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Polarity and gradients in lepidopteran wing epidermis

I. Changes in graft polarity, form, and cell density accompanying transpositions and reorientations

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SUMMARY

Lepidopteran wing epidermis has certain advantages for studying the spatial organization of cell populations: ease of accessibility and manipulation, large size, an essentially two-dimensional structure, and direct expression by the scale cells of their polarity. Grafting experiments reveal that polarity and density of graft cells as well as overall graft form are functions of a graft's source and its transplantation site; graft polarity is also determined by the orientation of the graft. The results are consistent with the existence of at least one morphogenetic gradient along the proximo-distal axis of the wing's upper epidermal layer. Various gradient models that might explain the experimental observations are considered.

INTRODUCTION

The cells of many animal and plant tissues express an overt polarity. Insect integuments are particularly noteworthy in this regard, for they are marked by polarized cuticular outgrowths which reflect the polarity of the underlying epidermal cells. For this and other reasons the insect epidermis has been an extremely useful system for the study of spatial differentiation.

In a pioneering study, Wigglesworth (1940), working with the bug, *Rhodnius*, observed that after an integumental square had been excised, rotated 180°, and reimplanted, the bristles which appeared on the graft following a molt displayed a reversed polarity. He inferred that each epidermal cell possessed a covert polarity at the time of excision, perhaps a 'cytoskeleton' which determined subsequent bristle orientation.

Wigglesworth considered polarity an autonomous property of each epidermal cell. Similarly, the morphogenetic gradients revealed in the fifth instar *Rhodnius* tergites (Locke, 1959, 1960) and in the *Rhodnius* leg segments (Locke, 1966)

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could be interpreted in terms of a cell-autonomous property. Certain transpositions of grafts yielded pattern deformations which could be interpreted as the result of the transposed cells migrating in an attempt to re-establish contact with other more compatible cells.

However, other work suggested that insect cell polarity might be under intercellular control. Extending Wigglesworth's experiment with *Galleria* larvae, Piepho and his students (Piepho, 1955; Piepho & Hintze-Podufal, 1971) showed an interaction between host and graft cells; scale cells at the periphery of the 180°-rotated graft, and host cells bordering the graft, both displayed marked changes in the normal axial orientation. Particularly influential were the proposals of Lawrence (1966) and Stumpf (1968) that the morphogenetic gradient in the abdominal segment of *Oncopeltus* and *Galleria*, respectively, is diffusion-based. According to these workers, the hair or scale polarity corresponds to the slope of the gradient; when two pieces of integument differing in axial level are joined, the resulting hair or scale polarity patterns can be interpreted as a consequence of movement of gradient substance from higher to lower levels, either until no singularity remains or until a maximum stable slope is produced. A further thorough study on the nature of the segmental gradient was carried out by Lawrence, Crick & Munro (1972). They matched results of 90° graft rotations in *Rhodnius* with computer simulated gradient models, and demonstrated that a diffusion-resistance factor is essential for any diffusion model.

In this and the following paper (Nardi & Kafatos, 1976), we have studied the behavior of transposed and reoriented grafts in the pupal wing epidermis of the tobacco hornworm, *Manduca sexta*. Like the insect integuments already discussed, the wing in Lepidoptera consists of a monolayer of epithelial cells. A significant advantage of our system is the large size: the *Manduca* wing is 2 cm long. The large size permits tests of gradient effects at many more sites than in the abdominal segment. In initial transplantation experiments, the color pattern appeared to behave as an autonomous cell property; this observation is not surprising considering the late developmental stage examined. Therefore, we focused our attention on the scale cell polarity, graft cell density, and graft shape which are still labile at this stage.

MATERIALS AND METHODS

Selection of pupae

Experimental animals were selected from recently pupated animals that had been reared on an artificial diet (Bell, personal communication) at 25 °C. Once the pupal cuticle has assumed a reasonable degree of rigidity (approximately 6 h after pupation), excision and transplantation of the cuticle plus the attached single epidermal layer (the upper layer of the forewing) can be done with facility; the cells of the lower wing surface remain *in situ*. When the epidermal cells retract from the pupal cuticle about 2 days later, grafting experiments again

become impossible; without the rigid support offered by the cuticle, the manipulation of epidermal squares is no longer feasible. Integument removed up to 15 h post-pupation consists of cuticle and a monolayer of epidermis associated with a basement membrane, hemocytes, tracheoles, and sensory nerves; after this time, the basement membrane plus associated structures are detached, and the integument consists of only cuticle and epidermis. (The absence of nerve cells was verified by a maceration procedure, and by methylene blue staining.) Results with both types of integument are identical, except that the fraction of successful grafts increases when the basement membrane is present; some damage is probably inflicted upon cells detached from the basement membrane. Therefore, unless otherwise indicated, all investigations utilized integument from 6- to 15-h-old pupae.

Anesthetization

Before operations the pupae were exposed to carbon dioxide gas for about 15 min. The exposure was sufficient to immobilize the animals for the duration of the operation (5–10 min).

Operational technique

With the aid of a low-power dissecting microscope, small squares of integument, often varying in size but usually 2 mm × 2 mm, were excised by making four shallow incisions with a razor blade fragment. A square of this size has approximately 100 cells along each margin or a total of about 10^4 cells. The squares were removed with forceps and then replaced in a reoriented manner. Alternatively, two squares were removed and exchanged before positioning (transposition experiments). Two animals were used for a given exchange when the transplantation sites were adjacent. All grafts were removed from and replaced in one of the eleven 3 × 3 mm regions outlined in Fig. 1 (A). To guard against infection and melanization, a small amount of streptomycin:penicillin:phenylthiourea (1:1:2) powder was applied to the wound with the tips of the forceps. After the graft had been properly positioned, a small, clear plastic disc was applied and sealed over the wound with low melting point paraffin. The operated animals were then individually placed on tissue paper in sealed plastic dessert cups, and remained in a 25 °C incubator until a few hours before adult emergence; at this time they were transferred to large jars in which wing spreading could occur properly. After the wings reached their maximum size, they were severed at the base and examined with a dissecting microscope.

Histological technique

For examining the graft shape and socket cell density, the cuticular morphology of the adult wing was studied after removal of the scales. Chlorazol black E (1 % aqueous) stains certain cuticular regions more intensely than others. The staining property of each region is maintained in grafts – i.e. at the time of

grafting the wing is already determined with respect to the adult cuticular staining properties. This was confirmed by Feulgen staining of male grafts implanted in a female host. In Lepidoptera, the nuclei of the two sexes can be distinguished by differences in the amount of heterochromatin (Marcus, 1962). It was shown that the putative demarcation between graft and host tissue, based on cuticular staining, corresponded to the demarcation between male and female nuclei. These findings allowed one to clearly delineate graft from host by simply staining with 1 % chlorazol black E whenever the two regions sufficiently differed in the degree of their cuticular staining.

Before cuticular staining, wings were dipped into 95 % ethanol and then placed in 10 % KOH for 24 h. The alkali treatment served three functions: bleaching of the cuticle, loosening of scale cells, and separation of the appressed upper and lower cuticular membranes. After removal from alkali, gentle washing with water removed most of the loose scales. The two cuticular membranes were separated by simply grasping each with forceps and slowly pulling in opposite directions. At this stage the upper cuticular layer was stained for 5 min in chlorazol black E and floated into position on a large glass slide; for each specimen of adult cuticle, the corresponding pupal cuticle was also preserved. A microscope slide projector was used to trace graft-host interfaces and socket cell positions.

RESULTS

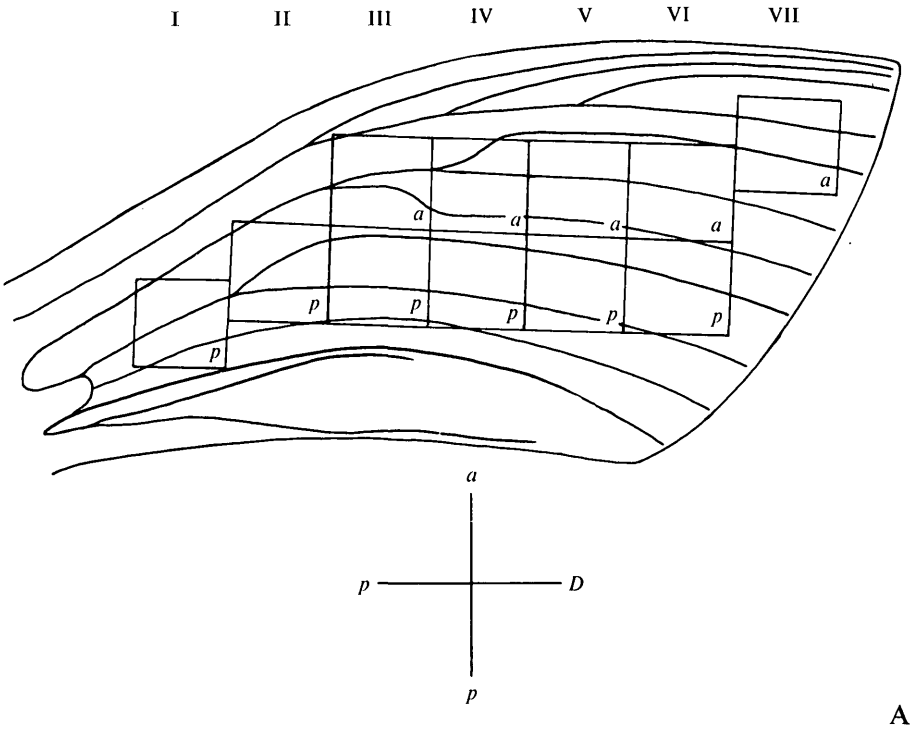
Definition of wing axes and transplantation sites

The proximo-distal axis of the pupal wing runs parallel to most of the tracheae that occupy the lacunae between the wing's two epidermal layers. In the adult wing the scales are all aligned parallel to this axis, pointing distally. The antero-posterior wing axis is perpendicular to the proximo-distal axis (Fig. 1 A).

Utilizing the wing tracheae as landmarks, eleven regions were defined in the pupal wing (Fig. 1 A): five *a* regions in the anterior half (III*a*–VII*a*) and six *p* regions in the posterior half (I*p*–VI*p*). Each pupal region corresponds to a well-defined site in the adult wing (Fig. 1 B).

Scoring of results

Grafts which have been excised, removed, and re-implanted in their original position and orientation, show relatively little alteration of the normal scale pattern (Fig. 2 A). By contrast, the cellular polarity may be distinctly different in transposed or rotated grafts; but no consistent and/or marked changes could be detected in the polarity of adjacent host scales. Three main modifications of graft polarity can be distinguished. (1) In the 'rosette' type the scales are radially arranged within the graft tissue (Fig. 2 B, 2 C). The locus from which the scales radiate out varies and depends upon the particular host-graft combination. (2) In some transpositions, this locus is at the proximal edge of the graft, and the scales assume a 'semi-rosette' configuration (Fig. 2 D). (3) All those grafts in



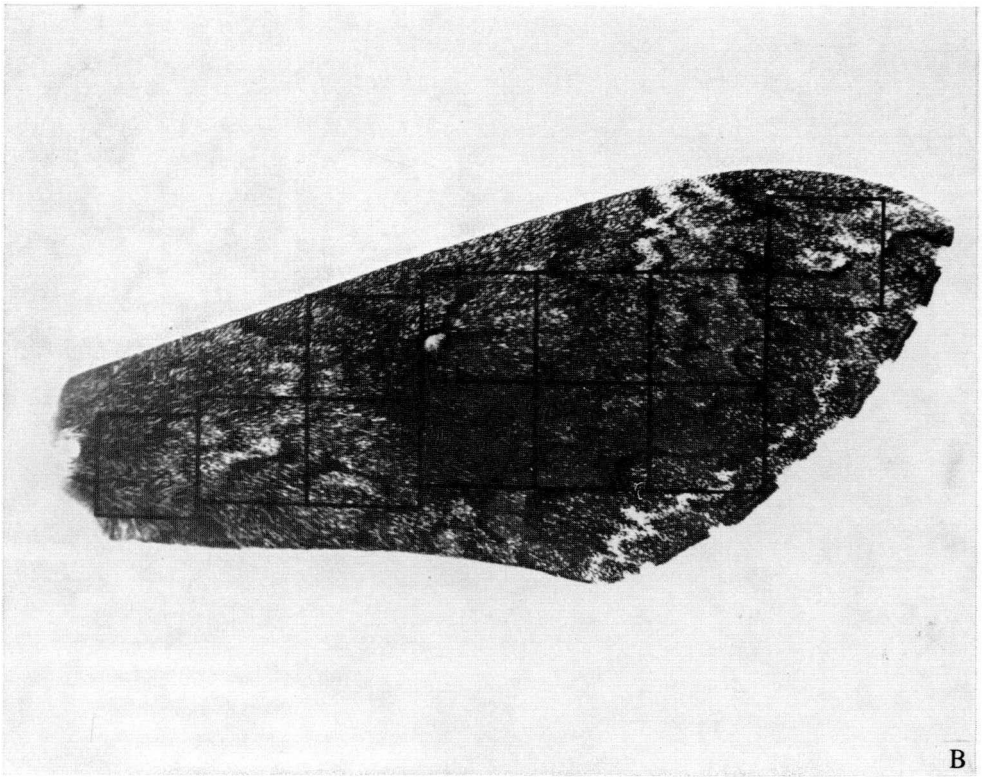
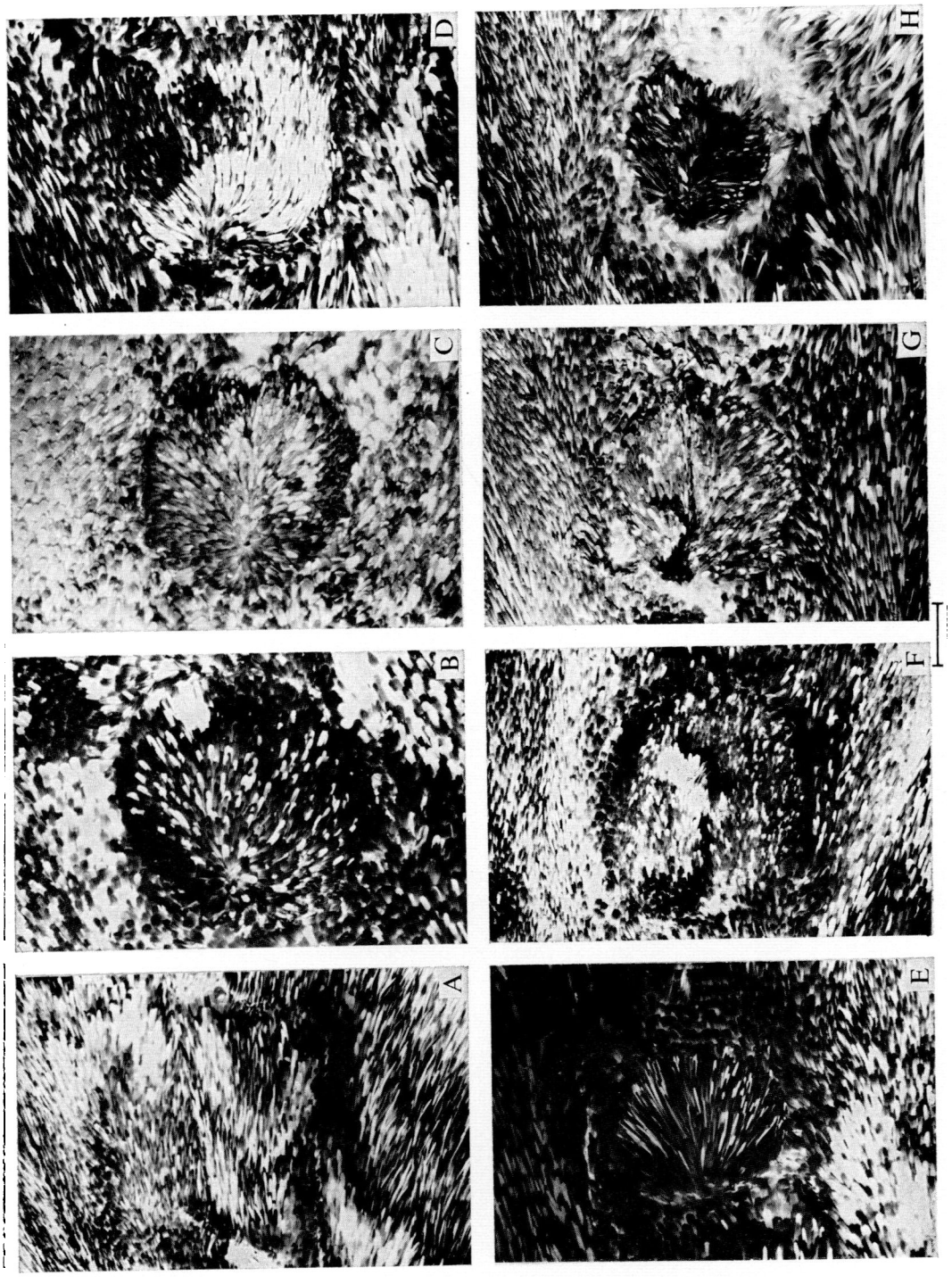


FIGURE 1

(A) Division of the pupal wing into 11 graft regions. The tracheae used as landmarks are shown as well as the two main wing axes.

(B) The regions of the adult wing corresponding to the 11 pupal wing regions.


A
B
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E
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H

which the maximum scale polarity shift is less extreme are included in this last category (Fig. 2E, 2F, 2G, 2H). Categories (2) and (3) cover a wide spectrum of intensities so that to avoid subjective interpretation, scoring was based on rosettes alone.

(A) *Independence from the polarity of the lower epidermal layer*

The wing is shaped like a flat sac, the two cellular layers being closely apposed. Might the lower cell layer of the forewing exert an influence on rosette formation in the upper layer?

In one set of experiments, epidermal region IV*a* (*P*) was left in its original position and orientation and was surrounded either partially or completely by epidermis from region VII*a* (*d*). The IV*a* region always forms a rosette scale pattern when transposed to region VII*a*. The results of seven different arrangements are diagrammed in Fig. 3. Several interesting features emerged from the results. First, the IV*a* scale cells only assumed a rosette arrangement when isolated on at least three sides (anterior, posterior and proximal) from their normal neighbors in the same (upper) layer. Second, such isolation was a necessary condition for rosette formation, but not a sufficient one; unless the IV*a* integument was also removed and then replaced, no polarity change ensued. If surrounding host tissue is treated in the same way, however, no effect is observed on host scale polarity, thus demonstrating that the absence of marked effects on host polarity in various site–source combinations cannot simply be attributed to failure to remove host integument from the underlying epidermal layer. (For discussion, see following paper.) These experiments show that only the position of the graft relative to the surrounding host epidermis in the same cell layer, and not relative to the underlying layer, is relevant for rosette formation. The independence of the two layers is also shown because the epidermal polarity and venation of the lower surface remains unaffected by overlying grafts.

FIGURE 2

Adult cuticles showing different site–source combinations. In each case the cuticles have been mounted with proximal side to the left.

(A) Graft from region I*p* which has been excised, removed, and reimplanted.

(B) Graft from region III*a* transposed to VII*a*.

(C) Graft from region III*a* transposed to V*p*.

(D) Graft from region III*a* transposed to III*p*.

(E) A I*p* graft positioned at V*a*.

(F) A VII*a* graft transposed to region IV*a*.

(G) The same site–source combination as shown in F, but note differences in graft polarity for the two figures.

(H) Graft from VI*p* transposed to region I*p*. The graft has undergone significant buckling.

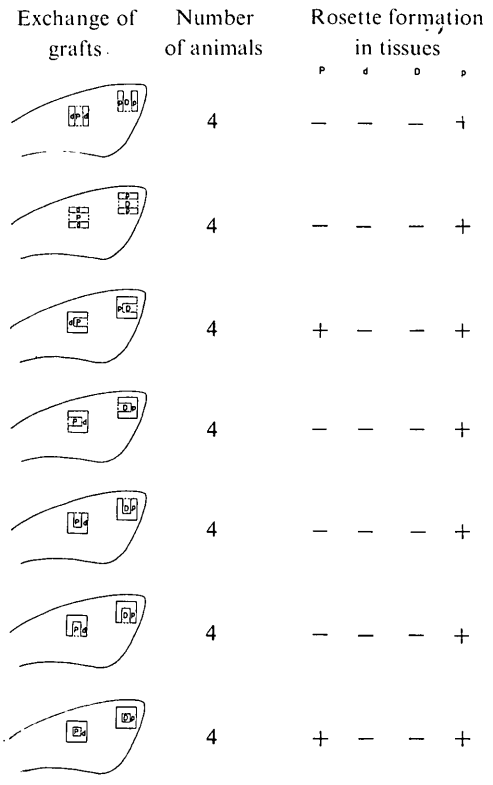


Fig. 3. The involvement of the upper and lower epidermal cell layers in the formation of rosettes. All grafts have been excised and removed before being replaced or transposed. *P* and *p* correspond to tissue from region IV*a*; *D* and *d* correspond to tissue for VII*a*. Each arrangement was tested in quadruplicate.

(B) *Evidence for a gradient*

Asymmetry of the proximo-distal wing axis

While the graft polarity for a particular host-graft combination may be of one type, the polarity for the reciprocal exchange is usually different. For example, a rosette pattern always appears whenever the proximal tissues III*a* and IV*a* are transferred to distal sites V*a*–VII*a* (Fig. 2B); in this direction of transposition, the locus of scale cell radiation shifts further distally as the grafts are displaced greater distances from their origin. In reciprocal transfers of V*a*–VII*a* grafts to either III*a* or IV*a*, the graft cells never assume the rosette pattern (Fig. 2F). Thus the interaction between host and graft is both asymmetric and graded; and this behavior could be a manifestation of an axial gradient(s). The results of several experiments which are discussed below further substantiate this interpretation.

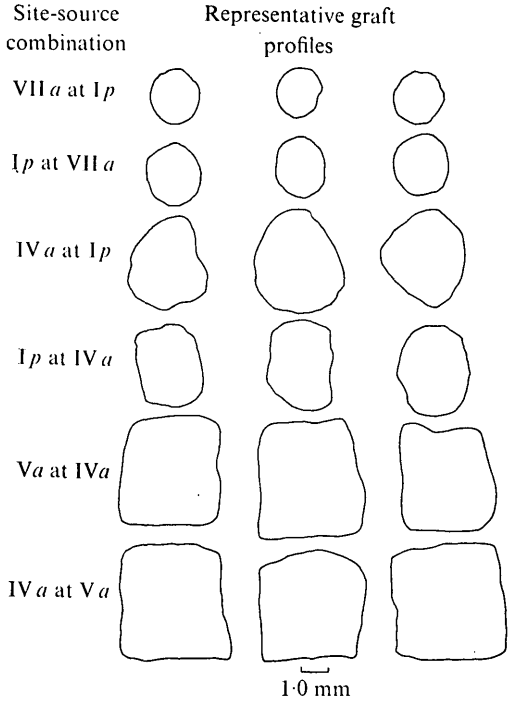


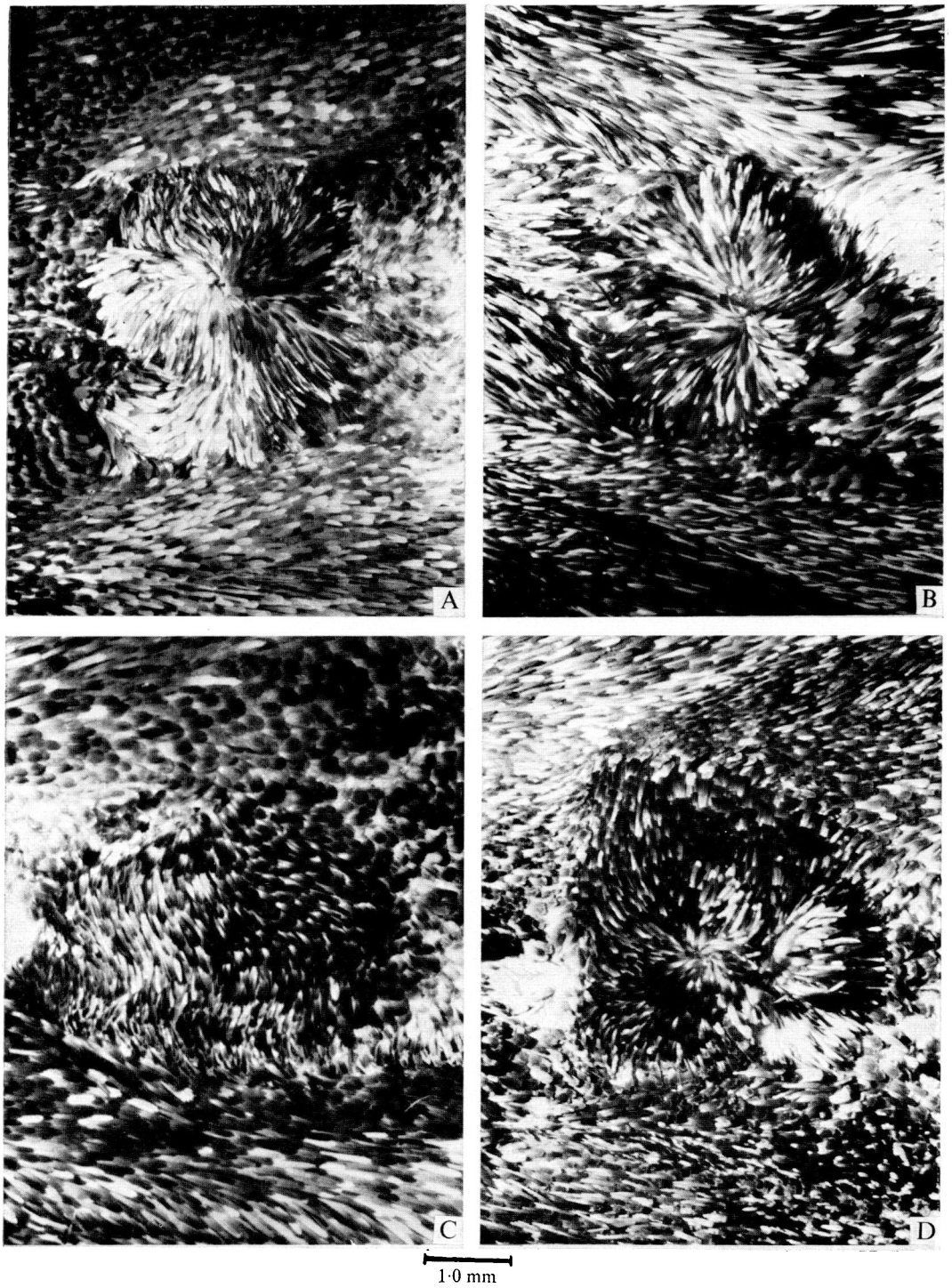
Fig. 4. Profiles of representative grafts demonstrating the relationship between configuration and distance of transposition along the proximo-distal axis. The initial sizes and shapes of all grafts were approximately the same.

Forms assumed by transposed grafts

An examination of the morphology of the wing cuticle beneath the scale layer (see Materials and Methods) showed that graft shape and size, as well as graft polarity, is a function of transplantation source and site. The greater the distance along the *PD* axis (*in either direction*) between the source of a graft and its transposition site, the more rounded and smaller the graft becomes. Fig. 4 shows representative grafts (originally of identical size and shape) from reciprocal exchanges across three different axial distances. Changes in graft cell polarity as well as graft form resulting from transposition of tissue demonstrate the presence of the *PD* wing gradient(s).

90° *rotation of grafts*

Three regions – III*a*, IV*a*, and VII*a* – were examined in this manner; for each, ten grafts were rotated clockwise and ten counterclockwise. Typical polarity patterns are shown in Fig. 5C and 5D. An S-shaped scale pattern arises in all three regions following clockwise rotation (Fig. 5C). A mirror-image S-shaped pattern was observed in VII*a* grafts rotated counterclockwise. By contrast, when rotated 90° counterclockwise, both III*a* and IV*a* grafts formed a more extreme


A
B
C
D
1·0 mm

type of pattern shown in Fig. 5D. The existence of a *PD* gradient is also implied by this 'handedness' of grafts rotated 90°.

(C) *Transposition results and the form of the gradient(s)*

The non-equivalent responses of transposed proximal and distal grafts were investigated extensively, in the hope of defining more precisely the shape of the wing gradient(s). Grafts from each of the 11 wing regions were transposed to each of the other ten sites. For each of the 121 combinations, at least ten animals were used. The slight variability in results for certain combinations, and the difficulties encountered in scoring some grafts argued against the significance of small differences in scored values. The scores were summarized into three categories, as follows.

Score	Fraction of total grafts showing rosettes
0	0·0–0·1
+	0·2–0·5
+ +	0·6–1·0

It should be noted that this system evaluates only the extreme, rosette response; partial polarity changes are classed as null responses. Most combinations which score 0 nevertheless manifest distinct shifts in polarity (Fig. 2D); presumably these are generated by the same factors responsible for rosette formation. Although a few exceptions occur, Table 1 clearly demonstrates the asymmetric behavior of reciprocal proximal–distal exchanges.

The nonlinear nature of the *PD* gradient in the anterior region is evidenced by the fact that some *a* grafts (e.g. III*a*, IV*a*) form rosettes when moved to any more distal *a* location, whereas others (e.g. V*a*) form rosettes only after being distally transposed a distance greater than the width of a graft region. The transpositions, like the rotation experiments, suggest that the *PD* gradient in the anterior region is steepest in the proximal regions, III*a* and IV*a*. When the rotation and transposition results are considered together, the gradient slope is judged to be intermediate in region V*a*, and relatively flat (but not zero) in regions VI*a* and VII*a*.

The shape of the *PD* gradient in the posterior region cannot be discerned from the information in Table 1; but the gradient slopes in the anterior and posterior

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FIGURE 5

Adult cuticles showing different graft-host combinations. In each case the cuticles have been mounted with the host's proximal side to the left.

(A, B) Transposition and 180° rotation of grafts.

(A) I*p* at V.

(B) III*p* at I*p*.

(C, D) 90° rotation of grafts.

(C) Clockwise rotation of IV*a*.

(D) Counterclockwise rotation of IV*a*.

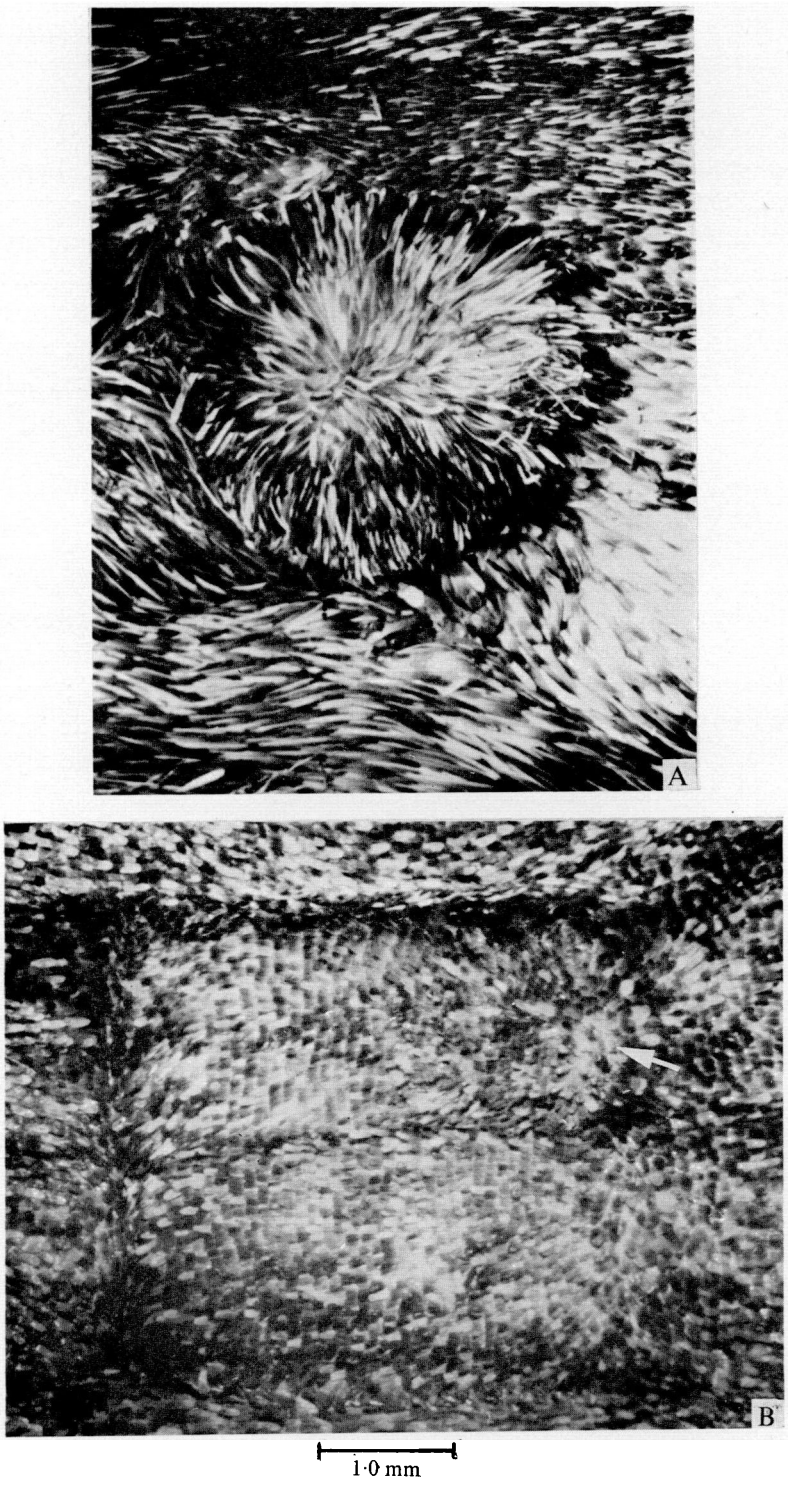
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Table 1. *Formation of rosettes following transposition, for various site–source combinations**

	Site										
Source	III*a*	IV*a*	V*a*	VI*a*	VII*a*	I*p*	II*p*	III*p*	IV*p*	V*p*	VI*p*
III*a*	0	++	++	++	++	+	++	++	++	++	++
IV*a*	++	0	++	++	++	+	+	+	+	++	++
V*a*	0	0	0	0	+	0	0	0	0	0	0
VI*a*	0	0	0	0	0	0	0	0	0	0	0
VII*a*	0	0	0	0	0	0	0	0	0	0	0
I*p*	+	++	++	++	++	0	0	0	0	0	++
II*p*	+	+	0	+	++	0	0	0	0	0	+
III*p*	0	0	0	0	+	0	0	0	0	0	0
IV*p*	0	0	0	0	0	0	0	0	0	0	0
V*p*	0	0	0	0	0	0	0	0	0	0	0
VI*p*	0	0	0	0	0	0	0	0	0	0	0

* For explanation, see text.

regions are evidently not the same, since I*p* is the only posterior graft that scores a ++ in transpositions between *p* regions and then only after being transposed a distance equal to the widths of five graft regions.

Transpositions only between *a* regions or only between *p* regions clearly point to the existence of a gradient along the *PD* axis. The results of transpositions between anterior and posterior regions are more difficult to interpret. Certain exchanges (i.e. among I*p*, IV*p*, IV*a*, V*p*) exhibit a non-transitive relationship that cannot be immediately explained in terms of a single wing gradient.

Another unexplained observation is that two proximal sources (III*a* and IV*a*) form rosettes when transposed either proximally or distally.

(D) 180° *rotation of grafts*

Squares from each of the eleven pupal wing regions were rotated 180°. In all cases, pronounced polarity changes resulted. The scale configurations formed by all rotated proximal grafts (I–V) were definitive rosettes (Fig. 6A), while rotated distal grafts (VI, VII) (Fig. 6B) only showed asymmetry, the originally proximal graft cells being reoriented more than the distal cells. In all cases,

FIGURE 6

Adult cuticles showing different graft-host combinations. In each case the cuticles have been mounted with the host's proximal side to the left. These illustrate examples of reorientations that were done at least in duplicate for each wing region; in some cases, more than ten animals were used for a particular rotation.

(A) 180° rotation of I*p*.

(B) 180° rotation of VI*a*. Compare the shape of this graft with the more rounded shape of the above graft. Arrow points to proximal edge of graft and a small rosette.

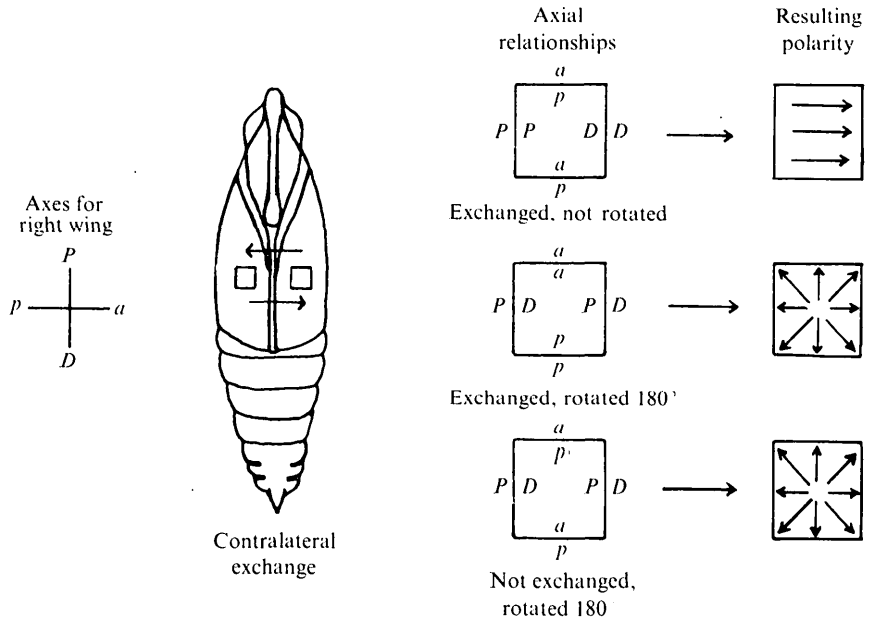

Fig. 7. Contralateral exchanges of epidermal grafts.

the polarity of a given graft was affected to a greater extent by 180° rotation than by transposition to any adjacent wing region. The more pronounced effect of rotation in proximal (Fig. 6A) as compared to distal (Fig. 6B) regions suggests that the gradient is steeper proximally.

Along which axis (or axes) of 180° rotated grafts is variation in a cell parameter sufficient for rosette formation? To answer this question we used contralateral exchanges between left and right wings, with or without rotation.

Three regions (II*p*, III*a*, IV*a*) were examined by this procedure, and consistent results were obtained. As Fig. 7 indicates, contralateral exchange alone did not lead to polarity change; in this case the *ap* axis of the graft was reversed relative to that of the host. On the other hand, contralateral exchange and concurrent 180° rotation led to alterations indistinguishable from those caused by rotation *in situ*; in both types of experiment, the *PD* axis was misaligned. The *ap* axis was reversed for rotations *in situ*, but not in the exchange-rotation experiments. One can therefore conclude that rosettes form (at least in 180° rotated grafts) because of differences along the *PD* rather than along the *ap* axis.

(E) *The role of nerves*

If the pupal integument was excised at a certain time after pupation (see Materials and Methods), the wing epidermal cells could be separated from nerve cells.

Ten IV*a* grafts consisting only of epidermal cells, and ten IV*a* grafts with both

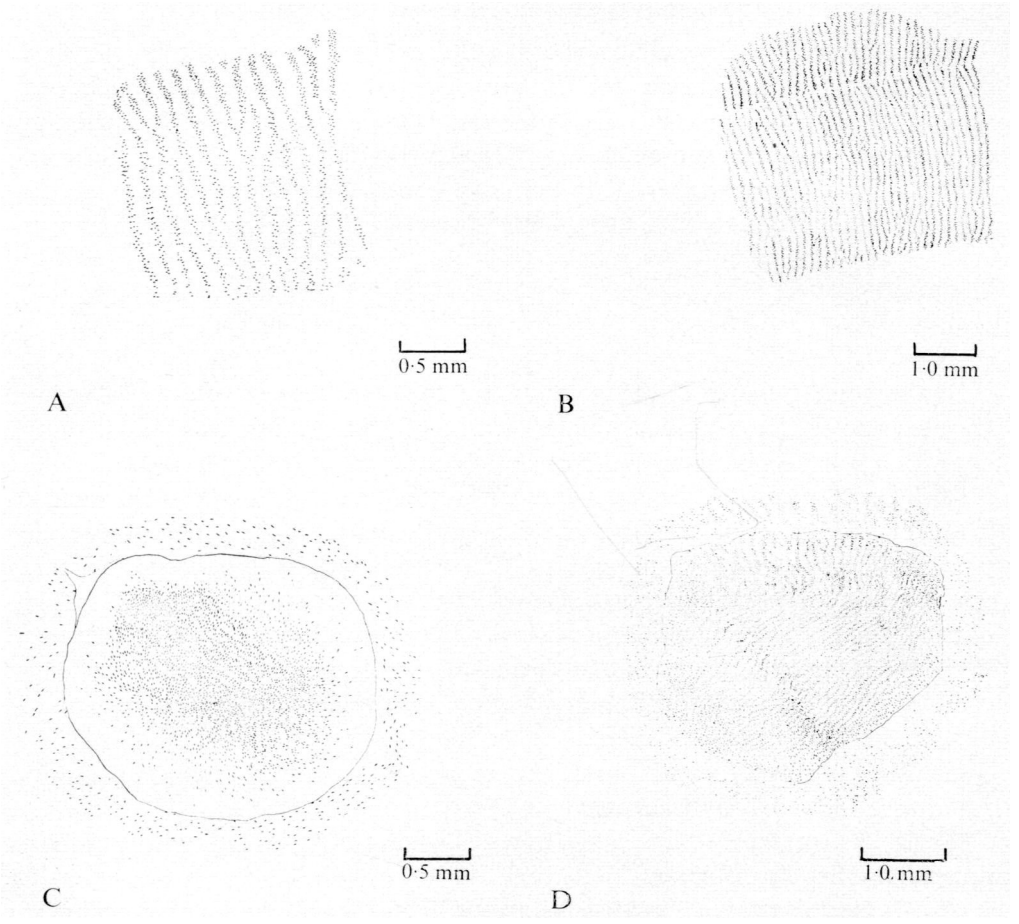

Fig. 8. Drawings showing scale cell distributions in different areas of the wing. Lines represent graft boundaries, and each dot represents the point of attachment for a scale cell. (A) Scale cell distribution in an undisturbed III*a* region. (C) Scale cell distribution for a III*a* graft transposed to VII*a*. (B) Scale cell distribution in an undisturbed VII*a* region. (D) Scale cell distribution for a VII*a* graft transposed to III*a*.

epidermal and nerve cells present, were rotated 180° without transposition. The effects on the adult scale pattern were indistinguishable in both groups, all grafts exhibiting radially symmetrical rosettes. Likewise, identical polarity patterns arose, following certain types of transpositions, regardless of whether the nerve cells were attached to the transplants or not. The nerve cells apparently do not directly participate in the specification of wing polarity.

(F) *The cell densities of transposed grafts*

Since scales, like other epidermal organules, exhibit a regular spacing in terms of cell diameters (Lawrence, 1973), their distribution in the adult cuticle can serve as a convenient measure of cell density. The distribution of scale sockets in two representative transposed grafts – III*a* at VII*a* and VII*a* at III*a* – are shown in Fig. 8C and 8D, respectively. In both cases, the cell density within the graft is higher than in the respective untransposed regions (Fig. 8A, 8B).

DISCUSSION

Summary of findings

By the pupal stage, both the venation and the color pattern of the adult wing have been covertly established, yet the polarity of the scales is still labile. Grafting experiments have disclosed the following features of wing epidermis.

(1) The cell polarities of the upper and lower cell layers develop independently.

(2) The direction of displacement of a graft along the *PD* axis governs the type of polarity change it will undergo. One distinctive change (rosette) appears when certain grafts are transposed distally, the distance required being a function of the source of the graft; but these same grafts (with the exception of III*a* and IV*a*), when transposed proximally, do not undergo a comparable polarity change. Thus, with respect to their effect on scale polarity, distal and proximal displacements are non-equivalent.

(3) The polarity of a given graft region is more markedly altered by simply rotating it 180° than by transposing it without rotation.

(4) Grafts rotated 180° can form rosettes when transposed either distally or proximally. See Fig. 5A and 5B.

(5) The scale patterns that arise following 90° clockwise and counterclockwise rotation are not always mirror-image patterns.

(6) Some distortions in host cell polarity appear near the graft-host interfaces, but there is no consistent change in presumptive polarity.

(7) A wing region does not assume a rosette configuration unless isolated by more distal tissue (on at least three sides) from cells of similar axial level and in the same cell layer.

(8) An experiment involving back-transplantation of transposed grafts, into their sites or origin, suggests that graft polarity remains labile until 5 days after pupation (Nardi, 1975). This is the time at which the first cuticle is secreted by the developing adult cells.

(9) The shapes and sizes of transposed grafts are correlated with the distance of *PD* axial displacement (irrespective of direction, unlike feature (2)).

(10) The cell density within a transposed graft is higher than that in the corresponding *in situ* wing region. This is true irrespective of the direction of axial displacement.

Requirements for pattern relaxation

In other systems, pattern relaxation is not continuous with time, but instead correlates with the occurrence of epidermal cell divisions; this was demonstrated first by Lawrence (1971) in the *Rhodnius* tergite, and then by Caveney (1973) in the larval sternite of *Tenebrio*. In *Tenebrio*, the larval proliferative divisions permit repolarization, whereas the pupal divisions, which are differentiative, do not.

Pattern regulation in the *Manduca* wing apparently proceeds via a route different from that followed in the *Tenebrio* sternite. At the time of pupation the wing cells have finished proliferating (Kühn, 1965, and personal observations), and only certain ones are destined to undergo two differential cell divisions. Cell proliferation does occur at the wounded margin of *Manduca* wing grafts; however, graft repolarization often extends throughout the graft tissue. In pupal *Tenebrio*, where proliferative divisions again occur at the wounded margin, only the graft edges repolarize. We conclude that in *Manduca* wing epidermis, the occurrence of proliferative cell divisions is not a prerequisite for pattern regulation. Instead, as suggested by Bohn (1974), Lawrence (1974), and Nübler-Jung (1974) for other insect systems, cell migration may be a crucial factor in the relaxation of the *Manduca* scale pattern (see Nardi & Kafatos, 1976).

Gradient models

The asymmetric behaviour of distally and proximally translocated grafts would be predicted by qualified diffusion models, such as the homeostatic model of Lawrence, Crick & Munro (1972) or the follow-up servo model of Wolpert (1971). These were developed specifically to account for results which depend on the direction of displacement in the gradient. In the terms of the homeostatic model, proximal cells would not be good homeostats when moved distally, i.e. down the gradient. Because of the homeostatic asymmetry, a graft which is moved down the gradient would lose morphogen and change its pattern to conform to the surroundings; on the other hand, a graft which is moved up the gradient would be able to maintain its morphogen concentration (e.g. by degrading the morphogen flowing into it through diffusion) and hence its original orientation.

Such models explain with equal ease the non-equivalent effects of proximal and distal translocations. With respect to the absence of marked polarity changes in the proximal host tissue surrounding a distal graft (Fig. 2F, 2G), the homeostatic model is somewhat less convincing. Normally, loss of gradient substance would be expected to occur from the host margins, leading to some local slope reversals. The absence of polarity changes in the host margin might be rationalized on the basis of the large mass of host tissue, as well as of the absolute values of the morphogen discontinuities; similar considerations might explain why some distally translocated grafts yield rosettes, while others yield less extreme polarity alterations.

Although in rigorous terms models involving diffusion cannot be eliminated for the *Manduca* wing, they do not readily explain three other features of our results. One is that grafts rotated 180° can form rosettes when transposed either distally or proximally. If most non-rotated grafts transposed proximally do not form rosettes for the reasons given above, then the results of rotation plus proximal translocation are difficult to explain with such models. Furthermore, no provisions are included in these models to account for the other two features of our results – changes in graft cell density and in graft form. The limitations of grafting experiments must be realized; but the properties of the gradient(s) which have been described cast some doubt on the viability of diffusion models for explaining the *Manduca* grafting phenomenology.

The next paper will consider a model that not only explains with facility all the experimental observations thus far described, but also predicts results of additional experiments.

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