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Polarity and gradients in lepidopteran wing epidermis

II. The differential adhesiveness model: gradient of a non-diffusible cell surface parameter

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SUMMARY

For explaining the *Manduca* wing gradient (Nardi & Kafatos, 1976) a model which postulates a proximo-distal gradient in cellular adhesiveness is considered. The model is based on Steinberg's (1963) differential adhesiveness hypothesis. Rosette formation in certain transposed and/or reoriented grafts can be adequately explained by this model. Several predictions, formulated by using the concept of surface free energy as a thermodynamic measure of adhesiveness, have been tested and proven correct. (1) Transposed grafts tend to assume circular forms, which are configurations of minimum free energy. (2) Because of the pressure difference expected across the interface of two cell populations with different surface free energies, cell densities increase in both distally and proximally transposed grafts. As a corollary to this rule, final size of a graft is a function of its distance from the original position. (3) Histological sections of host-graft boundaries suggest minimal cell contact at the interface. In proximal grafts placed in distal regions, cell density is far lower near the host-graft interface, as compared to the high interior density; the peripheries of distal grafts do not show this effect. (4) Juxtaposition of three different wing regions in all possible arrangements yields the expected two-dimensional configurations. (5) Differences in adhesiveness can be demonstrated by allowing two different wing grafts to interact in an essentially neutral environment (i.e. at a leg or antenna site). As the distance between two given graft regions increases, the extent of their final contact decreases.

When applied to other insect systems, the model not only offers an alternative interpretation for results currently explained by diffusible substance models, but also accounts for certain features that were unexplained by other models.

INTRODUCTION

According to the fundamental conceptual dichotomy of mosaic *v.* regulative development, most current insect pattern formation models, except for Locke's (1959, 1967), are regulative. They assume that intercellular communication operates over distances of several cell diameters, through a diffusible morphogen,

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and that the individual cells are not yet developmentally autonomous at the time of the experiment. However, in the wing of *Manduca* at the time of grafting the cells are autonomous in terms of the color, pattern and shape of the scales which they will generate; wing grafts construct autonomously their venation pattern and cuticle of characteristic staining properties (Nardi, 1975; Nardi & Kafatos, 1976). To explain the observed experimental perturbations in graft morphology and scale polarity, it may prove profitable to consider a pattern formation model which does not invoke diffusible morphogens, and which considers the individual cells autonomous at the time of the experiment. Such would be a model postulating that the morphological changes result from the repositioning of cells – a repositioning which is brought about because of differential adhesiveness inherent in the cells themselves.

Developmental autonomy in insect epidermal cells

As early as the blastoderm stage, the cells of the *Drosophila* embryo have been committed to either anterior or posterior structures (Chan & Gehring, 1971). Finer commitments are probably established somewhat later (Gehring & Nöthiger, 1973). Within individual imaginal discs, the available evidence implies, although it does not prove rigorously, that the developmental competence of cells becomes progressively restricted as the cells continue to divide during larval life. Marked clones derived from somatic crossing-over reveal the progressive emergence of cell lineage restrictions within a disc (Garcia-Bellido, 1972). By the third larval instar in *Drosophila*, fairly detailed maps of developmental commitment can be constructed for various discs (Gehring & Nöthiger, 1973). It appears that by that time individual cells can undergo autonomous development, even if their state of commitment is not irreversible (Garcia-Bellido, 1972). Cell autonomy is manifested in terms of cell recognition. Following reaggregation of disaggregated disc cells from different genetically marked discs and/or regions within a given disc, integrated mosaics usually fail to form; instead the cells segregate according to their region of origin (Garcia-Bellido, 1966, 1972).

The mechanism for segregation and positioning of cells

Numerous mechanisms can be offered to explain segregation of different cell types. Only three mechanisms have been offered, however, which can account for proper positioning of cells as well as for their segregation (Trinkhaus, 1969). One is the chemotactic hypothesis. Another is the timing hypothesis of Curtis. The third is the differential adhesiveness hypothesis. The indirect evidence which exists overwhelmingly favors the third alternative.

According to the differential adhesiveness model, the repatterning process which occurs during reaggregation of dissociated cells is the result of the cells' motility and of quantitative differences in inherent cellular adhesiveness (Steinberg, 1963, 1964). The hypothesis assumes that n-segregating cell populations can be considered analogous to an n-phase system of immiscible liquids. The two

systems share two very important properties. The units of the cell population, like the molecular units of the liquids, are mobile yet limited in their movements by the existence of attractive forces. All systems with these properties are subject to the thermodynamic principles of liquids: the configuration assumed by the units will be that in which the free energy of the system is minimal, and the work of adhesion and/or cohesion is maximal. For a homogeneous cell population a spherical shape is expected after sufficient time in liquid medium. The forms to be expected for mixtures of two cell types can vary, depending upon the relative differences in cellular adhesiveness. It should be noted that pattern in this model is based on adhesiveness, i.e. an autonomous cellular property.

Application of the differential adhesiveness hypothesis to insect epidermal cells

The differential cellular adhesiveness hypothesis has not been applied as yet to insects, perhaps because of the technical difficulties encountered in *in vitro* culturing of insect cells. While the final segregation pattern obtained after *in vivo* culturing may convey information regarding configurational equilibrium, the segregation process itself is not amenable to observation *in vivo*, and thus certain predictions of the hypothesis cannot be tested. Conceptually, difficulties are also encountered in dealing with an essentially two-dimensional array of cells, such as the insect epidermis, which lacks the apparent isotropic characteristics of many vertebrate tissues. In applying Steinberg's hypothesis to insect systems, one must remain aware of this major difference. Nevertheless, sorting out does occur in two-dimensional vertebrate aggregates as well as in three-dimensional ones (Garrod & Steinberg, 1973). As already indicated, reassociated insect cells do sort out according to cell type in *in vivo* culture.

Two features of the lepidopteran wing encourage application of the differential adhesiveness hypothesis. One is the unmistakable mosaic nature of the pupal wing, in terms of most aspects of cuticular differentiation (see above). The second feature is the rounded form which transposed grafts tend to assume, regardless of their initial shape. In the present paper, the differential adhesiveness hypothesis will be tested against the results of grafting experiments in the wing – both those presented in the preceding paper (Nardi & Kafatos, 1976) and those obtained as a specific test of the adhesiveness model. It should be noted that the existence of a gradient of an autonomous cell property at a particular developmental stage does not in itself establish whether the gradient was originally generated by a mosaic or a regulative mechanism.

MATERIALS AND METHODS

The rearing, selection, and treatment of experimental animals as well as the method for examining the cuticular structure of the adult moth wing has been previously described (Nardi & Kafatos, 1976). A planimeter was used for measuring graft perimeters.

For histological study of developing adult epidermal cells bordering the interfaces, wings were fixed either in Carnoy's fluid for whole mounts, or in 3% glutaraldehyde in cacodylate-sucrose buffer, pH 7·6 (Smith, Telfer & Neville, 1971) for araldite embedding and sectioning with an ultra-microtome. Whole mounts were stained with hematoxylin and sections with 1% toluidine blue in borax solution.

RESULTS

The model

The model which is presented and tested in this paper proposes that at least one proximo-distal gradient of a non-diffusible parameter, cellular adhesiveness, exists within the wing epidermis. As inferred from the transplantation data, the gradient is non-linear, being steeper in proximal wing regions (Nardi & Kafatos, 1976); and for reasons that will become evident in the following section, it is postulated that the high point of the gradient is proximal. For cells which differ in 'isotypic' adhesive strength (i.e. work of adhesion for each with its own type), the 'heterotypic' adhesiveness is postulated to be lower than the average of the isotypic values; and, for a given average, the heterotypic value is postulated to decrease as the cells become more disparate (i.e. as the difference between the isotypic values increases). For convenience, the two postulates of the preceding sentence will be referred to as the 'rule of adhesive averages'. Without introducing any additional gradient properties, the findings of the first paper in this series (Nardi & Kafatos, 1976) can be interpreted in light of this hypothesis.

The unexpected requirement for breaking a graft's attachment to the underlying cell layer before pattern reorganization can occur (Nardi & Kafatos, 1976) can now be rationalized: breaking the attachments may remove a barrier to cell motility, a barrier which can prevent the rearrangement of cells according to their differential adhesiveness. It should be noted that additional impediments may hinder motility – e.g. attachment of cells to their own basement membrane, or to the pupal and developing adult cuticle. Indeed, there are indications that pattern relaxation can only occur during an early brief portion of the pupa-to-adult metamorphosis (Nardi, 1975), when the cells are free of these attachments. Given these impediments, there is no *a priori* reason to expect that the patterns attained by wing grafts represent configurational equilibrium; the equilibrium configurations cannot even be predicted with certainty, since the exact shape of the postulated adhesiveness gradient is unknown. Nevertheless, the observed patterns should be configurations of reduced surface free energy, which are assumed by the interacting cell populations as they progress towards configurational equilibrium.

Shapes assumed by transposed grafts

By analogy with liquids, the attractive forces between neighboring cells in the wing epidermis can be considered to correspond to negative free energy. When an epidermal square is removed from the wing, the peripheral cells are no longer uniformly exposed to these attractive forces, and thus suffer an increase in potential energy. The cells of the explant will 'attempt' to minimize this surface energy, and hence the exposed peripheral area, by formation of a hollow sphere. In grafts removed but replaced in their original positions, configurational changes (excluding wounding) should not occur. Transposed and/or reoriented grafts, on the other hand, establish contact with new neighbors. If the adhesive properties (as measured by the work of adhesion, W) of the newly adjacent cell populations differ, at equilibrium either host and graft will intermix or the graft will adopt one of three possible rounded forms: (*a*) a rounded planar shape, (*b*) a rounded dome shape, or (*c*) the form of an isolated sphere. The graft will tend toward forms (*a*) or (*b*) if

$$W_{GH} < \frac{W_G + W_H}{2} \tag{1}$$

as predicted from the rule of adhesive averages, and if any one of the following four sets of adhesive relationships holds for graft (G) and host (H) cells.

$$W_G > W_{GH} \geqslant W_H, \tag{2}$$

$$W_H > W_{GH} \geqslant W_G, \tag{3}$$

$$W_G > W_H > W_{GH}, \quad \text{where } W_H \approx W_{GH}, \tag{4}$$

$$W_H > W_G > W_{GH}, \quad \text{where } W_G \approx W_{GH}. \tag{5}$$

The rounded planar graft forms are in fact the ones which are most commonly observed in wing transplants. If the mobility barriers are substantial, the grafts will approximate rather than actually assume the predicted shapes. If cell associations are reversible, the importance of a given barrier will depend on the free energy differences (ΔF) between initial and final adhesive states. Without forgetting the distinction between kinetics and thermodynamics it can be seen that if the mobility barrier for the average cell is constant, an absolutely larger, negative ΔF value will lead to a closer approximation to equilibrium – i.e. to a more rounded form.

When confluence is established between host and graft, heterotypic cell associations ($G \cdot H$) are formed at the graft periphery. As the square graft becomes rounded, some of these heterotypic associations are converted to isotypic ones ($H \cdot H$ and $G \cdot G$). The equation is

$$2\, G \cdot H \rightleftarrows H \cdot H + G \cdot G + \Delta F, \tag{6}$$

where

$$\Delta F \propto 2W_{GH} - (W_G + W_H). \tag{7}$$

As the absolute value of the (negative) ΔF increases, the number of isotypic cell

contacts increases at the expense of heterotypic ones. This can only be accomplished by circularization and contraction of the host-graft interface. The graft becomes progressively more round and smaller and ultimately invaginates or evaginates as a rounded dome.

According to (7), the value of ΔF and hence the final shape and size of the graft should depend on the nature of the cells involved, rather than on the direction of their exchange. In contrast to rosette formation, which is observed only when graft transposition is in the distal direction, graft rounding and graft contraction should occur both in distal and in proximal transpositions. Moreover, according to (7) and the rule of adhesive averages the absolute value of ΔF will increase with increased disparity in the adhesiveness of graft and host, i.e. with the distance of transposition. Dome formation has been observed in intertissue exchanges (Nardi, 1975) and, very infrequently, in exchanges involving very widely separated wing regions (I*p* and VI*p*; I*p* and VII*a*). Planar contraction of grafts is the rule in most wing grafting experiments.

The preceding three paragraphs lead to a consistent set of predictions. The smaller a graft becomes upon transposition, the more rounded its periphery should be. If a particular exchange leads to a small, rounded final graft shape, the reciprocal exchange should also do the same. Also, the greater the distance along the gradient (in either direction) between the source of a graft and its transposition site, the more rounded and smaller the graft should become. These predictions are borne out by the graft outlines depicted in Fig. 4 of the previous paper.

The observed decrease in graft radius can be described as the consequence of a pressure difference between graft and host. Unfortunately, this pressure difference cannot be inferred simply from the initial pupal and final adult graft measurements (see Appendix I), because the expansion of the moth wing at the time of eclosion is differential, being greater in distal as compared to proximal regions. Nevertheless, this expansion makes even more meaningful the relative decrease in final graft size which is observed when a proximal graft is translocated to progressively more distal sites (cf. results for I*p* grafts in Fig. 4 of the previous paper). In this case, differential expansion reduces the magnitude of the expected effects of distance; moreover, the size of the graft cannot be limited by the extent of host expansion, since the expansion is even more limited at the original graft source.

Table 1 presents size measurements for I*p* grafts transposed to three progressively more distal sites. This experiment took advantage of the fact that the adult cuticle of I*p* is very dark, and can be distinguished readily from the cuticles of all regions distal to III*a*, after staining with chlorazol black E. The initial sizes and shapes of all I*p* grafts were approximately the same; in each case the pupal cuticle was saved and the final graft perimeter in the adult was standardized against the initial pupal perimeter. The expansion upon eclosion was reflected in the *increased* perimeter of grafts implanted in region IV*a*. However, as the grafts

Table 1. *Change in perimeters of Ip grafts as a function of transplantation site*

Site	Number of grafts measured	Average percentage change in graft perimeter	Range of values for percentage change
IV*a*	10	+18·9	+5·0 → +35·4
Distal half of IV*a*+ proximal half of V*a*	10	+6·5	−8·7 → +27·8
VII*a*	10	−18·6	−36·1 → −17·2

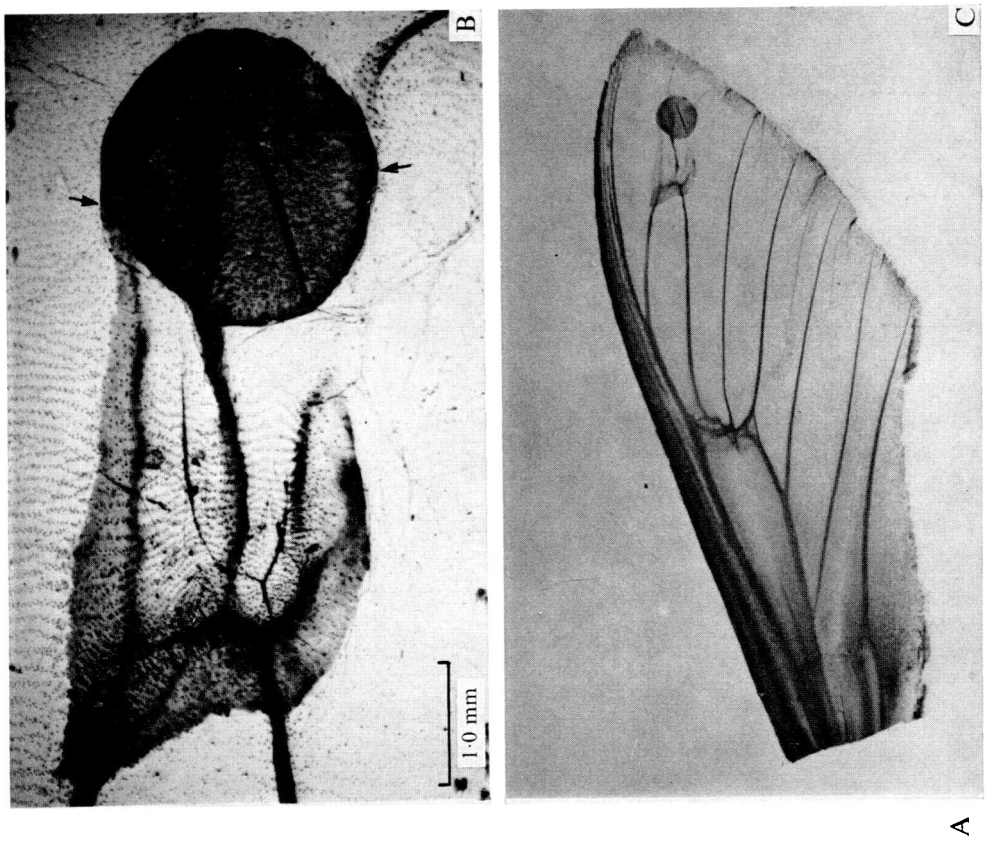
were transplanted further distally, the perimeter change became less positive and finally negative, as predicted. Consistent results were obtained in the reciprocal exchanges, but the data are not presented, since the differential wing expansion magnified the differences in this case.

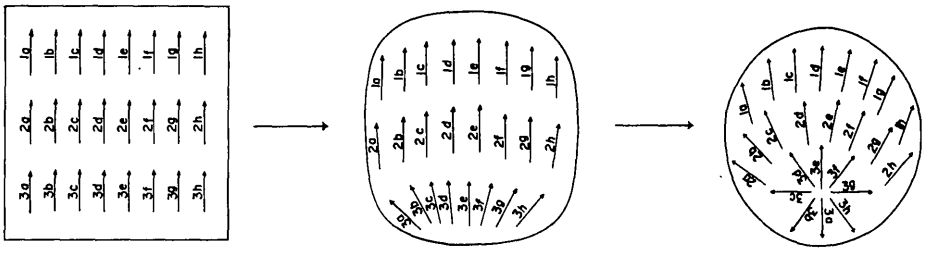
Rosette arrangement of scales: interpretation of the findings

Transposition of proximal grafts to distal positions

It is important to realize that a gradient exists within a given graft. The cells along the distal edge are postulated to be the least adhesive cells of that graft. Cell populations repositioned among less adhesive cells should tend to minimize their contacts with the new neighbors; this can be achieved by active rounding of the graft, as already discussed. Maximization of the work of adhesion can also be accomplished through exposure of the least adhesive cells of the graft to the surrounding host tissue. Relocation and reorientation of the cells in the distal graft margin may occur, so that these cells come to occupy more of the graft periphery, while the most proximal cells sink toward the graft interior. This process is diagrammed in Fig. 1 A. If the cells move as a more or less confluent layer, changes in cell orientation will result, and hence changes in scale polarity, without any alteration in the adhesiveness of individual cells. Partial progression along the path diagrammed in Fig. 1 A will yield a semi-rosette; further progression will yield a distally eccentric rosette, and finally a rosette with radial symmetry.

The postulated cell movements could not be easily documented within a single graft, although repositioning of certain pattern elements in rosette-forming grafts was highly suggestive of such intragraft migration of cells. An analogue was therefore constructed (Fig. 1 B, 1 C) by juxtaposing square I*p* and IV*a* pupal grafts in a VII*a* site. The orientation did not matter. In every experiment the most adhesive graft cells (I*p*) formed a tight round cluster, whereas the less adhesive graft cells (IV*a*) extended around this cluster, enveloping and separating it partially from the even less adhesive host cells (VII*a*). The analogy to the events postulated for a single graft is clear. In a single distally transposed graft, the most adhesive cells (proximal margin) are thought to form a tight


B
1·0 mm
C
A



cluster which moves toward the graft interior, while the less adhesive graft cells (distal margin) spread around this cluster, separating it more or less completely from the surrounding host cells, which are least adhesive (Fig. 1A).

It should be noted that the type of reorganization which a particular type of graft will exhibit cannot be predicted rigorously, in the absence of detailed information about the migratory abilities and adhesive values of the wing cells, as well as about the time during which repatterning can occur. The evidence of Fig. 1B indicates that envelopment by the least adhesive graft cells can occur; if it proceeds far enough, under certain conditions, a rosette can be generated, as in Fig. 1A.

Transposition of distal grafts to proximal positions

Grafts transposed proximally, according to the model, are encircled by more adhesive cells. The host cells in this case will tend to minimize their contacts with the graft cells, by actively converting the square interface to a rounded form. Because of this change in overall form, the graft cells will undergo some polarity changes (see Nardi & Kafatos, 1976). However, if the adhesive relationships are

$$W_H > W_{GH} \geqslant W_G \tag{8}$$

the intragraft cell movements depicted in Fig. 1A will not occur, since the cells of the proximal graft margin will form energetically favorable contacts if they remain exposed to the host; the converse of Fig. 1A, i.e. the spreading of proximal cells around the least adhesive (distal margin) cells, would require their forfeiting of some contacts with the most adhesive host cells and may be hindered kinetically as well. It is not intuitively obvious what the equilibrium configuration should be, but absence of a rosette is not unexpected. However, if the adhesive relationships are

$$W_H \geqslant W_G > W_{GH}, \quad \text{where } W_G \approx W_{GH} \tag{9}$$

the spreading of proximal margin graft cells could proceed as far as rosette formation. Non-rosette polarity changes are observed in most proximally transplanted grafts, and rosettes in some III*a* or IV*a* proximal transpositions

FIGURE 1

(A) A schematic interpretation of cell movements and repolarization during rosette formation. The polarity of graft cells is represented by the arrows. The proximo-distal axial position is designated by numbers (3 being the most proximal graft level) and the antero-posterior position by letters *a–h*.

(B, C) The cuticular pattern in the adult wing after removal of scales. The three juxtaposed wing regions have been distinguished from one another by Chlorazol black E staining. Arrows mark the points where the three cell populations meet.

(B) The darkest graft I*p* is partially engulfed by the cells of the IV*a* graft when both are surrounded by VII*a* tissue.

(C) Position of the grafts in the wing.

(Nardi & Kafatos, 1976). We may surmise that relationships (8) and (9) are applicable, respectively.

180° rotations

Following graft rotation, the adhesiveness gradient within the graft is oriented in a direction opposite to that of the host gradient. In addition, one half of the graft is encompassed on three sides by less adhesive cells. Due to this combination of factors lowering the adhesive strength of the heterotypic cell associations, it is not unexpected that the graft response will be more pronounced than when the same graft is distally transposed without concurrent rotation. As the system attempts to return to configurational equilibrium, a rosette could be easily generated – for example, in a manner analogous to that of Fig. 1A, by migration of the least adhesive cells of the original distal margin around the rest of the graft with concomitant sinking in of the most adhesive cells of the original proximal margin. The pattern reorganization is more intense in rotations of proximal as compared to distal grafts. This would be predicted by the rule of adhesive averages, since the gradient is steepest, and hence the destabilizing adhesive disparities are greatest, in the proximal wing regions.

180° rotations, accompanied by transpositions

As in the *in situ* rotations, misalignment of the adhesiveness gradient in host and graft could generate a rosette, irrespective of the direction of transposition. In distally transposed grafts, an additional condition favoring rosette formation is the presence of less adhesive cells in the surrounding host tissue. The latter condition alone is sufficient to cause rosette formation in some distally transposed non-rotated grafts; when gradient misalignment is superimposed by rotation of the graft, the rosette should accentuate. This is in fact what is observed. Compare Figs. 2E and 5A in previous paper.

90° rotations

In general, 90° rotations should not generate as intense adhesive disparities as 180° rotations do. In the three regions examined (Nardi & Kafatos, 1976), rosettes form only for counterclockwise rotations of the proximal regions, III*a* and IV*a*. This may be explainable by the gradient shape: the difference in gradient level is postulated to be greater between the proximal and posterior parts of these wing regions than between the proximal and anterior parts (Nardi & Kafatos, 1976).

Rosette formation in untransposed, unrotated tissue surrounded by more distal wing epidermis: the failure to observe pronounced and consistent changes in host cell polarity

It has been reported (Nardi & Kafatos, 1976) that an untransposed, unrotated IV*a* region can give a rosette under certain conditions. The requirements are that the tissue must be (1) removed from its attachment to the underlying wing layer before being reimplanted in the same position and orientation, and (2) bordered on at least three sides by more distal tissue.

As already discussed, the first requirement can be explained by assuming that attachment to the underlayer represents a major barrier to the motility of wing cells. The second requirement can also be understood readily: unless adhesive disparities exist on at least three sides, the enveloping cell movements which are needed for rosette formation cannot take place.

Of the above two requirements, only the second cannot be met by the host tissue surrounding any graft. It is not surprising, then, that pronounced and consistent polarity changes are not observed in the host tissue.

Changes in graft cell density

As documented in Nardi & Kafatos (1976) (Fig. 4) a relative decrease in graft radius occurs when the graft is translocated to a distant site, in either direction. According to the equation derived in Appendix I, this decrease in radius can be considered the result of a pressure difference across the interface. When the graft is more adhesive than the host, there will be a strong attraction of the graft cells toward the graft center; in this case the graft will contract actively. Due to epidermal abhorrence of a free edge, the graft will always retain contact with host cells unless the edges of the graft actually fuse to form a hollow spherical vesicle (Wigglesworth, 1972). When the host is more adhesive than the graft, the host cells will decrease their lateral contacts with the graft and in so doing will force contraction of the graft. In either case, the cell density is expected to increase within the body of the graft, as in fact it does (Fig. 8, Nardi & Kafatos, 1976). However, a difference can be expected at the interface. In the first case, the peripheral graft cells will minimize contacts with the host cells in the face of the overall centripetal tendency within the graft. In the latter case, this behavior should not occur within the graft; by contrast, the host cells near the interface should be stretched out, as they attempt to minimize interfacial contact by constricting the graft. The expected density non-uniformities are in fact observed near the interface (see Fig. 8 of preceding paper): on the graft side when the graft is more adhesive (Fig. 8C), and on the host side when the host is more adhesive (Fig. 8D). One cannot argue that the non-uniform distribution of scale cells within distally transposed grafts is due simply to wounding, since this same distribution pattern is not observed in those grafts transposed in the opposite direction.

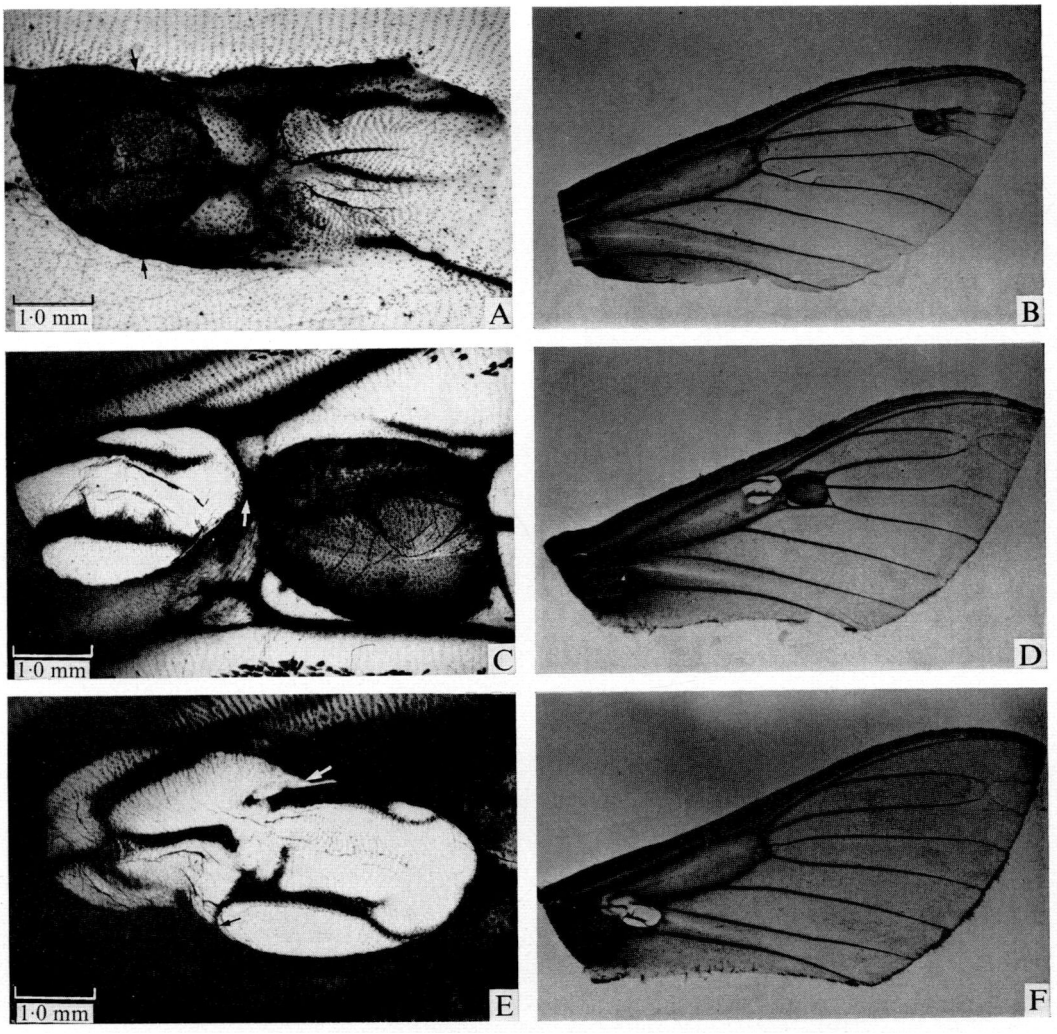


FIGURE 2

The cuticular pattern in the adult wing after removal of scales. The three juxtaposed wing regions have been distinguished from one another by Chlorazol black E staining. Arrows mark the points where the three cell populations meet.

(A) The darkest area, I*p*, is partially engulfed by IV*a*. Both I*p* and IV*a* are surrounded by VII*a*.

(B) Position of the grafts in the wing.

(C) The lightest area (VII*a*) and the darkest area (I*p*) make contact at only one point indicated by the white arrow. Both are located in region IV*a*.

(D) Position of the grafts in the wing.

(E) The darker of the two grafts (IV*a*) slightly engulfs the VII*a* graft when these regions are juxtaposed at I*p*.

(F) Position of the grafts in the wing.

From whole mounts and transverse histological sections of wing epidermis, regions of lower cell density along the host-graft interface have been shown to be occupied by very elongate cells whose large exposed surface area is attained at the expense of lateral intercellular contacts. By contrast, the more interior graft cells, which become closely packed, decrease their surface exposure, but correspondingly increase their lateral contacts.

Juxtaposition of three different wing regions

According to the proposed model, an adhesive hierarchy should exist among wing cells. In order to reduce their surface free energy, three region combinations would be expected to yield certain configurations, which should depend on the levels of the tissues in the adhesiveness hierarchy.

In a series of experiments, three regions were used to test this prediction (I*p*, IV*a*, VII*a*). In each of three types of experiment, two of these regions were represented by square grafts implanted contiguously within the third region. Sixteen animals were used for case I, and six each for cases II and III.

Case I: Grafts Ip and IVa positioned in region VIIa

Four different positionings of I*p* and IV*a* were examined (four animals for each). Each graft was either placed distally, proximally, anteriorly, or posteriorly relative to the other. The final configuration assumed was not influenced significantly by the initial positioning (compare Figs. 1B, C and 2A, B).

The initially square forms of I*p* and IV*a* were modified, as I*p* rounded up and was partly enveloped by IV*a*. Cells of IV*a* were interposed between I*p* and VII*a* (Figs. 1B, C and 2A, B), I*p*–VII*a* contacts being exchanged for supposedly more stable I*p*–IV*a* contacts.

Case II: Grafts Ip and VIIa positioned in region IVa

Although one-fourth of each graft's perimeter initially contacted the other graft, in the final configuration (Fig. 2C, D) the two grafts only contacted at one point. I*p*–VII*a* contacts were replaced by I*p*–IV*a* cell contacts, which are postulated to be more stable.

Case III: Grafts IVa and VIIa positioned in region Ip

The initial extent of contact between IV*a* and VII*a* was not noticeably reduced (Fig. 2E, F). The IV*a* graft slightly engulfed VII*a*. I*p* cells minimized their contacts with both grafts.

These experiments are in full agreement with the predictions of the differential adhesiveness model. They can be treated formally as follows (see Fig. 3).

The interfacial free energy, γ, is proportional to the interfacial tension which acts vectorially along the interface. At each three-region contact point, the three binary interfacial tensions can be represented as shown in Fig. 3. According to Lamy's Theorem (Thompson, 1917): 'For three forces acting at a

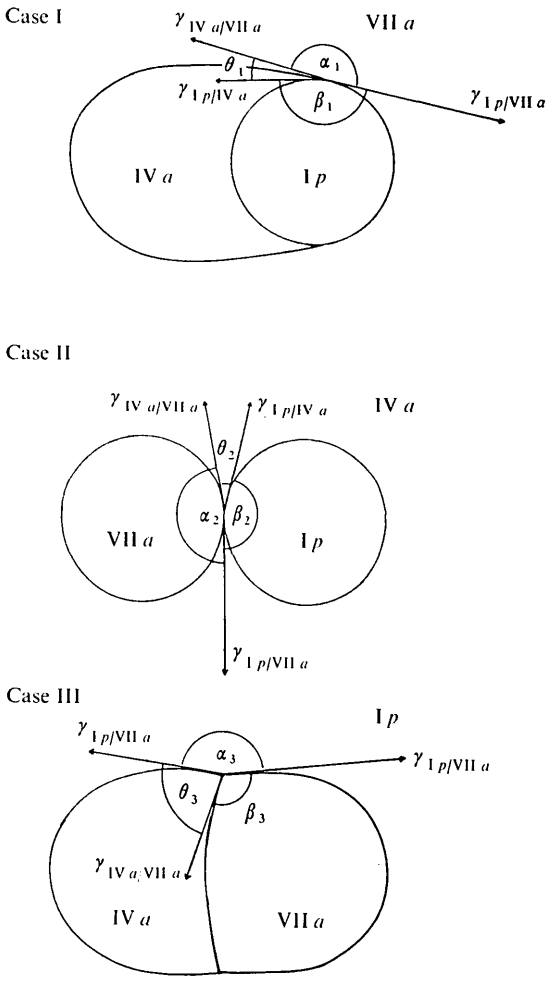


Fig. 3. Schematic representation of results obtained from experiments involving the juxtaposition of three different wing cell populations. Angles of contact between different populations are related to the interfacial tensions.

point, each force is proportional to the sine of the angle contained between the directions of the other two.' For all three cases,

$$\text{sine}\theta > \text{sine}\beta, \text{ sine } \alpha$$

Therefore,

$$\gamma \text{ I}p/\text{VII}a > (\gamma \text{ IV}a/\text{VII}a, \gamma \text{ I}p/\text{IV}a). \tag{10}$$

Interfacial free energies are negatively related to works of adhesion (Fig. 4). Therefore, the adhesive strengths can be ordered as

$$(\text{I}p\text{–IV}a, \text{IV}a\text{–VII}a) > (\text{I}p\text{–VII}a), \tag{11}$$

According to the rule of adhesive averages, either Ip or VIIa should be the most

Interfacial free energy = $\gamma_{A/B}$

Total interfacial free energy = $\gamma_{A/C}+\gamma_{B/C}$

Work of adhesion between A and B ($W_{A/B}$) = $\gamma_{A/C}+\gamma_{B/C}-\gamma_{A/B}$

Therefore, for the different wing cell populations:

$W_{Ip/VIIa} = \gamma_{Ip/IVa}+\gamma_{VIIa/IVa}-\gamma_{Ip/VIIa}$,

$W_{Ip/IVa} = \gamma_{Ip/VIIa}+\gamma_{IVa/VIIa}-\gamma_{Ip/IVa}$,

$W_{IVa/VIIa} = \gamma_{IVa/Ip}+\gamma_{VIIa/Ip}-\gamma_{IVa/VIIa}$.

Fig. 4. The physical interpretation of equations relating interfacial free energies to works of adhesion.

adhesive region. This is confirmed by the fact that these regions never engulf one another, or region IV*a*. Therefore, one of the following two sets of relationships holds:

$$(Ip\text{–}Ip) > (Ip\text{–}IVa,\ IVa\text{–}VIIa) > (Ip\text{–}VIIa)$$

plus

$$(Ip\text{–}Ip) > (IVa\text{–}IVa) > (VIIa\text{–}VIIa) \tag{12}$$

or, alternatively,

$$(VIIa\text{–}VIIa) > (Ip\text{–}IVa,\ IVa\text{–}VIIa) > (Ip\text{–}VIIa)$$

plus

$$(VIIa\text{–}VIIa) > (IVa\text{–}IVa) > (Ip\text{–}Ip). \tag{13}$$

Of the two alternatives, the former (12) is consistent with the other evidence discussed in this paper, and can be considered to represent the true adhesiveness hierarchies.

Interaction of two different cell populations in an essentially neutral environment

The interactions which have so far been examined transpire in one plane, and within the wing environment. It was discovered by chance that pupal wing epidermis positioned in the epidermis of other appendages quite often failed to be assimilated and instead formed isolated, differentiated vesicles. This observation permitted us to study the interaction of normally non-adjacent wing regions in an essentially neutral environment: disparate wing grafts were placed together at a site spanning the pupal antenna and mesothoracic leg, and the form assumed by the grafts was observed in the adult moth.

Three wing regions (I*p*, IV*a*, and VII*a*) were examined with five replicates for each of the six binary combinations. The results demonstrated that the different wing regions display preferential affinities even in a neutral environment. The

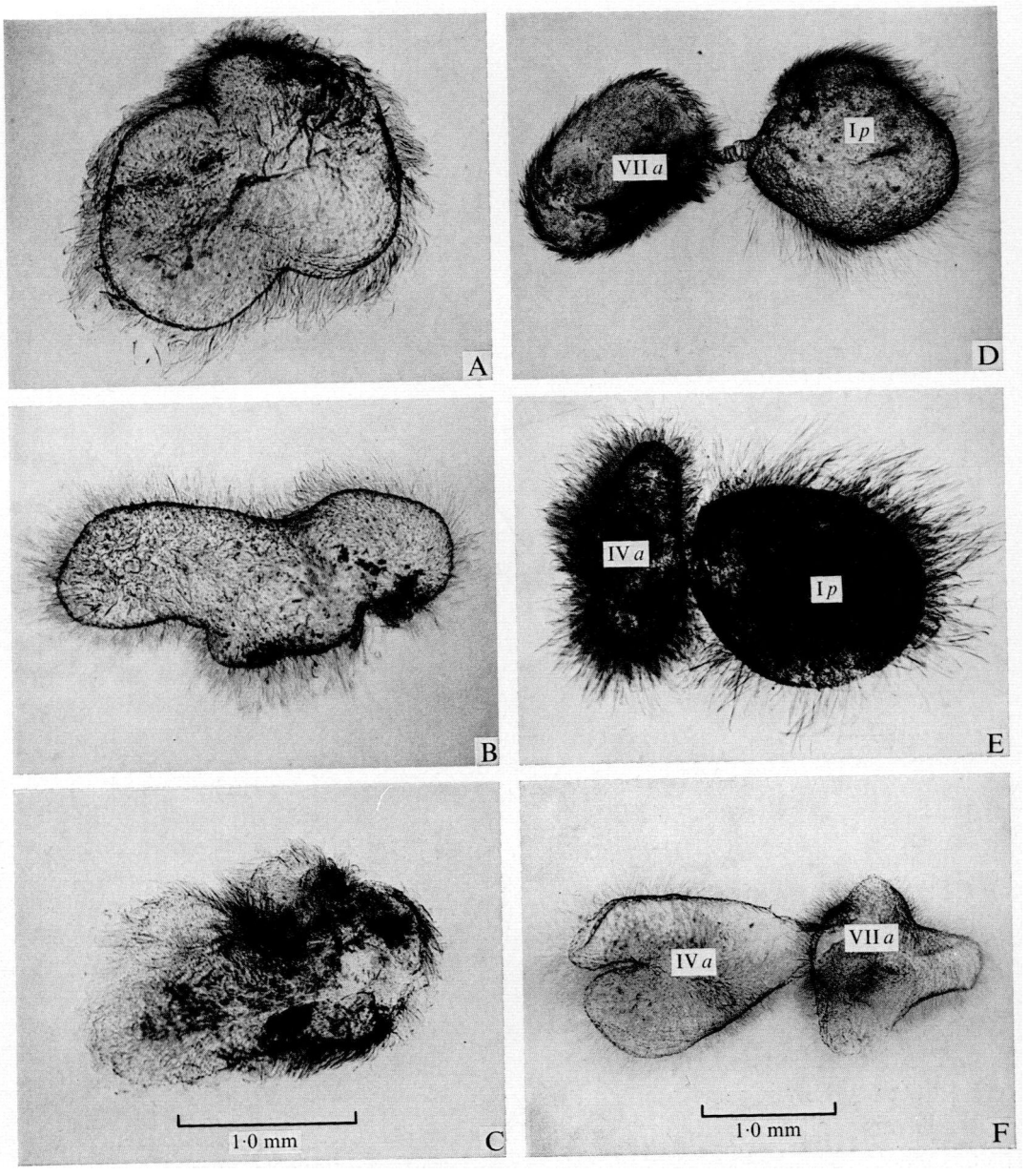


Fig. 5. (A–C) Adult cuticle formed by two identical pupal wing grafts that have been juxtaposed in the leg-antenna epidermis.

(A) Two I*p* grafts.

(B) Two IV*a* grafts.

(C) Two VII*a* grafts.

(D–F) Adult cuticle formed by two different pupal wing grafts that have been juxtaposed in the leg-antenna epidermis. Note the extent of contact between two given regions.

(D) Grafts I*p* and VII*a*.

(E) Grafts I*p* and IV*a*.

(F) Grafts IV*a* and VII*a*.

control vesicles formed by juxtaposing two identical grafts, from the same wing region, are shown in Fig. 5A–5C. In these controls, the two grafts fused and formed a single vesicle. By contrast, tissues from different wing regions formed distinct vesicles, which were connected to each other via a conspicuously constricted region (Fig. 5D–5F). The diameter of the constriction decreased as the distance between the sites of origin increased.

Recall that heterotypic cell associations will be exchanged for isotypic ones to a greater extent as the difference in adhesiveness between two groups of cells (which, according to the gradient model presented, is also proportional to the distance between them) increases. In this experiment, therefore, one can establish the relative differences in adhesiveness for the three wing regions to be:

$$(Ip\text{–}Ip),\ IVa\text{–}IVa,\ VIIa\text{–}VIIa) < (Ip\text{–}IVa,\ IVa\text{–}VIIa) < (Ip\text{–}VIIa). \quad (14)$$

DISCUSSION

Applicability of the differential adhesiveness hypothesis

Although the differential adhesiveness hypothesis can explain the phenomena associated with wing epidermis grafting, can it likewise account for the behavior of grafts in other insect systems? Before diffusion gradients were proposed to exist in the insect segment, Locke (1959) interpreted his observations as follows. 'The tergal epithelium has a capacity for maintaining transverse continuity within similar levels in the axis. There is an axial gradient within each segment, the anterior cells showing greatest facility in maintaining continuity. This could be described as an affinity between the cells at each level in the axis, the strength of the affinity being greatest anteriorly.' This is tantamount to the adhesiveness gradient model proposed in the present paper. Locke also stated that although 'nothing could be inferred from [his] experiments about the cellular mechanism of this response, it could be due to the migration of cells.' Recent findings in a number of systems (Bohn, 1974; Lawrence, 1974; Nübler-Jung, 1974) indicate a role for cell migration in pattern regulation. The overemphasis on diffusion models may now be balanced with a proper appreciation of the role autonomous cell properties can play in pattern regulation, provided the cells are motile. In fact, Bohn (1971) has already argued that the morphogenetic gradient in the cockroach leg can be interpreted in terms of cell surface properties, rather than diffusible morphogens. Therefore, any changes (besides wounding effects) in polarity and pattern of epidermal cells surrounding a graft (Marcus, 1962; Lawrence, 1973*b*) could simply be due to the occurrence of intercalary regeneration and movement of cell sheets rather than the response of cells to changes in a diffusion gradient landscape.

Many results of insect grafting are consistent with both diffusion and non-diffusion gradient models. However, diffusion models have not offered an explanation for one consistent feature: the increased cellular density of trans-

posed grafts. According to the adhesiveness gradient model, cell densities for grafts transposed in either direction along the gradient should be higher than densities for control grafts, because of the pressure difference expected across the interface of two cell populations differing in surface free energy (see fig. 8 in Nardi & Kafatos, 1976 and Fig. 6 in Appendix I). For *Rhodnius*, Lawrence, Crick & Munro (1972) state that: 'The tubercles on the graft are smaller and more dense due to closer packing of the cells.' This same phenomenon can likewise be observed in pictures of transposed grafts from Marcus (1962).

A configuration of minimum free energy – a circular mono-layer – would be predicted by the differential adhesiveness model for grafts which have been relocated or rotated. Locke (1966) remarked that a graft of leg integument rotated 180° 'became more or less circular, with radially oriented bristles'. It may be objected that the roundness may result from the greater degree of wounding at the corner of square grafts relative to the sides. The different shapes of *Manduca* grafts transposed for variable distances along the gradient argue against this interpretation (see fig. 4 in Nardi & Kafatos, 1976). In *Oncopeltus*, a 90° rotated graft was shown to continue changing towards a circular shape long after the wound had healed (Lawrence, 1974).

In summary, we may note that the differential adhesiveness model is in agreement with results obtained in a variety of insect grafting studies – and that it fits the *Manduca* grafting results with fewer qualifications than appear to be necessary for diffusion models (Lawrence *et al.* 1972).

If adhesiveness gradients exist in insect epidermis, it still remains to be determined when and how they become established. Conceivably, they may have their origin in blastoderm mosaicism and thus be initially related to ooplasmic determinants. Even if that is the case, however, it seems inevitable that the final gradient shape will be attained through a process of intercellular communication and regulation. *A priori*, this process could be mediated by cell contacts, although the involvement of diffusible morphogens remains a most attractive hypothesis (Lawrence *et al.* 1972). Because of technical difficulties and the lack of genetic markers, this question cannot be studied profitably in *Manduca*. Whatever the mechanism which sets up the gradient, a plausible and parsimonious interpretation of the grafting results is that by the pupal stage the wing gradient is in fact a gradient of cellular adhesiveness, rather than a gradient of diffusible morphogen.

The possible functions of an adhesiveness gradient

(A) *The control of growth*

Some additional insight into the scheme for gradient establishment and a possible function for the gradient can be derived from other insect systems. In *Oncopeltus* (Lawrence, 1973*a*), the boundaries between segmental gradients are established as early as the blastoderm stage. If subsequently this boundary is removed, as when two adjoining sternites become partially fused because of an

interruption in the intervening intersegmental membrane, then in the fused region the amount of growth for the two segments is reduced to that for a single segment. This suggests that the segmental gradient entails a mechanism for growth control (Lawrence, 1970*a*). In *Drosophila*, the proximal and distal wing boundaries are established when the wing disc encompasses 50–100 cells (Garcia-Bellido, 1972). By analogy with the above two systems, we can envisage that in the *Manduca* wing the proximal and distal boundaries acquire fixed values of cellular adhesiveness at an early stage, and that cell divisions proceed between these fixed reference points until a pre-determined gradient steepness is attained, whereupon growth ceases (see Lawrence, 1973*a*). One could envisage the gradient in cell surface adhesiveness being generated by an orderly partitioning of surface properties during these divisions. The steepness would be monitored locally by the cells through contacts with their immediate neighbors. An attractive feature of this model is that it offers a simple explanation for the independent growth of different wing compartments postulated to have separate gradients (Crick & Lawrence, 1975).

Support for the growth control model is provided by experiments on regeneration in the leg of *Leucophaea* (Bohn, 1971). The extent of intercalary regeneration in the tibia between a distal graft and a proximal stump appears to be controlled not by the total length of graft plus stump, but by the local steepness of the inferred gradient. Additional experiments indicate that the tibiae of different legs, which vary in length, differ in terms of the steepness of the gradient, but not in terms of the boundary values. This was shown by reasoning that regardless of the absolute tibial length, cells located at a given fractional length should have the same gradient levels; in fact, regeneration occurs only when the stump and graft from different legs are cut at different fractional levels. Apparently, while the cells cannot tolerate discontinuities in gradient levels, they are unaffected by the disparate slope of a neighboring graft. This observation emphasizes the local nature of growth control, which in principle requires only that the difference in a graded parameter between two adjacent cells be measured – quite possibly through cell surface contacts. This may be analogous to the mutual stimulation of growth and proliferation in mixed lymphocyte cultures (Bach, 1968).

(B) *The directionality of sensory nerve outgrowth in the wing: another possible function for the epidermal adhesiveness gradient*

Clever (1959, 1960) noted that the sensory nerve processes of the wing always grow toward the wing base along lacunar routes. The choice of route can be explained by 'contact guidance' (Weiss, 1934), i.e. by the existence of environmental, oriented physical features – the lacunae; however, the choice of direction along this route cannot be so explained. Therefore, Clever postulated a proximo-distal wing gradient, in order to account for the directed movement of the

processes toward the wing base. Being established well before the sensory cells of the adult wing differentiate, the gradient of adhesiveness of the epidermal cells could provide directional information for the underlying sensory cell processes. It should be noted that the direction of nerve growth, as predicted by this model, is towards the wing base, i.e. towards a region of greater epidermal cell adhesiveness.

A comparable suggestion has been made on theoretical grounds by Gustafson & Wolpert (1963); similarly, DeHaan (1963) has speculated that the directed migration of chick precardiac mesoderm cells over the endoderm is governed by a gradient of endodermal cell adhesiveness. Carter (1965) and Letourneau (1975*a*, *b*) have demonstrated in model systems the significance of adhesiveness gradients for orienting the movement of cultured mammalian cells and embryonic chick sensory neurons, respectively.

(C) *Positional specificity*

Gradients may convey the positional information that underlies pattern formation (Lawrence, 1970*b*, 1971; Wolpert, 1969, 1971). Across the lepidopteran wing, for example, the color and shape of the scales vary in intricate but highly repeatable ways; this complex, albeit two-dimensional, normal pattern might be explained, at least in part, by the wing gradient.

It should be noted that positional information need not be highly detailed in order for an elaborate pattern to form. The positional specification conferred by a given gradient level may be broad, and the fine-tuning of the pattern may be finalized by inhibitory or competitive mechanisms. Certainly, such mechanisms appear to function in the uniform spacing of epidermal organules such as hairs, bristles, etc. (Lawrence, 1973*b*), and in the localization of the mesothoracic bristle, *adc*, in *Drosophila* (Claxton, 1969). Similarly, in the retinotectal system the one-axon-to-one-tectal cell specificity originally postulated (Sperry, 1963) does not appear to exist (Gaze & Keating, 1972; Feldman, Keating & Gaze, 1975); the actual specificity can be accounted for by the existence of multiple cell surface labels, which allow cells to establish contacts with many other cells – some of which are optimal (i.e. most stable) matches.

Regardless of how the spatial pattern of the scales is specified, the large number of scale types, defined by color and shape, corresponds to an amazingly high number of distinct differentiation states within a single lepidopteran wing. As Gierer (1973) has emphasized, combinatorial models can account for very large numbers of differentiation states, without unduly taxing the informational content of the genome: each of the numerous scale types could be easily specified by a combination of a small number of regulatory polypeptides.

Appendix I

If cell populations from various regions of the wing differ in their adhesive (namely, surface free energy) properties, then the behavior of any two of these cell populations may be treated by the equation of Young & Laplace (Hutchinson, 1962; Adamson, 1960) which was originally derived to relate the surface free energy of a curved liquid surface to the pressure difference between the liquid and vapor phase or between two liquid phases. When applied to the biological system, the equation, which is derived below, predicts certain changes in graft size and cell density.

(A) in Fig. 6 represents a section in the adult cuticle of the final host (*H*)-graft (*G*) interface. (B) represents an element of the initial pupal host-graft interface.

Displacement of the section B a distance dz results in the following change in area:

$$xy-(x-dx)\,(y-dy) \approx xdy+ydx.$$

If γ is the interfacial free energy, the work done is

$$W = \gamma\,(xdy+ydx).$$

Since the change in area arising from the displacement of B to A is negative, the work done is also negative. Relating the pressure difference across the interface to the work,

$$\Delta P = (P_G-P_H),$$
$$W = \Delta P\, xydz.$$

At equilibrium the two work terms must be equal. In order to express P in terms of the radii of curvature, R_1 and R_2, comparison of similar triangles gives the following expressions:

$$(x-dx)/R_1 = x/(R_1+dz),$$
$$dx = xdz/R_1,$$
$$(y-dy)/R_2 = y/(R_2+dz),$$
$$dy = ydz/R_2.$$

The final expression obtained is

$$(P_G-P_H) = \gamma(1/R_1+1/R_2).$$

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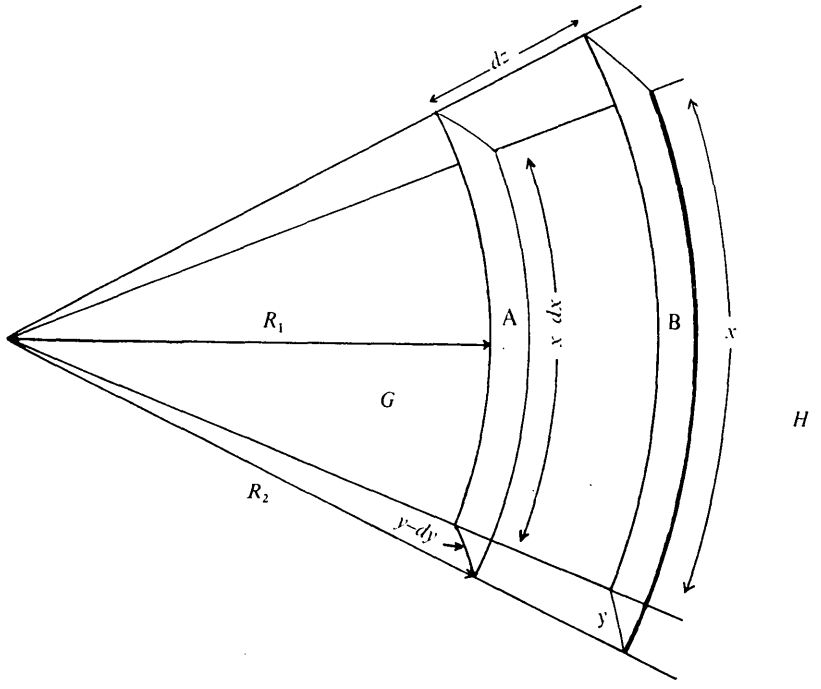

Fig. 6. Schematic drawing of a small section of the curved boundary separating graft (*G*) and host (*H*) cells. (A) A section of the host–graft interface in the adult; and (B) the same interface in the pupa.

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