur within the leg disc one would expect all regions of the legs to be chimaeric, but if determination was already so established that different regions of the disc sorted by migration one would only expect to find mosiac patterns in those regions actually near the cut where tissues of one type are likely to be donated from both halves. Tobler found that 64% of the implants were mosaic for tarsus, whereas none were mosaic for femur, which confirmed that cells sort out from the different regions of the leg disc, and do not change their determined status.

García-Bellido (1966a) refined these methods further, and succeeded in dissociating the disc to about 90% single cells with the remainder as small clumps consisting of 2-20 cells. These cells were then mixed and reassociated by centrifugation, and were then implanted into an adult for two days because "reconstruction of integrated structures proved to be more efficient" before final transplantation into a larva. Using the wing disc (Hadorn and Buck, 1962) he confirmed that cells from different regions of the disc would only form chimaeras with cells from the same regions and not from others.

Generally each part of the wing disc only formed structures appropriate to their origin, but there was one case where cells of the anterior part of the disc (marked with *y*) combined with those from a posterior half (marked with *e*) to form a row of integrated bristles normally found along the wing-edge. At the overlap two bristles of the posterior type were formed by anterior cells. García-Bellido proposed that this resulted from growth and "restitutive regeneration" of the anterior cells. Restitutive regeneration must clearly mean, in this case, a change in the prospective fate of the cells, possibly dependent on the interaction between cell types at the junction.

The patterns formed by completely dissociated leg disc cells were not usually as finely integrated as those formed by partially dissociated discs. Often single sex-comb teeth or claw elements would appear in an alien environment. García-Bellido argued that this implies that their formative cells were precisely determined prior to dissociation, and that the basis for reconstruction of pattern in these mixed cells is cellular migration. Moreover, since occasionally bristles in a row were quite inappropriately oriented to the others, he argued that cellular

polarity was also a persistent feature of these cells, and was not epigenetically determined after reaggregation. These hypotheses would demand that the cells not only normally migrate to their precise site in a developing pattern but also rotate until their polarity is appropriate to the other cells. He also combined fore- and middle-leg discs which did not sort out from each other. In the male fore-leg there are neat transverse rows of closely packed bristles that are lacking in the middle-leg. He found that only fore-leg cells made transverse rows; they were never chimaeric.

García-Bellido (1966a) argues that this observation implies that "the determined cells carry not only information which enables them to join in regions, but also more specific information which enables them to reconstruct the patterns they come from". García-Bellido's argument for highly specific determination of single cells is based on the differentiation of errant cells in the abnormal patterns he gets after complete dissociation. If a sex-comb tooth is formed in isolation, this, he argues, implies that the sex-comb mother cell was determined as such prior to dissociation. But even if the determination were less precise, what would one predict an isolated cell determined as, say, "tarsus" would do when commanded to differentiate and metamorphose? It must make something, and as such a cell could make anything on the tarsus there seems no reason why it should not spontaneously develop into a sex-comb tooth. In my opinion the reconstruction of pattern reported previously is not likely to result from the migration of each cell to its precise place in the developing pattern, and that at the time of dissociation, determination had not reached down to the single cell. This is clearly so for the cells which will make the little bracts which form nearby some kinds of leg bristles and are never found in isolation. After dissociation and reaggregation it is common to find a bristle of one genotype whereas its associated bract is of another.

This conclusion is underwritten by the incisive observation of Nöthiger (1964) who considered the earlier work on regulation in the genital disc by Hadorn *et al.* (1949) (p. 237). On each of the claspers there is one characteristic long bristle. These authors cut the disc into three sections and sometimes each section made two claspers, each with one such bristle. The cells which prospectively were to make only one pair of bristles cannot have been located in all three pieces and therefore in this case at least two bristles must have been made by cells not pre-determined to make them. Furthermore Schubiger (1968) examined development of the leg and constructed a fate map

of exceptional detail. On the trochanter in particular there is invariably one large "edgebristle" of characteristic form. The effect of cutting the disc into four pieces and reimplanting the pieces into a mature larva was to reduce the appearance of this edgebristle to only 60% of the discs. This seemed to be due to damaging the presumptive bristle cells, for if the cut was oriented even closer to the presumptive bristle the frequency of appearance dropped to 15%. When portions of the disc were reared in young larvae or kept for some days in an adult the frequency of appearance of the edgebristle increased and sometimes two were formed, one in each half: even in the cut portions there must be cells which can recover the ability to make edgebristle if they have time. Halves of younger discs are more likely to make two edgebristles than fragments of older discs; this implies that the area competent to form edgebristle contracts with the age of the disc. These experiments make clear that the capacity to form an edgebristle is not restricted to a single cell until the process of metamorphosis itself; and may not be so restricted even then. In *Oncopeltus*, even after metamorphosis and development of hairs, the remnant epidermal cells have not lost their ability to make hairs, and under experimental conditions will do so again (Lawrence, 1966c).

D. CHANGES IN THE DETERMINED STATE

Hadorn (1963) investigated the ability of the imaginal disc cell to maintain the same determined state through many cell generations. A half disc was cultured in an adult female abdomen, and after about two weeks growth was removed, bisected and one piece was implanted into another adult, while the other was implanted into mature larva to see what structures it could make during metamorphosis. After culture in several successive adults the proliferative rate of the cells increased rapidly and from then on several implants could be made, and new sublines initiated. It is a consequence of the continued section of the growing disc that later test implants descend from smaller and smaller regions of the original disc. If determination in the original half disc were to the single cell level, one would expect eventually to have cultures producing only one cell type. There is in fact some narrowing of the repertoire of those sublines producing genital structures over many transfer generations (Hadorn, 1963, 1965, 1966), which confirms that the genital disc is subdivided into districts of different determination. However this specialization does not continue indefinitely, and even

after many generations the narrowest of sublines still produced, for instance, anal plates (with characteristic bristles and cuticle) and hindgut. Hadorn (1966) noted that the test implants from lines bearing anal plates and hindgut nearly always contained either both organs, or only anal plates, but very rarely (6/966) hindgut alone. This and studies of the lineage suggested that the capacity to make hindgut was often lost from the line, but was repeatedly being regenerated from presumptive anal plate cells. Such a "regulative" change is reminiscent of that reported by Gehring for the neogenesis of palpus (p. 244) and is termed regional specific transdetermination. During the first five transfer generations genital disc cells gave only parts of the genital system in test implants, but later perfectly formed structures appropriate to other discs made a sudden appearance (Hadorn, 1963). Use of differently marked hosts and implants (Gehring, 1966) showed that these cells had not come from the host and resulted instead from a change in the determined state of the genital disc cells. The relevance of such transdetermination to normal development is not yet clear but there are certain features which are of particular interest. We may note:

(i) the change to the new state is abrupt and complete, and intermediate cell types are not formed, or at least not recognized;

(ii) the cell affinities of the transdetermined cells become appropriate to their new identity so that sorting occurs (García-Bellido, 1966b);

(iii) the change is probably to a wide repertoire such as "leg" or "wing" and not just to "trochanter" or "wing-fringe". Gehring (1966) has shown that in one case of transdetermination from palpus to antenna, at first an antennal third segment is made, which later develops into an arista and a second segment. Hadorn (1966) examined a number of test implants in his genital disc lines in which transdetermination had only recently occurred to antenna. These showed no predominant bias to be one particular segment of the antenna;

(iv) the probability of transdetermination occurring is directly related to the proliferative rate (Tobler, 1966; Wildermuth, 1968a);

(v) some transdeterminations occur with greater frequency than others, and transdetermination may be both reversible and irreversible (Hadorn, 1966);

(vi) transdetermination is a communal act by a small group of cells.

This key fact was shown by irradiating larvae of the genotype y^+sn^+/ysn, mwh^+/mwh with X-rays so as to induce somatic cross-over and mutations (Gehring, 1967, 1968). Such changes happen only with a low probability in a single cell and from this a clone of cells marked with, say, yellow and singed integument will grow.

Gehering found, as was to be expected, transdetermined elements within a clone, but he also found regions of transdetermined tissue

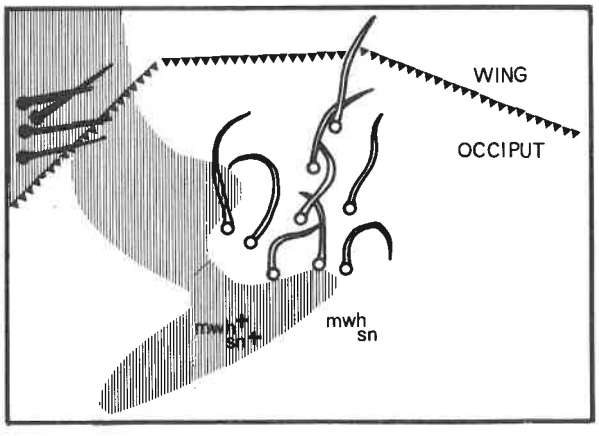

Fig. 36. Gehring's experiments on transdetermination. Note that the two tissue patches (wing and occiput) and the patches of two genotypes (*mwh*$^+$*sn*$^+$ and *mwh sn*) are spatially unrelated. (After Gehring, 1967.)

that were genetically mosaic. This could imply either that transdetermination occurred spontaneously in a group of adjacent cells; or that transdetermination occurred independently in regions of two genotypes and that migration and sorting had brought these two groups of cells together. A detailed examination of such a mosaic showed, however, that both the genetic boundary and the tissue boundary were continuous and definite, and independent from each other (Fig. 36). Yet, if the area of wing tissue had been formed by two populations of cells migrating together there would have been a random assortment of the two genotypes in the wing tissue. The observed pattern could best be explained if transdetermination was a co-operative event and happened synchronously in two or more cells.

As transdetermination is a co-operative event, could the normal determined state also depend on cellular interaction?Attempts to

start imaginal disc cell lines with single cells have not been successful (Gateff, personal communication), but in vertebrate cell lines the determined state can be transmitted via a single cell (Coon, 1966).

In summary, these experiments on imaginal discs have confirmed Hadorn and Gloor's (1946) original description of the mature larval disc as being divided into fields or anlagen. Each anlage contains cells that are determined for a particular organ, and through growth can compensate for loss of part of the anlage. Occasional spontaneous changes of the determined state do occur, but normally the cells of one anlage cannot replace lost cells from another. Mixed cells from different anlagen will normally sort themselves out, but if a few cells are trapped in an alien environment they will retain their determined state and develop autonomously. In my opinion the formation of integrated patterns after dissociation and reaggregation depends on some supercellular organization process, rather than migration of previously determined cells to their appropriate site in the pattern under reconstruction.

V. CELLULAR DIFFERENTIATION

Cellular differentiation is a constant feature of postembryonic life; even in adult insects there is continuous regeneration of intestinal cells and haemocytes (Krishnakumaran *et al.*, 1967). Most typical examples concern cases where proliferating cells cease dividing, acquire characteristic organelles and make specialized products. Of course dividing cells are also synthesizing many characteristic products and assembling them into conspicuous organelles but cell differentiation begins essentially with the transition from this set of syntheses to another. Often this will result in the creation of a mature, stable cell type, and the idea has therefore grown up that differentiation involves a loss of developmental plasticity (Grobstein, 1966), but so many cases of "dedifferentiation" have been established that this part of the definition could well be dropped and the attention turned to the acquisition of particular syntheses (Hay, 1966, 1968).

The transformation from larval to adult syntheses which occurs in the epidermal cells of many insects at metamorphosis (Wigglesworth, 1934, 1940b; Lawrence, 1966c, 1969) is an example of differentiation. One lesson to be culled from such insects is the real distinction between determination and differentiation, for here these processes are often widely separated in time. The adult pattern of

Rhodnius is determined very early—although it can be evoked in the first-stage larva if it is parabiosed to a fifth stage (Wigglesworth, 1934) it normally does not appear until metamorphosis, many cell divisions later. During this growth the particular characteristics of the latent adult pattern are passed on to daughter cells. If wounding results in excessive divisions of cells determined to form a pigment spot, or a particular bristle type (Wigglesworth, 1940b), then at metamorphosis a larger pigment spot or more bristles of that type are formed in the adult. The same system is responsible for the maintenance of determination in discs, and such latent but propagable determined states are probably usual in developing systems (Baker, 1967).

The controlling factor in cellular metamorphosis is juvenile hormone; in its presence the cells make larval cuticle and in its absence adult cuticle. If, in *Oncopeltus,* juvenile hormone is applied to cells undergoing cellular metamorphosis the cells will make cuticle which contains both adult and larval components. The synthesis of melanin changes from the larval to the adult pattern and becomes insensitive to the juvenile hormone early in the moult; in some areas this change involves the initiation, and in others the cessation, of melanin production. The surface sculpturing of the cuticle remains sensitive to the hormone for much longer, and all intergradations of cuticle between larval and adult can be made (Lawrence, 1969). This production of intermediate cuticle shows that the transformation can be broken down into components, which illustrates that the synthetic pathways may be autonomous, and may differ in sensitivity to the juvenile hormone, or in their moment of commitment.

Little is known about the intercellular switches which control the synthetic pathways involved in differentiation in any organism. One feature of insects, which is certain to help, is puffing in polytene chromosomes (Ashburner, 1967, 1970), but the simplest systems for studying the biochemistry of differentiation employ cells which make a great deal of one product. Kafatos has explored one such system in an insect. During metamorphosis of the pupa of *Antheraea* particular epidermal cells of the galea differentiate and diversify into three types. This diversification stems from unusually oriented mitoses (vertical division illustrated in Fig. 1(b), Kafatos and Feder, 1968) which probably can be regarded as differentiative divisions like those found in bristle or dermal gland development (Lawrence, 1966b). One cell becomes highly polyploid and manufactures a large

quantity of a pure enzyme (cocoonase) which is deposited as crystals of active substance on the outside surface of the pupal galeae (Kafatos and Feder, 1968) and later is dissolved in a buffer solution secreted by the labial glands (Kafatos and Williams, 1964; Kafatos, 1968). The active enzyme then digests the hard proteinaceous cocoon to allow escape of the emergent moth.

Kafatos and Feder (1968) have shown that DNA synthesis associated with increasing polyploidy of the secretory cell nucleus occurs concurrently with rapid synthesis of cocoonase. This is of particular interest because of the rather widely held view that DNA synthesis and differentiated cell function are mutually exclusive (Holtzer, Abbott, Lash and Holtzer, 1960). As the cocoonase is synthesized it is sequestered in a large vacuole in the cytoplasm, and pulse chase studies with explanted galeae have shown that about 70% of labelled protein enters the vacuole within 2 hr of labelling (Kafatos and Reich, 1968). The remaining 30% of synthesized protein stays in the cytoplasm. In the presence of 60 μg/ml of Actinomycin D *in vitro,* uptake of labelled uridine into nuclear RNA drops to only about 1% of its value in paired control galeae. After 12 hr incubation in Actinomycin D rate of uptake of amino acids into cocoonase is about 80% of controls, whereas the uptake into cytoplasmic protein has dropped to about 10% of its former level. Kafatos and Reich interpret these interesting results as evidence that the messenger RNA for cocoonase, in contrast to that specifying the indefinite proteins of the cytoplasm, is long-lived. More will, no doubt, come from this system.

VI. OUTLOOK

If one is interested in biological phenomena rather than in a particular species it is well to choose one's experimental organism carefully. There is a case for using insects to study all branches of developmental biology, but it is in the study of pattern formation that they offer outstanding advantages. The two-dimensional nature of the cuticle and the precisely local differentiation that occurs, as well as the orientation of cuticular structures themselves, has already offered a tantalizing glimpse at methods of intercellular communication, and may well take us further than the traditional systems have done. In studying intercellular communication we need to know what chemical molecules and ionic interchange occur between the cells of a developing tissue and how information is encoded. By this

route it may be possible to escape from hazy fields and gradients to a more mechanistic understanding.

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