FURTHER STUDIES ON GRADIENT-DEPENDENT DIVERSIFICATION IN THE PUPAL CUTICLE OF *GALLERIA MELLONELLA*

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INTRODUCTION

Many facts indicate that gradients may determine the developmental fate of cells. Recently a study of the last abdominal segments in the pupa of *Galleria* has provided further evidence in favour of this concept (Stumpf, 1966*b*, 1967*c*, *d*). This study was based on earlier observations concerning the orientation of certain integumental structures, such as scales of *Galleria* (Piepho, 1955*a*, *b*; Marcus, 1962; Stumpf, 1966*a*), cuticular ripples of *Rhodnius* (Locke, 1959, 1960, 1964) and hairs of *Oncopeltus* (Lawrence, 1966) and *Rhodnius* (Locke, 1966). These findings revealed that within the epidermis of insects there exists a segmental gradient by which integumental structures become oriented. Locke (1959) first discovered that the gradient is repeated in each segment; skin grafting from one segment to another resulted in deviations from the normal ripple pattern only if the graft was transplanted to a new location in the segment (Locke, 1959; Marcus, 1962).

The gradient apparently forms intermediate values where breaks in the slope result from experimental interference. The gradient probably consists of a concentration gradient of some diffusible substance (Stumpf, 1966*a*, 1967*a*, *b*). It exists already in the penultimate larval instar (Locke, 1959; Marcus, verbal communication; Lawrence, 1966). It has been suggested (Stumpf, 1967*b*) that the gradient substance may be produced at one segment margin and destroyed at the other.

Neither the previous data nor the results reported here exclude the possibility of a double gradient system as assumed for the development of sea urchins (Runnström, 1931), the insect *Euscelis* (Sander, 1959, 1960) and *Hydra* (Webster, 1966*a*, *b*). But as long as no facts point to the existence of a second gradient in the insect segment, only one gradient need be assumed.

The gradient model, supported previously mainly by the correspondence between predictions derived from it and the experimental orientation patterns of scales, hairs and ripples, holds also for the axial diversification† of the segment (Stumpf, 1966*b*, 1967*c*, *d*). Each of the fifth, sixth and the seventh abdominal segments of the pupa of *Galleria* is subdivided into three regions of different cuticular structure (Pl. 1, fig. 1). As a working hypothesis the diversification of the segment had been assumed to be

‖ Deceased, October 1967. See editor's note, p. 59.
† Since the term differentiation is being increasingly used to mean the acquisition, by cells, of specific characteristics during development, diversification was defined (Stumpf, 1967*d*) as the process involved in the initiation of qualitative differences between cells.

brought about by different regions of the gradient (Text-fig. 1). The direction in which the gradient declines has been chosen arbitrarily.

The middle region, a narrow ridge of thickened cuticle, indicates most clearly the correspondence between the location of a particular structure and the expected location of a particular concentration (Stumpf, 1966, 1967c, d). If a piece of larval integument from the region of the presumptive ridge is rotated about 180° (Text-fig. 2a, b), the result (Pl. 1, fig. 3) conforms with the predictions of the gradient model, and the ridge pattern follows the line of appropriate concentration (Text-fig. 2c). However, the same pattern could result if the ridge formed as an interaction between cuticle of types 1 and 3 rather than autonomously.
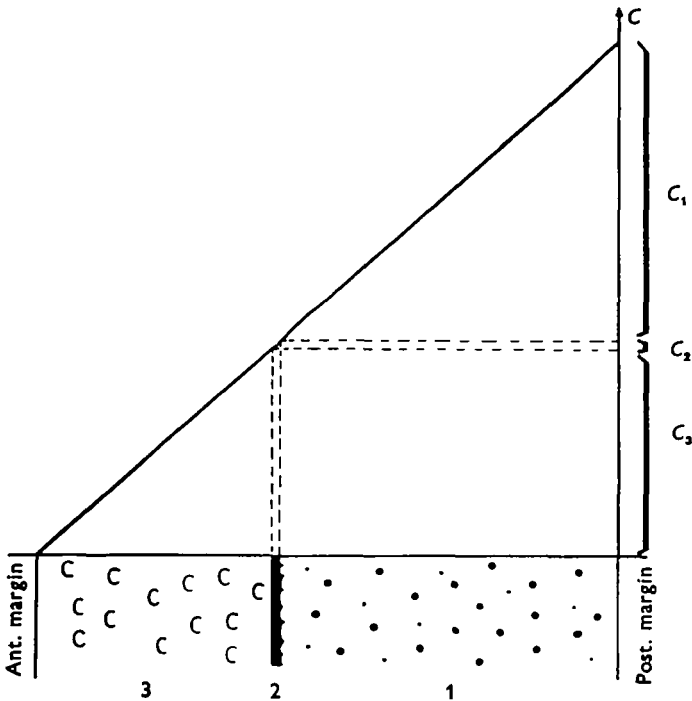

Text-fig. 1. Diagram showing the hypothetical correspondence between assumed gradient and cuticular region. The direction of the gradient from the posterior to the anterior margin is arbitrarily chosen. Ordinate: concentration of the gradient substance; abscissa: cuticular regions.

One purpose of the present investigation is to exclude this possibility. The diversification into three regions occurs only in the fifth to seventh abdominal segments. The first four segments show only that cuticular pattern which is seen in region 1 of the rear segments (Pl. 1, fig. 1, 2); these four segments lack ridges and region-3 cuticle. By making appropriate transplants from a posterior segment into an anterior segment it is possible to investigate whether the ridge occurs only where it is expected according to the gradient model, or whether it occurs at every border between region-3 cuticle and region-1 cuticle irrespective of the gradient level. But first it must be established how a graft from a posterior segment behaves in the epidermis of an anterior segment.

Another purpose of the present investigation is to see why the first four abdominal segments differ from the posterior ones, the latter being subdivided into three cuti-

cular regions while the former develop only region-1 cuticle. The difference could be due to a difference of their reacting systems or to a difference in quality of their gradients, both gradients having the same effect upon the orientation of scales in the adult. By means of transplantations from posterior into anterior segments it is possible to distinguish between these alternatives.
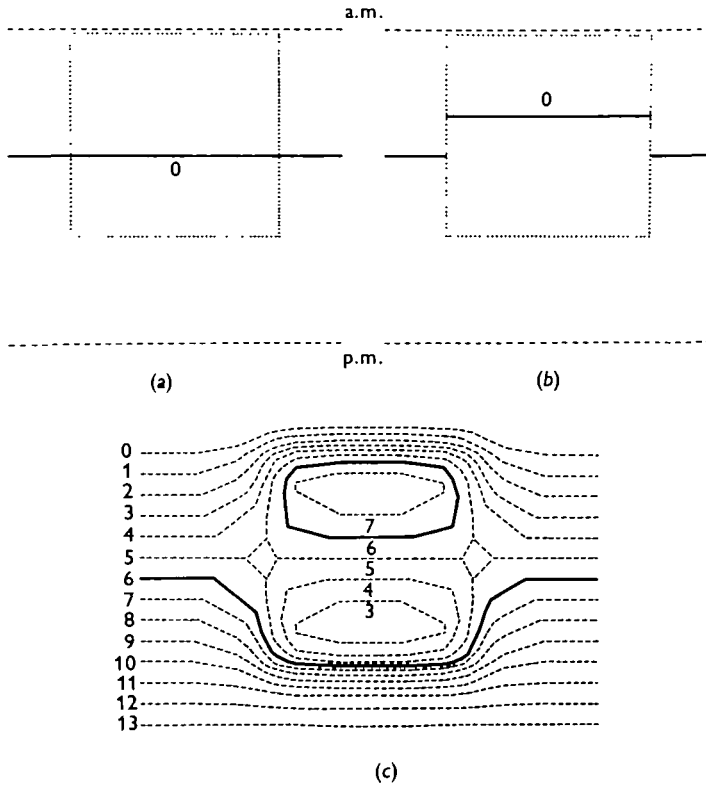
Text-fig. 2. Effect of rotation of a graft by about 180° in the sixth larval segment. (*a*) Excised graft before rotation. (*b*) After rotating. Broken lines: presumptive segment margins of the pupa; solid line: presumptive ridge; circle: bristle B_1; dotted line: line of cutting. *a.m.*: anterior segment margin; *p.m.*: posterior segment margin. (*c*) Pattern of isomixes expected for this operation. Solid line: isomix corresponding to the ridge.

MATERIAL AND METHODS

Culture, experimental treatment and preparation of the operated animals followed the method of Marcus (1962) and was the same as earlier described (Stumpf, 1967 *d*).

Late last instar larvae were chosen for the transplantations. In all experiments the graft originated from the sixth abdominal segment and was transplanted into the fourth abdominal segment. The pattern of the larval segment is given in Text-fig. 3. The bristle B_1 persists in the pupa, being located immediately posterior to the ridge. Thus this bristle marks the presumptive ridge region in the larva. The excised grafts were rectangles which extended along a third to one half of the larval segment axis.

In the following, the term 'segment margin' refers to the border between an inter-segmental membrane and the main part of the segment. Since the intersegmental

membranes occupy a greater part of the segment in the pupa than in the larva, mostly at the expense of the posterior part, the segment margins of the larva and pupa are not homologous. The relative positions are given in Text-fig. 3.
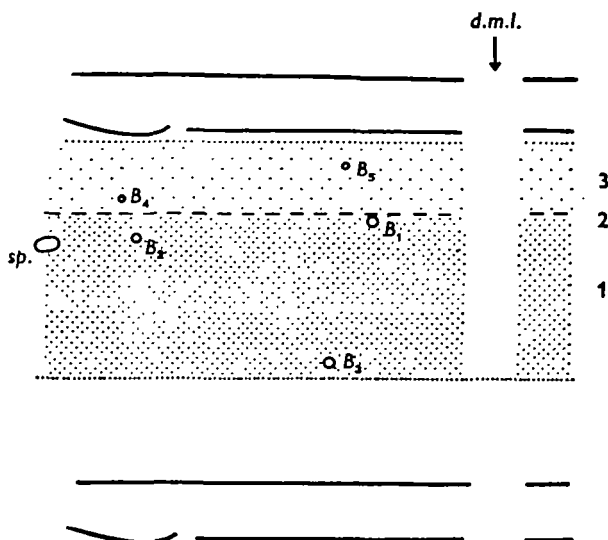
Text-fig. 3. Diagram of the corresponding areas of the sixth segment in the larva and in the pupa. Solid lines: lines of muscle insertion representing the border between segment area and intersegmental membrane in the larva; dotted lines: presumptive border between segment area and intersegmental membrane of the pupal segment; broken line: presumptive ridge (pupa); dotted areas anterior and posterior to the broken line: presumptive region 3 and region 1 (pupa); circles: bristles, the most posterior bristle B_2 appears only in the larva; $sp.$: spiracle; $d.m.l.$: dorsal midline.

EXPERIMENTS AND RESULTS

Experiment I

This experiment was performed in order to see whether a graft from the sixth segment retains its ability to develop a ridge and region-3 cuticle in the environment of an anterior segment. Pieces of integument from the sixth abdominal segment containing presumptive ridge, region 3, and region 1 were transplanted to an equivalent place on the fourth abdominal segment (Text-fig. 4).

From twenty grafts six formed only a very thin abnormal cuticle. Probably they had been operated on too late. Prepupae always exhibit this sort of cuticle after grafting, and it seems that wounding in the prepupal stage inhibits the formation of a normal pupal cuticle. The remaining fourteen grafts showed a well-developed ridge and, anterior to it, region-3 cuticle. This region-3 cuticle adjoined anteriorly and at the sides to the region 1 cuticle of the host without a ridge being formed between the two regions (Pl. 1, fig. 4). In five of the fourteen cases the bristle B_1 had disappeared. In the remaining nine grafts the ridge was located normally with respect to the bristle, i.e. slightly anterior to it.

Thus the graft containing the presumptive ridge region and presumptive region 3 develops both of these structures when transplanted into an equivalent position in the fourth segment.

As controls, grafts taken from the B_1-region of segment 4 have been transplanted into the ridge region of segment 6. They did not form a ridge.

Experiment II

This experiment consists of two transplantations: (*a*) the transplantation of a graft from the posterior part of segment 6 (presumptive region 1) into the anterior region of segment 4, and (*b*) the transplantation of a graft from the anterior part of segment 6 (presumptive region 3) into the posterior region of segment 4. It was designed in an attempt to change the regional character of the graft from its presumptive regional character by transplanting it to an appropriate place in the fourth segment.

4th segment

4th segment

6th segment

6th segment

Fig. 4   Fig. 5
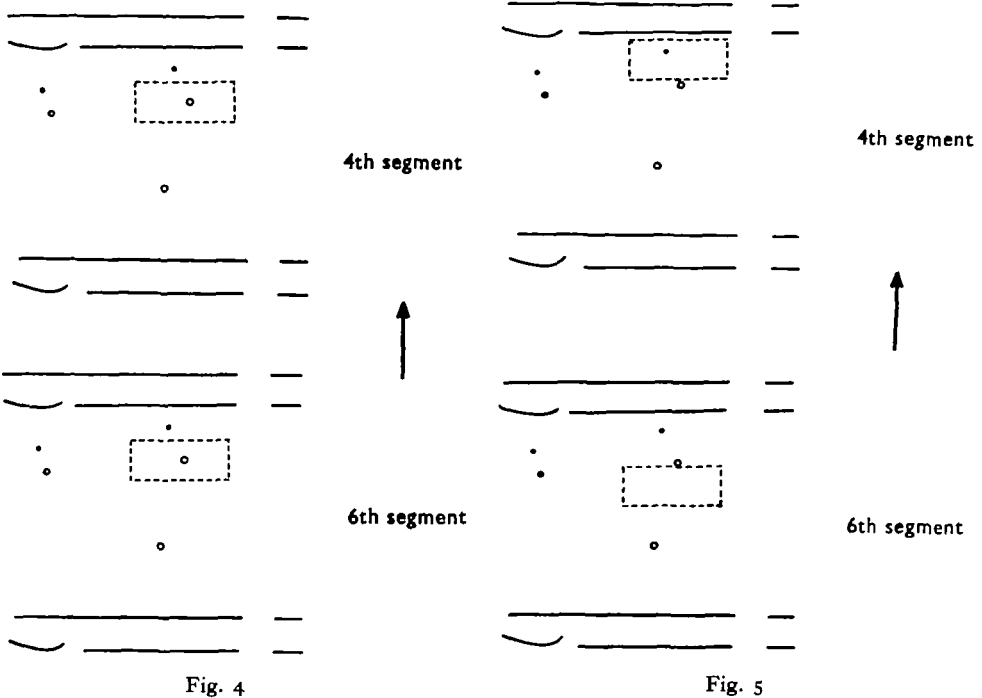
Text-fig 4. Experiment I. The marked piece from the sixth segment has been transplanted into the place in the fourth segment marked by the broken lines.

Text-fig. 5. Experiment II*a*. The marked piece from the sixth segment has been transplanted into the position marked by the broken lines in the fourth segment.

This procedure is designed to reveal whether the difference between anterior and posterior segments is brought about by gradients of a different nature, or whether the gradients are of the same quality but the competence of the reacting system is different.

If the gradients are of a different nature, that is, if they depend upon different diffusible substances, there will be no interaction between host and transplant. The possibility does exist that the transplanted tissue, which, because of the different nature of its gradient is competent to form a ridge, might lose some of its substance by diffusion into the host, and thereby change its own gradient level. Thus in the case

where the transplant is taken from a more concentrated gradient area than the ridge, this loss of substance might lead to ridge development. This possibility could only apply to one of the Expts. II*a* or II*b*, which one it might be would depend on the direction of gradient decline. In the other case the graft would be of a lower gradient concentration than is appropriate for ridge formation, and in that case there would still be diffusion out from the graft. Although the host substance would diffuse into the graft it would of course not be capable of inducing a ridge in the graft.

However, if the gradients in segments 4 and 6 depend upon the same diffusible substance, there will be interaction according to the gradient model and in both experiments (see Pl. 1, fig. 5, 6) a ridge will be formed.
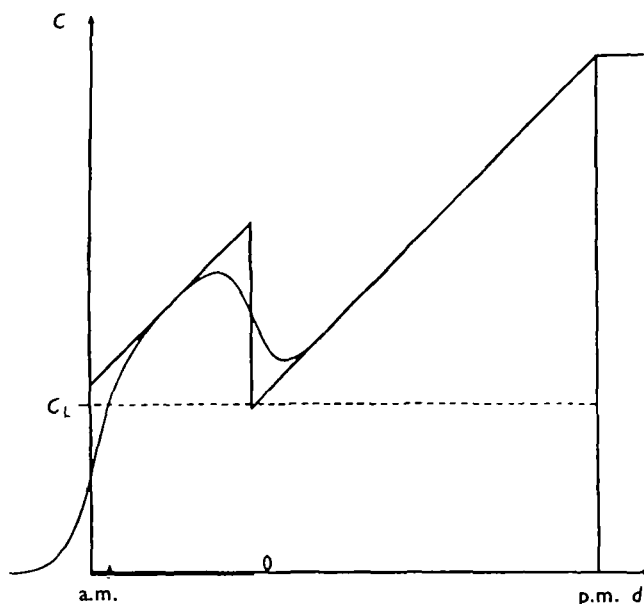
Text-fig. 6. Predicted result of Expt. II*a*, assuming gradients in segments 4 and 6 are of the same substance. The straight line marks the concentration at the time of transplantation and the curved, concentration after some time for diffusion. Note the concentration (C_L) which determines the development of a ridge is formed near the anterior margin of the segment. Ordinate: concentration of the gradient substance; broken line: concentration C_L corresponding to the ridge; abscissa: distance, d, from the anterior margin; *a.m.*: presumptive anterior segment margin of the pupa; *p.m.*: presumptive posterior segment margin of the pupa; broad section of the abscissa: location of the graft; hump on the abscissa: region where the ridge is expected to occur (where the line C_L crosses the curved line of the gradient): circle on the abscissa: location of the bristle B_1.

Experiment II*a*

A rectangular graft consisting only of presumptive region 1 was removed from the region posterior to the bristle B_1 of the sixth segment and was transplanted into the region anterior to the bristle B_1 of the fourth segment (Text-fig. 5). Actually the grafts were larger than one third of the segment in axial direction. (It was assumed that no disturbance resulted when a segment region was grafted partly into the intersegmental membrane of the host.)

Out of fifteen grafts only one turned black. Each of the remaining fourteen grafts showed a distinct ridge and region-3 cuticle anterior to this ridge (Pl. 1, fig. 5).

As controls, grafts taken from a position anterior to the bristle B_1 of the sixth segment have been transplanted into the equivalent position of the fourth segment. They did not form a ridge.

Experiment II b

A rectangular transplant consisting only of presumptive region 3 of the sixth segment was excised from the region anterior to the bristle B_1 and was grafted into the region posterior to the bristle B_1 of the fourth segment (Text-figs. 7, 8). In this experiment the grafts were smaller than in Expt. II a in order not to include intersegmental membrane in the graft.

4th segment

6th segment
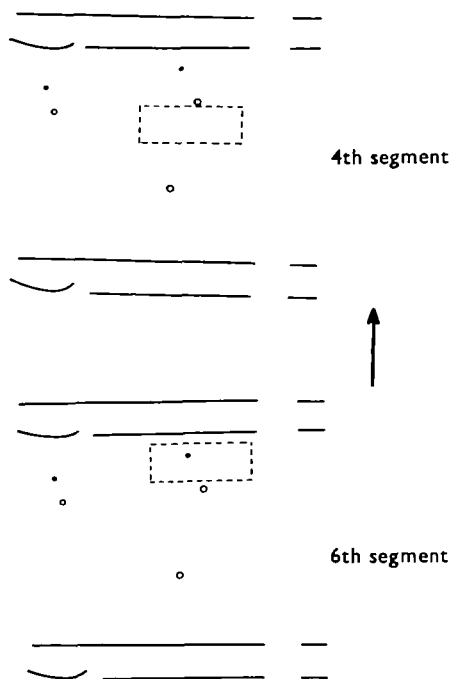
Text-fig. 7. Experiment II b. Explanation as in Text-fig. 5.

Since smaller grafts easily turn black, out of twenty-two grafts only three survived. All three showed a distinct ridge and region-3 cuticle (Pl. 1, fig. 6).

As controls, grafts taken from a position posterior to the bristle B_1 of the sixth segment have been transplanted into the equivalent position of the fourth segment. These controls did not form a ridge.

As ridges are formed in both Expts. II a and II b we can conclude that the gradients are of the same nature, and presumably depend upon the same diffusible substance.

DISCUSSION

The results show that the regional character of a piece of integument from a posterior segment can be changed not only by rotating it in its own segment (Stumpf, 1966 b, 1967 c, d), but also by transplanting it to a suitable place in one of the anterior segments. Presumptive region 1 of the sixth segment, for instance, when transplanted to a region

anterior to the bristle B_1, becomes capable of forming a ridge and region-3 cuticle, as predicted by the gradient model. Here a ridge and region-3 cuticle appear in a segment which by itself never forms those structures.

Experiments I and II b show that the ridge is not a structure caused by the border between region-1 cuticle and region-3 cuticle since these regions can adjoin each other without a ridge being formed between them. Furthermore, we can conclude from Expts. II a and II b that neither region 1 nor region 3 has been firmly determined at

Text-fig. 8. Expected course of the gradient after the operation according to Expt. II b, for a gradient assumed to decline from the posterior to the anterior margin. Explanation see Text-fig. 6.

he time of the operation. Thus the formation of all three regions seems to depend upon the concentration of the gradient substance, in accord with the gradient model.

Only if the gradient in the anterior and posterior segments is of the same qualitative nature is it possible to predict from the gradient model which regions will form in a graft which has been taken from a posterior segment and transplanted into an anterior segment. The results of the Expts. II a and II b conform to these predictions and reveal that the gradients are of the same nature. The regional character can be changed to the definitive region 2 (ridge) either from the presumptive region 1 or from the presumptive region 3.

In one of these cases the graft can be assumed to achieve that particular concentration of the gradient substance corresponding to the ridge by diffusion from the host tissue into the graft. Hence it can be concluded that the gradients in both sorts of segment are the same or at least that their effects add to each other. As likewise shown by Locke (1960) in *Rhodnius* the gradients can be assumed to be of the same nature even though in different segments different cuticular patterns are formed. Thus the difference between anterior and posterior segments must be due to a difference in competence. The anterior segments are not competent to react to the same gradient by diversification into three different regions.

The ridges formed in Expt. II are not straight as in Expt. I but are somewhat bent. The curve is oriented differently in Expt. II*a* and Expt. II*b*. This is to be expected since in Expt. II*a* a shift occurred from a posterior to an anterior position in a segment, whereas in Expt. II*b* this shift took place in the opposite direction. The contour lines of concentration (isomixes) expected for these cases are given in Text-fig. 9. In Expt. II*a* (Text-fig. 9*a*) the isomix corresponding to the definitive ridge is located in
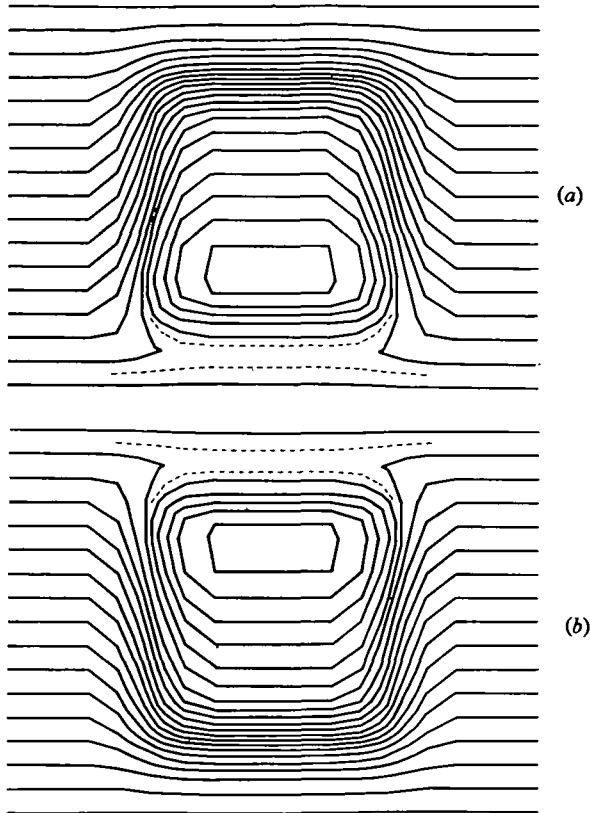
Text-fig. 9. Pattern of isomixes expected after transplantation. (*a*) For a shift from a posterior to an anterior location, (*b*) for a shift from an anterior to a posterior location.

the anterior part of the graft and is concave posteriorly. The ridge exhibits the same form. In Expt. II*b* (Text-fig. 9*b*) the isomix corresponding to the definitive ridge is located in the posterior part of the graft and the ridge is concave anteriorly, in accord with the calculated isomixes for this experiment.

The regional character of the epidermis of the sixth segment is retained when a piece of this epidermis is transplanted to an equivalent position in the fourth segment, as shown by Expt. I. The presumptive regions of the graft are formed. We could suppose that the three regions had been determined prior to the operation. On the other hand we know from Expt. II that the fate of a graft can still be changed from its presumptive fate to that characteristic of another region. So, while the gradient, as the inductive stimulus for the diversification, exists within the epidermis during at

least two larval periods, it obviously can still be changed under certain conditions. Therefore, whether or not we call the epidermis 'determined' at the time of the operation depends on the definition we give the term 'determination'.

The reaction of the tissue to the stimulus occurs only during a very restricted period, the period of competence. (The term 'competence' appears here in two different connections. In some instances it is related to the incompetence of epidermis in the anterior segments, i.e. to a spatial difference. In other cases it is related to the competence or incompetence of one area of epidermis during different developmental states, i.e. to a temporal difference.) The exact time during which the epidermis is competent is not known. Until the prepupal stage the regional character can still be changed, but any incision in the prepupal stage causes the surrounding area to develop only an abnormal thin cuticle. Thus, we can say only that the epidermis is competent to react to the gradient during some period of the prepupal stage.

The question now remains: shall we designate a tissue as 'determined' only after it has reacted to the stimulus, or rather only as long as the stimulus exists within it? In the case of induction phenomena the time of competence and the occurrence of the inductive stimulus normally coincide. But in the case of the diversification of the pupal segment the factors to which the tissue reacts already exist within it a long time before the reaction. It we were to define a tissue as 'determined' as soon as the substance to which it later reacts exists within it, these experiments would provide another example of transdetermination as exhibited by the work of Hadorn (1963, 1966). This definition would correspond to that given by Spemann (1936, p. 131) who demanded that a tissue, in order to be called 'determined', possess in itself the specific conditions which lead to its further fate. Only then would explantation experiments provide a criterion for the state of determination. That would mean, however, that we would have to consider such phenomena as, for example, the presumptive epidermis of certain amphibian early neurulae as determined, although we know that it can still be induced to form a lens when transplanted over the eyecup. So I would propose instead that we use the term 'determination' only after the tissue has already reacted to the stimulus, even though this usage would be close to the meaning of the term 'differentiation'.

SUMMARY

1. In last instar larvae pieces of integument from different regions of the sixth segment have been transplanted to the fourth segment. The formation by the grafts of a ridge (region 2) and of region-3 cuticle, normally occurring only in the fifth, sixth and seventh abdominal segments, has been studied in the pupa.

2. Grafts containing presumptive regions 1, 2 and 3 developed these regions when transplanted to an equivalent position in the fourth segment.

3. Grafts containing only presumptive region 1 formed also region-2 cuticle and region-3 cuticle when transplanted to the anterior part of the fourth segment.

4. Grafts containing only presumptive region 3 formed also region-2 cuticle and region-1 cuticle when transplanted immediately posterior to the bristle B_1 in the fourth segment.

5. The results support the concentration-gradient concept. They reveal that the gradients in the fourth and the sixth segments are of the same nature and that the

difference in the cuticular structure of these segments must result from a difference in competence. The concept of determination is discussed in relation to these results.

EDITOR'S NOTE

Dr Hildegard Stumpf met her tragic death shortly after this paper had been submitted and before necessary alterations in the text could be made. Dr P. A. Lawrence, who had helped Dr Stumpf with the English of the original paper, kindly made himself responsible for these small alterations. Reprints can be obtained from Dr Lawrence, Department of Genetics, Milton Rd, Cambridge, England.

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EXPLANATION OF PLATE

PLATE I

Fig. 1. Normal sixth segment with its three regions of different cuticular structure, numbered from the posterior to the anterior; vertical dark structure: dorsal midline.

Fig. 2. Normal fourth segment.

Fig. 3. Experimental ridge pattern: see Text-fig. 2.

Fig. 4. Result of Expt. I: fourth segment with graft exhibiting ridge and region-3 cuticle.

Fig. 5. Result of Expt. II a: fourth segment with graft exhibiting ridge and region-3 cuticle.

Fig. 6. Result of Expt. II b: fourth segment with graft exhibiting ridge and region-3 cuticle.

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