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A GRADIENT OF POSITIONAL INFORMATION IN AN INSECT, *RHODNIUS*

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SUMMARY

Locke discovered a segmental gradient in *Rhodnius* which controls the polarity of the epidermal cell and gives positional information. The polarity is expressed by the orientation of folds in the adult epicuticle, which are aligned parallel to the contours in the gradient. It was later suggested that this gradient could be of a concentration of a diffusible substance. Because concentration gradients could be maintained in various ways we have simulated several models in the computer, and examined the results of rotating square pieces of model landscape through 90° and allowing diffusion. The gradient landscapes after different times and at equilibrium are plotted as contour maps and are compared with cuticle patterns from adult insects after rotation of square pieces of epidermis in larvae.

One simple model, where the gradient depends only on the activities of a line of source cells at one end of the segment and a line of sink cells at the other, is eliminated by 2 observations: (1) the theoretical and experimental patterns are consistently different; and (2) when adults developing from operated larvae are made to form a supernumerary cuticle the first and second cuticles have almost identical patterns. This suggests that the gradient landscape has reached a steady state.

In another model the cells are considered to act as homeostatic units in the gradient, and when moved to a new position they each attempt to maintain their original or 'set' concentration. Simulation of this model gives equilibrium patterns which are similar to the experimental results. It is suggested that the cells become 'set' at some stage in the cell cycle to the ambient concentration. This hypothesis predicts that after reaching initial equilibrium the pattern should change only if there are cell divisions. Adult insects are made to moult again under different conditions and it is found that pattern change is correlated with cell divisions.

Locke also observed an asymmetry in the patterns after rotation of squares through 180°. Simulation showed that such asymmetry would result from each cell acting as a better homeostatic unit when moved one way in the gradient (for example when acting as a sink) than when moved the other (acting as a source).

We do not claim that these comparisons eliminate all other classes of model, and present our conclusions in as general a form as possible.

INTRODUCTION

Transplantation experiments on many embryos and regenerates have shown that early in development the fate of each part is subject to its position in the whole; the proper organization of the whole depending therefore on exchange of information between the parts. Thus, cells in a developing organ may have access to *positional information* (Wolpert, 1969), information which tells the cells where they are. Analysis of several developing structures has shown that the 2 or 3 main axes of the organ become determined at different times (e.g. Harrison, 1921; Copenhaver, 1926;

Székely, 1954) and it is therefore likely that positional information is set up independently in those axes.

Experiments on the insect segment have demonstrated an axial gradient of positional information which determines both polarity and the developmental fate of the epidermal cell (reviewed in Lawrence, 1970, 1971, 1972). This gradient was discovered by Locke (1959, 1960) who investigated the control of orientation of folds or 'ripples' in the adult cuticle of *Rhodnius*. By means of a series of transplantation experiments he demonstrated that the cells forming the ripples showed a graded difference down the axis of the segment. When cells from different positions in the segment were experimentally placed together, they interacted in relation to the amount and the sign of this difference. These interactions were expressed in the altered orientation of the ripples. Locke's discovery has been followed by studies on other insects and led to hypotheses as to the nature of the gradient. In particular, it has been shown that the gradient behaves similarly to a concentration gradient of a diffusible substance (Stumpf, 1965*a*, *b*, 1966, 1967, 1968; Lawrence, 1966). In this view the ripples are laid down approximately parallel to contours of constant concentration in the gradient. The ripples are not themselves contour lines but indicate the local polarity. As they do not express the *value* associated with each contour, a circular set of ripples may represent, for example, a gradient landscape shaped either like a hill, a hole or a volcano.

Our approach has been to repeat and extend Locke's experiments on *Rhodnius* and to compare our results with computer simulations of diffusion gradients maintained in different ways. Our aim is to show that a diffusion gradient is an adequate model, and to illustrate that a comparison between theory and experimental results may eliminate classes of model, and suggest new types of experiments. We present evidence against a simple model where the concentration gradient is maintained solely by the activities of the cells at the segment margins, and propose that in addition the cells of the segment are set to a certain value of concentration and attempt to maintain that level when moved. Each cell becomes reset to the local value of the concentration at some stage in the cell cycle. Here there are 2 interdependent gradients; one of concentration of a diffusing substance, and the other of the 'set' or determined values in the cells.

MATERIALS AND METHODS

The insects were kept at 29 ± 0·5 °C and fed on a rabbit. *Rhodnius* is a blood-sucking insect, each large meal initiating a moult cycle, during which the epidermal cells divide and then construct the cuticle of the next stage (Wigglesworth, 1933). In the 5th-stage larva the mitotic period lasts from about the 5th to the 9th day after feeding. The cuticle of the larva does not bear oriented ripples like the adult, but has numerous bristles. Most operations were performed on the tergal integument, where almost all the bristles degenerate at metamorphosis. In the unfed 5th larval stage the abdominal segments are about 1 mm from anterior to posterior margin; that is equivalent to a line of about 100 cells. The adult segments are about 1·8 mm across.

Fig. 1 summarizes the experimental procedures: when described as *before feeding* the operation was performed on a 5th-stage larva (which was given a small meal) one week before feeding a large blood meal. Ecdysis (the casting of the old cuticle) to adult followed the large meal in 17 ± 0·3 days (S.E.M.). Operations performed *after feeding* delayed the time of ecdysis by about 2 days (19 ± 0·5 days from the meal). Operations on 3rd- and 4th-stage larvae were carried out 1 week before feeding a large blood meal.

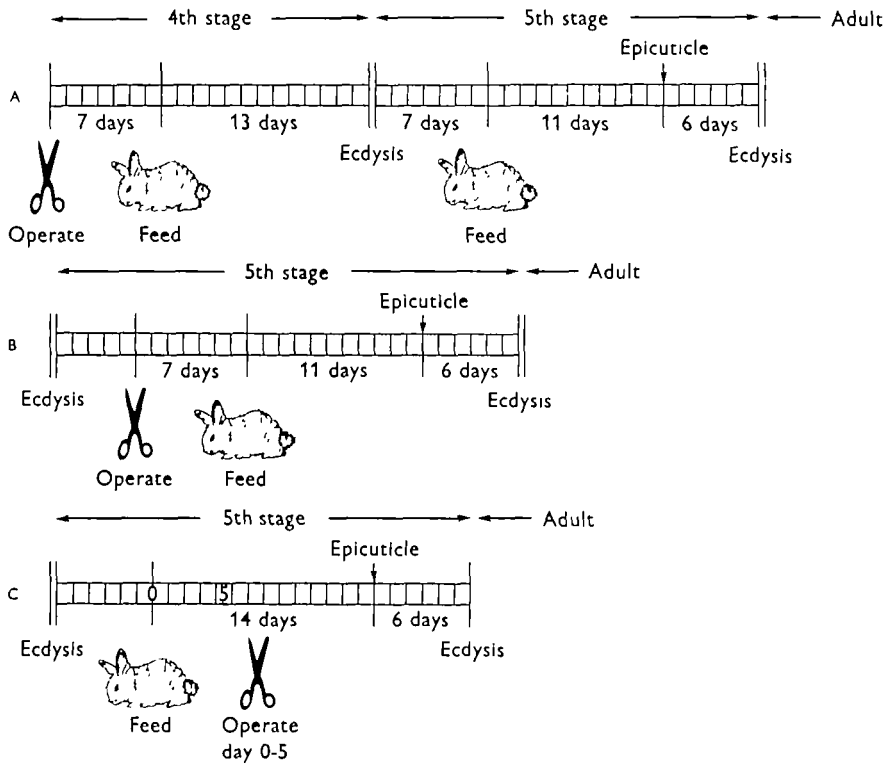

Fig. 1. Experimental procedures: A, operations on 4th-stage larvae; B, operations *before feeding* on 5th-stage larvae; and C, operations *after feeding* on 5th-stage larvae.

The insects were anaesthetized by immersion in water for about 5 min, and square pieces of cuticle and attached epidermis were cut with a piece of broken razor blade and fine Trident scissors (Curtin Scientific Co., Texas). The cut squares were rotated or transplanted without disturbing the underlying fat body and became sealed in place with dried blood. The cast cuticles were soaked in 70% ethanol, the adult integument boiled in 10% potassium hydroxide and both were mounted in Euparal. In some cases the epidermis and cuticle were fixed in Carnoy's fluid and stained in Hansen's trioxyhaematin.

Ecdysterone (Rohto Pharmaceuticals) was dissolved in 10% ethanol and 1 μl of the solution injected into the body cavity via a leg. The density of the epidermal cells in adult *Rhodnius* was measured by counting all nuclei in 6 sample areas (each of $6{\cdot}38 \times 10^{-3}$ mm^2) in particular regions of the graft, and 4 such areas in the host.

The angles (α and θ, see Fig. 6, p. 824) of the adult ripple patterns and from simulations were measured with a ruler and protractor; in order to reduce bias in this rather subjective method, all data were gathered from both natural and simulated patterns before comparative graphs were drawn.

COMPUTER METHODS

Diffusion alone (*Model* 1). An array of 100 × 100 points was set up, a line at one end being held at unit concentration and a line at the other at zero concentration. Diffusion results in a linear gradient of concentration. The experiment is simulated by, for example, rotating a 21 × 21 square section in the middle of the gradient through 90° or 180°. The set of points in this square

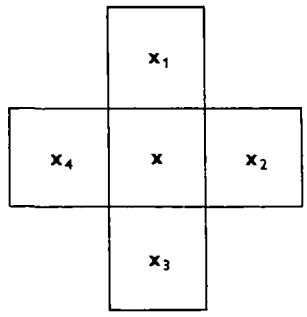

Fig. 2. Orthogonal neighbours of a point $\mathbf{x}$.

will be denoted by S. An analytic solution is available for the concentration F at the point $\mathbf{x}$, from an instantaneous point source of amplitude M at the point $\mathbf{z}$:

$$F(\mathbf{x}-\mathbf{z},\, M,\, t) = \frac{M}{4\pi Dt}\exp(-|\mathbf{x}-\mathbf{z}|^2/4Dt), \tag{1}$$

where D is the diffusion constant, and t is the time (Crank, 1956).

For calculation, the background linear gradient λ is removed to give an initial concentration landscape ϕ which is zero everywhere, except in S. The concentration at $\mathbf{x}$ at time t is then found by summing (1) over all points in S and adding back the linear gradient:

$$C(\mathbf{x},\, t) = \lambda\,(\mathbf{x}) + \sum_{\mathbf{z}\,\epsilon\,S} F(\mathbf{x}-\mathbf{z},\, \phi\,(\mathbf{z}),\, t), \tag{2}$$

where $\phi\,(\mathbf{z})$ is the initial concentration at the point $\mathbf{z}$. From this a contour map is plotted.

Diffusion plus homeostasis (*Model* 2). Each cell is now considered as a homeostatic unit, capable of acting as a local source or sink attempting to maintain its original or 'set' concentration. The diffusion equation applying is

$$\frac{\partial C}{\partial t} = D\left(\frac{\partial^2 C}{\partial x^2} + \frac{\partial^2 C}{\partial y^2}\right) - K(C - C_0), \tag{3}$$

where K is a constant and $C_0\,(\mathbf{x})$ is the set concentration of the cell at $\mathbf{x}$. This gives Model 2.

The equation (3) can be modified to give 2 equations with K_1 for $C > C_0$ and K_2 for $C < C_0$, corresponding to a model where the source and sink activity of a cell are not equal (Model 2A). We think this approximation is sufficient, even though it is likely that K would, in fact, vary continuously with C.

No simple analytic solution is available when $K \neq 0$, so the following reiterative method of solution is used: Let $\mathbf{x}_1, \ldots, \mathbf{x}_4$ denote the 4 orthogonal neighbours of a point $\mathbf{x}$ (Fig. 2) and let δt denote the time interval between successive recomputations. Then

$$C(\mathbf{x},\, t+\delta t) = C(\mathbf{x},\, t) + \delta t\left[\frac{D}{h^2}\sum_{i=1}^{4}\{C(\mathbf{x}_i,\, t) - C(\mathbf{x},\, t)\} - K(C(\mathbf{x},\, t) - C_0\,(\mathbf{x}))\right], \tag{4}$$

where h is the distance between neighbouring points in the 100×100 array.

The validity of this finite difference method was tested by comparison with the analytic solution for the special case where $K = 0$. For a sufficiently small δt very good agreement was obtained.

Cell division. According to Model 2, the effect of cell division is to cause the resetting of the homeostatic mechanisms of the daughter cells, so that the set concentrations (C_0) are made equal to the ambient concentration at the time of division. This is simulated by taking a sample of points and setting $C_0\,(\mathbf{x}) = C\,(\mathbf{x},\, T)$ for all $\mathbf{x}$ in this sample, where T is the time of cell division, and then calculating as above.

In the calculations no attempt was made to allow for the growth of the graft (which varied according to how much it stretched with the host), migration of cells towards the cut, or uneven distribution of mitoses.

Diffusion plus active transport (*Model* 3). For this model each cell moves a certain quantity of material to its anterior neighbour in each time interval, and the diffusion calculations are then made as above.

RESULTS

The adult cuticle of *Rhodnius* bears several types of oriented structures. Most of the dorsal surface of the abdominal cuticle is covered by mediolaterally oriented folds or ripples, 20–40 μm wide (Fig. 11). V. B. Wigglesworth (personal communication) is investigating the formation of cuticle in *Rhodnius*. He finds that the sheet of outer epicuticle is sculptured by changes of shape in the apices of the epidermal cells, and by the distension of vacuoles which locally deform the underlying cytoplasm. This structure is then stabilized by the secretion of the more substantial inner epicuticle. The orientation of ripples in the adult is first detectable as an elongation of the apices of the cells in the mediolateral axis. It may be sufficient to regard the orientation of the ripples as resulting from the polarized but independent action of the epidermal cells.

We have also used a mid-ventral strip of abdominal cuticle, which bears small epicuticular tubercles which point posteriorly; one tubercle is secreted by each cell (Fig. 16). There may be other more inconspicuous indicators of polarity, for example the orientation of the first unidirectional layer of endocuticular fibres (Neville, 1967; Caveney, 1971), or possibly the orientation of organelles such as microtubules in the cell.

Polarity and the local slope of the gradient

It is our central thesis that the polarity of the epidermal cell, as expressed in the orientation of the epicuticle, is locally determined by the steepest slope of the segmental gradient (Lawrence, 1966). The 2 following experiments illustrate how this local slope can be altered.

In an unfed 5th-stage larva an anterior rectangle from one ventral segment was exchanged with a posterior rectangle from an adjacent segment. The grafts were placed in their normal orientation (Fig. 3). The model predicts that diffusion will produce 2 areas where the direction of slope is reversed and where the tubercles should therefore point towards the anterior margin of the segment (Fig. 3). When this operation was done 2 such regions were found in the adult at the host-graft junction as predicted (Fig. 17). In control experiments the exchange of squares at equal levels in 2 segments caused no alteration of polarity.

In the second experiment a central square was rotated through 180° *before feeding*. If the anterior margin of the segment is the top of the gradient (say) the theory predicts that after rotation and diffusion the gradient landscape in the graft will consist of an anterior hollow and a posterior hill. The tubercles should point into the hollow and out from the hill. The operations, when performed both on 4th- and 5th-stage larvae, gave adult tubercle patterns very similar to the predicted pattern (Fig. 4). These results illustrate the dependence of polarity on the local slope; they provide a basis for our subsequent analysis of ripple patterns.

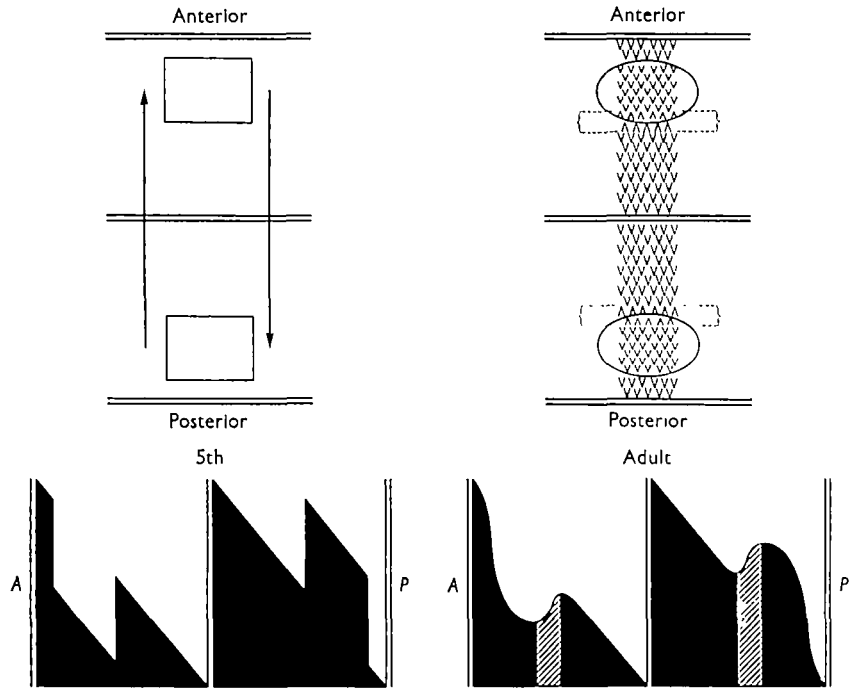

Fig. 3. Experiment illustrating the dependence of polarity on the direction of gradient slope. The operation was performed on the sternite of a 5th-stage larva (left) and the result shown diagrammatically on the right. Cross-sections of the gradient landscapes are indicated below. Note the regions where the gradient slope is reversed as a result of local diffusion. Brackets indicate where the oriented tubercles point towards the anterior margin (*A*) instead of towards the posterior (*P*). (Compare Figs. 16, 17.)

Temporary alterations in polarity

Cuts. The orientation of ripples can be altered if small areas of epidermis are killed shortly before cuticle deposition. The effect on the adult ripple pattern was found to correlate best with the number of days elapsing between wounding and ecdysis. The period from feeding to ecdysis in wounded insects was $18 \pm 0{\cdot}3$ days. As described by Wigglesworth (1937) we found that the cells migrate rapidly to a cut and accumulate along the line of damage in about 24 h. The cells nearby become oriented with their long axes towards the cut, and mitoses occur later in these rather sparse regions. Cuts within the segment done 6–9 days before ecdysis resulted only in large areas of wound cuticle, but interesting changes in the orientation of ripples occurred when the cuts were made 9–15 days before ecdysis. Cuts parallel to the mediolateral axis had little effect on the pattern: usually there was a narrow strip of wound cuticle flanked by ripples in the normal orientation (Fig. 12). Cuts in the direction of the antero-posterior axis, however, affected some ripples nearby which tended to turn to run parallel to the antero-posterior axis and to the cut (Fig. 13).

When squares were removed after feeding and replaced without change in orientation, these effects of wounding were also seen as a tendency for some ripples to run parallel

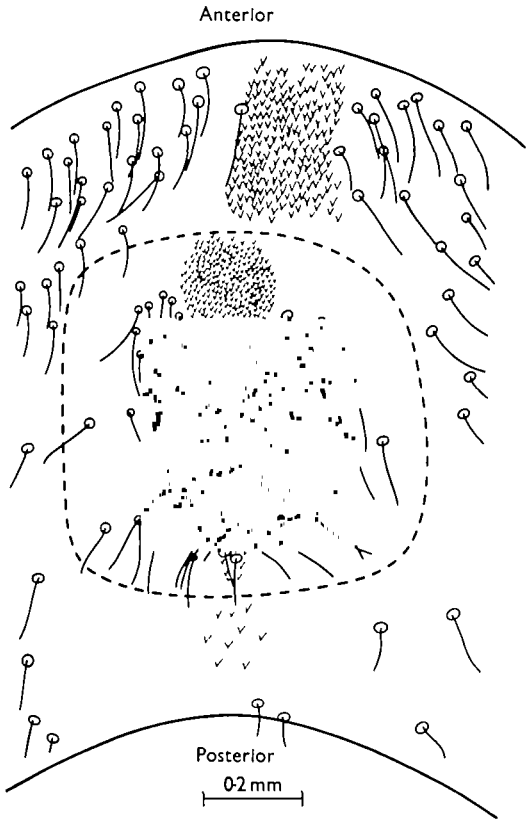

Fig. 4. Scale drawing of mid-ventral region of an adult segment after 180° rotation of a graft in 5th-stage larva *before feeding*. The host graft border is indicated by the dashed line. Note orientation of the graft bristles has returned to normal, but tubercles (indicated, not drawn individually) in the centre of the graft point anteriorly. In 2 regions of the graft one anterior and the other posterior, the graft tubercles point posteriorly.

with the antero-posterior cuts. Such control operations performed either before feeding or within 2 days after feeding had only a trace effect on the adult cuticle.

The effect of cuts across the intersegmental membrane was also studied. Again, cuts performed before feeding had little effect on the adult pattern, but after feeding they altered the orientation of ripples over a large area. Ripples were deflected towards the cut part of the intersegmental membrane, whether the cut extended completely between 2 segments (Figs. 14, 27) or whether it only included one margin of the segment. The maximum effect on ripple orientation occurred following cuts made 9–12 days before ecdysis. When the cut was made less than 9 days before ecdysis only wound cuticle was formed, and cuts made earlier than 12 days before ecdysis healed almost completely. In spite of the much more extensive effects of cuts across the intersegmental membranes, they healed about as fast as cuts within the segments, and if made within 2–4 days after feeding left almost no trace in the adult cuticle.

Burns. Following Locke (1967) regions (100–400 μm in diameter) of the epidermis were burnt at different times after a moulting meal. The epidermal cells react

differently to cuts and burns, responding only slowly to a burn, migrating into the killed region for 2–3 days. Mitoses were found mostly among these migrating cells (compare Wigglesworth, 1937). Burns performed less than 6 days before ecdysis had no effect on the epicuticle, which had already been deposited by then. Burns more than 9 days before ecdysis left very little trace. Burns between these 2 times resulted in a pattern of centrally converging ripples at the periphery of the affected region, these becoming thinner nearer the centre where only wound cuticle was found (Locke, 1967). The same pattern is found when such burns are made in the centre of rotated grafts. One or two burns at a critical time of about 6·5 days before ecdysis produced cuticle with an abrupt transition from wound cuticle to ripple cuticle, there being no deflection of the ripples.

90°-*rotation experiments*

Experimental results. Square regions of cuticle and epidermis were excised, rotated through 90° and reimplanted. The resulting S-shaped ripple patterns were examined in the adult. We find that when the operation is done later in the life cycle the S-shaped curve in the adult is steep, with a small angle (α, Fig. 6) between the central ripples and the antero-posterior axis. The earlier the larval stage in which the operation is performed the smoother the S-shaped curve and the larger the angle α in the adult. During the period intervening between the operation and the adult stage, the polarities of the cells in the graft and the host nearby are progressively changing towards the normal situation. This process can properly be termed *regulation.*

When the 90° rotation was performed more than 6 days *after feeding* on 5th-stage larvae, the results were complicated by wounding effects. There was a region of wound cuticle, which formed no ripples at all around the host-graft junction. The central region had ripples which were almost parallel to the antero-posterior axis. Following operations performed 0–5 days *after feeding* the area of wound cuticle was often small, and sometimes lacking. Here the patterns were well formed (Fig. 19) and measurements made of the angle α in 41 cases gave a mean of $11 \pm 2°$.

When the 90° operation was made *before feeding* the patterns were more regulated (Figs. 20, 15A, 18A) and from 51 cases the mean angle α was $26 \pm 1°$.

90°-rotation operations on 4th-stage larvae produced more regulated adult patterns, 11 cases having a mean angle α of $43 \pm 3°$ (Fig. 21). Following rotation in the 4th, the amount of regulation could be estimated in the 5th-stage larvae, by measuring the angle of bristle orientation in the centre of the graft in the cast skins (Fig. 5). *In situ* the bristles mostly point posteriorly, that is at about 90° to the ripples. In nine 5th-stage cast skins resulting from operated 4th-stage larvae, the mean angle made between the long axes of the bristles and the antero-posterior axis was 67°, corresponding to an angle, α, of $23 \pm 4°$. In one case of a 90°-rotation operation performed on a 3rd-stage larva, the orientation of the bristles in the 4th-stage corresponded to an α of 20°, and in the 5th stage to 35°. The angle α for the ripple pattern of the adult was 63° (Fig. 22). This experiment confirmed that the polarity changes progressively with each larval stage.

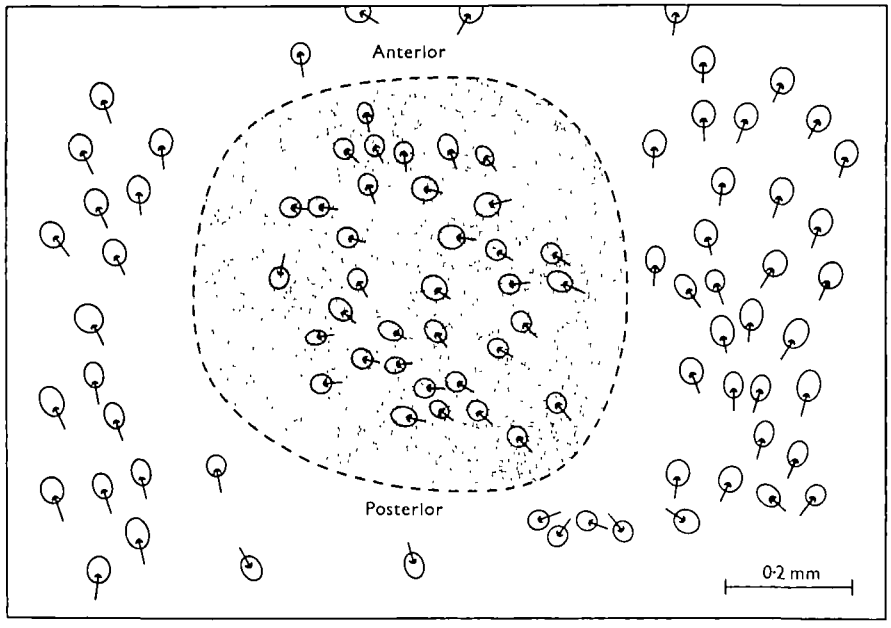

Fig. 5. 5th-stage cuticle following 90° rotation of graft anticlockwise on dorsal cuticle of 4th-stage larva. Drawing traced from a photograph to show the orientation of bristles in the graft (shaded) and the host. The bristle-free region near the host-graft border (indicated by the dashed line) is due to wounding.

Computer simulations – a comparison with the experimental patterns. Stumpf (1965*a*, *b*, 1966) and Lawrence (1966) both independently pointed out that the insect segmental gradient behaves as a concentration gradient of diffusible substance. The changing patterns produced by 90°-rotation operations could represent continuing diffusion. We report here comparisons between the results of simulations of diffusion gradients of different kinds, and the experimentally produced ripple patterns.

Concentration gradients might be maintained in a number of ways; for example (Model 1), there might be a source at one margin of the segment and a sink at the other (Stumpf, 1967; Crick, 1970). Here the source cells maintain the concentration of the substance (the morphogen) at a high level, and the sink cells, at the other end of the segment, destroy it. The morphogen could diffuse passively through the epidermal cells. Rotation of a square section of this gradient results in diffusion until the original smooth linear gradient is re-established, a process which passes through a continuous succession of characteristic landscapes of concentration, which can be expressed as a series of contour maps (see Computer methods).

Because the effects of rotation operations persist for such a very long time, Lawrence (1966) postulated that the cells themselves generate 'some resistance to the diffusion of the substance' and we have examined a model (Model 2) in which, in addition to the source and the sink at the margins, we have postulated that each epidermal cell attempts actively to maintain its own internal or 'set' concentration of the morphogen. When transposed, the cell can be either a source or a sink depending on the ambient

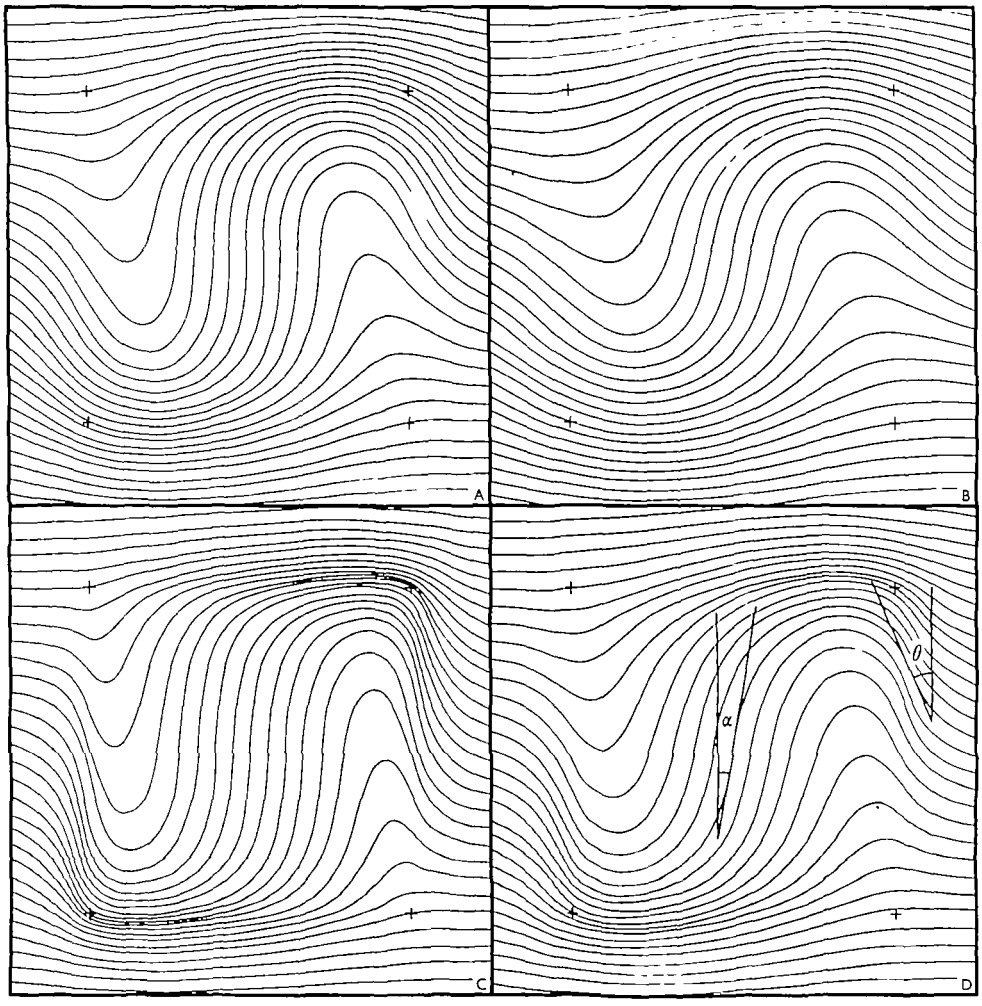

Fig. 6. Results of computer simulations. Contour maps after 90° rotation anticlockwise of the piece of gradient landscape delimited by the 4 crosses.

6A, B. Model 1, pattern after 2 different time intervals: A, $t = 60$; B, $t = 100$.

6C, D. Model 2, equilibrium patterns: C where the homeostatic ability of the cells is high ($K = 20$) and D where it is lower ($K = 10$). 6D, the 2 angles used are indicated, α being the angle between the central contour and the antero-posterior axis and θ that between the same contour at the periphery and the axis.

concentration in its new situation. It will reach an equilibrium concentration somewhere between its former level and the ambient concentration in its new environment. Over the whole rotated area, and in the host nearby, a new stable concentration landscape will become established. The nature of this equilibrium pattern depends on the value of the homeostatic constant K (see Computer methods). Where K is high the transposed cells will tend to maintain their original concentration, either because they are strong synthesizers or destroyers of the morphogen, or because the intercellular flux of morphogen is low. Where K is low the cells will take up a concentration more

nearly appropriate to their new position and the equilibrium pattern will approach the original linear gradient. In our computations we have assumed that the time available is long enough for equilibrium to have been reached.

The effect of a 90° rotation of a square region of the gradient of type Model 1 is not stable and diffusion begins immediately to modify it so that the contour map changes continuously until the original gradient is re-established. Contour maps of the gradient landscape at different time intervals were provided by the computer and compared with steady-state patterns produced by different levels of the homeostatic constant K in Model 2 and with the experimental results (compare Figs. 6 and 20). In particular, 2 angles were measured and the relationship between them plotted (Fig. 7). Model 1 results in patterns in which the centre of the pattern remains unchanged at first ($\alpha = 0$, Fig. 6A), while the peripheral contours change direction rapidly (measured by angle θ) (Fig. 7). By contrast, the dynamic equilibrium patterns from Model 2 show an alteration in the angle made between the central ripples and the antero-posterior axis (α), concomitant with small changes in the direction of ripples at the periphery (θ). When the points from the patterns in *Rhodnius* adults are entered in the graph (Fig. 7A) it becomes quite clear that these patterns are significantly different from those produced by Model 1 and more closely resemble those produced by Model 2 (see Table 3 for a detailed comparison).

Regulation of patterns. We have attempted to distinguish between transitory and steady-state patterns in another way. Adults which had been operated on as 5th-stage larvae were kept for some weeks, fed and then injected with 5 μg of a moulting hormone, ecdysterone. These individuals produced a second adult cuticle underneath the first. Any disturbance in a gradient maintained as in Model 1 would diffuse away with time, so that a comparison between the first and second adult cuticles should show some difference. It was found, however, that the 2 cuticles from an individual always bore remarkably similar patterns (Fig. 15) and measurement of the angles α and θ confirmed that there was very little difference between the first and second cuticles (Table 1). Between 3 and 6 weeks elapsed between the deposition of the 2 cuticles. The simplest interpretation of these experiments is that the patterns indicate a steady-state in the gradient landscape. They show that regulation is not solely a function of time and that other events are required.

It was noted by Locke (1959) that the patterns in the adult resulting from operations made in 4th-stage larvae were different from those resulting from later operations. This observation has been quantitatively confirmed: the measured angle of the central ripples (α) is $43 \pm 3°$ for operations in 4th-stage larvae, $26 \pm 1°$ for operations *before feeding* in 5th-stage larvae, and $11 \pm 2°$ for operations made *after feeding* in 5ths. If, as we suggested, the patterns represent a steady-state in the gradient landscape, we must ask what causes this regulation. One explanation might be that the operations themselves are not comparable; the wounding might be more extensive in operations done in the 4th stage, and certainly the graft is smaller. However, the angle of the bristles in the 5th stage after 90° rotation experiments in the 4th stage (equivalent to an α of about $23 \pm 4°$) suggested that regulation does continue in the moult from the 5th stage to adult (where $\alpha = 43 \pm 3°$). It seems that there are events in the 4th–5th and 5th–

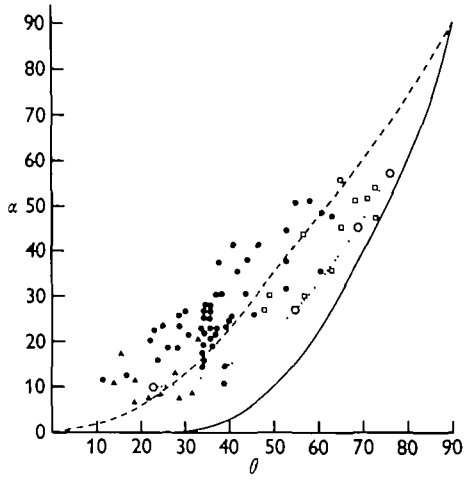

Fig. 7A

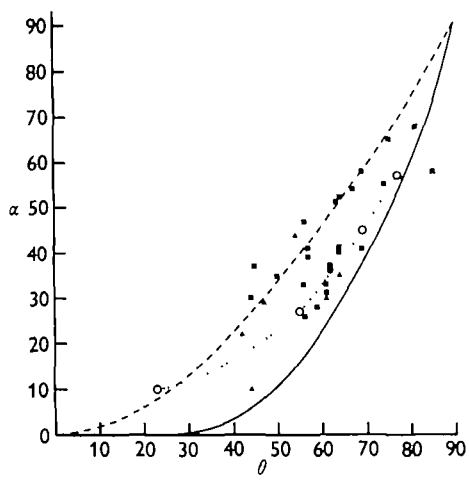

Fig. 7B

Fig. 7 A, B. The relationship between angles α and θ (see Fig. 6 D) in patterns simulated from the models and in the experimental results.

Theory: Solid line is drawn through a number of points taken from simulations for Model 1; dashed line the same for Model 2. The dotted line shows the results of simulating Model 2A where K has 2 values (14 when $C > C_0$ and 9 when $C < C_0$). The first point on this curve (open circle), with a minimal value for α, is the basic equilibrium pattern and the other 3 points are successively derived from it by resetting the cells in the calculated area (see Computer methods). Since each asymmetric pattern has 2 angles θ, the mean of both is plotted.

Experiments: The points are from individual patterns.

Fig. 7A, patterns in adult cuticle after operations on larvae. △, operated as 5th-stage larvae *after feeding*; ●, operated as 5th-stage larvae *before feeding*; □, operated as 4th-stage larvae.

Fig. 7B, patterns in adult cuticle after supernumerary moult with mitoses. ▲, second adult cuticle when operated as 5th-stage larva *after feeding*; ■, second adult cuticle when operated as 5th-stage larva *before feeding*.

adult moult cycles which lead to regulation; events which are lacking in the adult–second adult supernumerary moult.

Because the induction of moulting in adult insects by injection of ecdysone has not been reported before, little is known about the processes occurring in the epidermal cells. The epidermis of adult insects was therefore fixed and stained on different days after injection of 5 μg ecdysterone. By 1 day after injection the cells had deeply staining cytoplasm and enlarged nucleoli, and by 48–72 h deposition of the new cuticle had begun. No cell divisions were observed and no increase of cell density occurred (Table 2); apparently the very high dose of ecdysterone had led to premature separation of the epidermis from the old cuticle prior to cell division (Williams, 1968).

Cell division is a feature of all normal moult cycles in *Rhodnius* (Wigglesworth, 1940), although because of cell death (Wigglesworth, 1942) and the brevity of mitosis (Lawrence, 1968) it is difficult to estimate how much occurs. The most conspicuous difference between the normal moult cycle and the supernumerary adult moult was the absence of cell divisions in the latter. *We therefore postulate that pattern regulation is*

Table 1. *Results of* 90°*-rotation operations* (*means* ± S.E.M.)

Time of operation		*n*	Mean α	Mean θ	Change in α in individuals	Change in θ in individuals
5th stage *after feeding*		41	11 ± 2°	27 ± 2°		
5th stage *before feeding*		51	26 ± 1°	37 ± 1°		
4th stage		11	43 ± 3°	63 ± 3°		
5th-stages operated *after feeding* injected as adults	First cuticle	6	14 ± 3°	27 ± 5°	1·5 ± 4°	6 ± 3°
	No mitoses					
	Second cuticle		16 ± 5°	33 ± 6°		
	First cuticle	6	11 ± 2°	28 ± 3°	17 ± 4°	24 ± 5°
	Mitoses					
	Second cuticle		28 ± 3°	52 ± 4°		
5th-stages operated *before feeding* injected as adults	First cuticle	11	20 ± 3°	37 ± 2°	−1 ± 2°	2 ± 1°
	No mitoses					
	Second cuticle		19 ± 3°	38 ± 2°		
	First cuticle	26	25 ± 2°	42 ± 2°	18 ± 2°	21 ± 1°
	Mitoses					
	Second cuticle		43 ± 2°	63 ± 2°		

n = number of individuals.

Table 2. *Means* (± S.E.M.) *of cell density* (*cells*/*mm*^{2} × 10^{-2}) *in graft and host regions of adults*

	Controls first adult cuticle		Second adult cuticle after NO MITOSES		Second adult cuticle after MITOSES	
	n		*n*		*n*	
Density in graft	16	80 ± 3	12	78 ± 8	16	88 ± 3
Density in host		74 ± 3		78 ± 2		86 ± 3

All adults suffered 90°-rotation experiments as 5th-stage larvae *before feeding*.
n = number of individuals.

dependent on cell divisions. One simple hypothesis is that intercellular diffusion could only occur in association with cell divisions. But this situation would produce patterns identical to those predicted for Model 1. We therefore return to Model 2 and *suggest that the homeostatic level of each cell is reset at some stage in the cell cycle to the ambient concentration at that time.*

Wigglesworth (1940) by joining adults to moulting 5th-stage larvae in parabiosis, succeeded in making adults moult again, with cell divisions. An attempt was therefore made to achieve the right balance of ecdysterone in the supernumerary moult. From tests of a wide range of doses we found that mitoses could be induced by 2 successive injections of 0·4 μg on the first and fourth day after feeding the adults. Numerous mitoses (mitotic index being up to 10 %) were seen in such individuals when fixed on the fifth or sixth day. An injection of 2 μg of ecdysterone on the seventh day after

Table 3. *Comparison between resetting in theory and regulation in the experiments*

	Precursor pattern		Basic pattern		1st reset		2nd reset	
	α	θ	α	θ	α	θ	α	θ
Theory								
Model 2 $K = 15$	6	20	11	28	25	52	39	66
Model 2 $K = 11$	—	—	11	26	29	55	48	68
Model 2A when $C < C_0$ $K = 9$ when $C > C_0$ $K = 14$	—	—	10	23	27	55	45	69
Experiments								
Time of 90° rotation	5th stage *after feeding*		5th stage *before feeding*		4th stage			
	α	θ	α	θ	α	θ		
Mean angle	11 ± 2	27 ± 2	26 ± 1	37 ± 1*	43 ± 3	63 ± 3†		
Adults made to moult again with cell divisions	1st cuticle		2nd cuticle					
	11 ± 2	28 ± 3	28 ± 3	52 ± 4†				
			1st cuticle		2nd cuticle			
			25 ± 2	42 ± 2*	43 ± 2	63 ± 2†		

* We have a weak explanation for the poor fit between θ for operations done *before feeding* in 5th-stage larvae and the theory (compare Fig. 7A). A cut in the antero-posterior axis tends to cause ripples to run parallel to it (p. 820) which makes θ difficult to measure (Figs. 19, 20) and tends to reduce it. Notice that where an extra moult cycle has intervened (†) there is good agreement for θ. Stated angle values are in degrees.

feeding caused deposition of the cuticle. The insects were fixed and stained on the eleventh day and the first and second cuticles compared. After this regimen the pattern on the second cuticle was always different from the first (Fig. 18), the mean change in the angle α (in adults operated as 5th-stage larvae *before feeding*) being $18 \pm 2°$ (Table 1, Fig. 7B). This change brings the patterns into the same range as those produced when 90° rotation operations are made on 4th-stage larvae (Table 1). Similarly, comparison of the first and second cuticles from adults operated as 5th-stage larvae *after feeding* gave a mean change in the angle α of $17 \pm 4°$, which brought this angle into the same range as when operations were made *before feeding* (Table 1, Fig. 7B). During and after the mitotic period many dying cells were seen, which may well explain why the numerous cell divisions did not significantly raise the cell density (Table 2). This observation supports Wigglesworth's suggestion (1942) that cell density is kept stable by means of controlled cell death.

These experiments thus show that the pattern change is correlated with cell divisions although they do not implicate any particular phase of the cell cycle.

Returning to the theory it becomes necessary to show that if the homeostatic level of the cells were reset at cell divisions to the ambient concentration, this could result in the observed change in pattern. To test this we first needed to estimate the number of cell divisions and the basic K value from which the later patterns are derived by resetting.

Both figures can be estimated only approximately, so there is little point in trying to see whether the theoretical change in α and θ fit precisely with the observed regulation of the patterns. We estimate that there is on average 1 mitosis per cell per larval moult cycle. In *Oncopeltus* the epidermal cell number increases by a little over twice per moult cycle (Lawrence, 1968) and the growth pattern in *Rhodnius* is probably similar. Our initial estimate of K is open to considerable doubt; there is certainly some cell division following the operation when done *after feeding* and this is difficult to measure accurately. Nor do all the cell divisions occur synchronously and yet this has been assumed, for simplicity, in our calculations.

In a series of simulations we began with an equilibrium pattern for $K = 11$ (Model 2) which resembled that produced by operating on a 5th-stage larva *after feeding* and had a value for α of 11°. Resetting each cell in the area to the ambient concentration and keeping K constant, a new equilibrium pattern was derived ($\alpha = 29°$). Taking this pattern and repeating the resetting calculations we derived a further pattern ($\alpha = 48°$). It is difficult to estimate the proportion of cells which divide following an operation made *after feeding*, but examination of stained preparations suggested a figure of less than 50%. If we are to include these divisions in our calculations we must begin with a higher value for K. Starting with a K of 15 and resetting 50% of the cells gave us a pattern rather similar to the equilibrium pattern for $K = 11$ with an angle α of 11°. Repeating the resetting steps as above, but keeping K at 15 gave us the angles of 25° and 39° (Table 3). These 2 series of angles can be compared with the means of α obtained from the results of 90° operations on the 2 periods in the 5th-larval stage, in the 4th-stage larvae and where adults undergo a supernumerary moult (Table 3, Fig. 7B). This comparison shows that, allowing for all the approximations involved, the resetting mechanism postulated is capable of causing the changes observed.

Asymmetry

180°-rotation operations. These operations, when performed *after feeding* in 5th-stage larvae produced ripple patterns usually consisting of 2 whorls of ripples which were either side of the midline of the graft. The anterior whorl was larger than the posterior. Following operations performed *before feeding* in 5th-stage larvae the effects of wounding had apparently disappeared, but as noted by Locke (1959), the anterior whorl was usually more conspicuous (Fig. 23).

When the operation was performed on 4th-stage larvae, the pattern was even more asymmetric, the posterior whorl having diminished considerably (Fig. 24) and sometimes being completely absent.

Our model of the gradient must clearly be compatible with the results of both the 90° and the 180° rotation experiments. The asymmetry of the patterns produced after 180° rotation presents a new problem. If for the purpose of the argument we let the

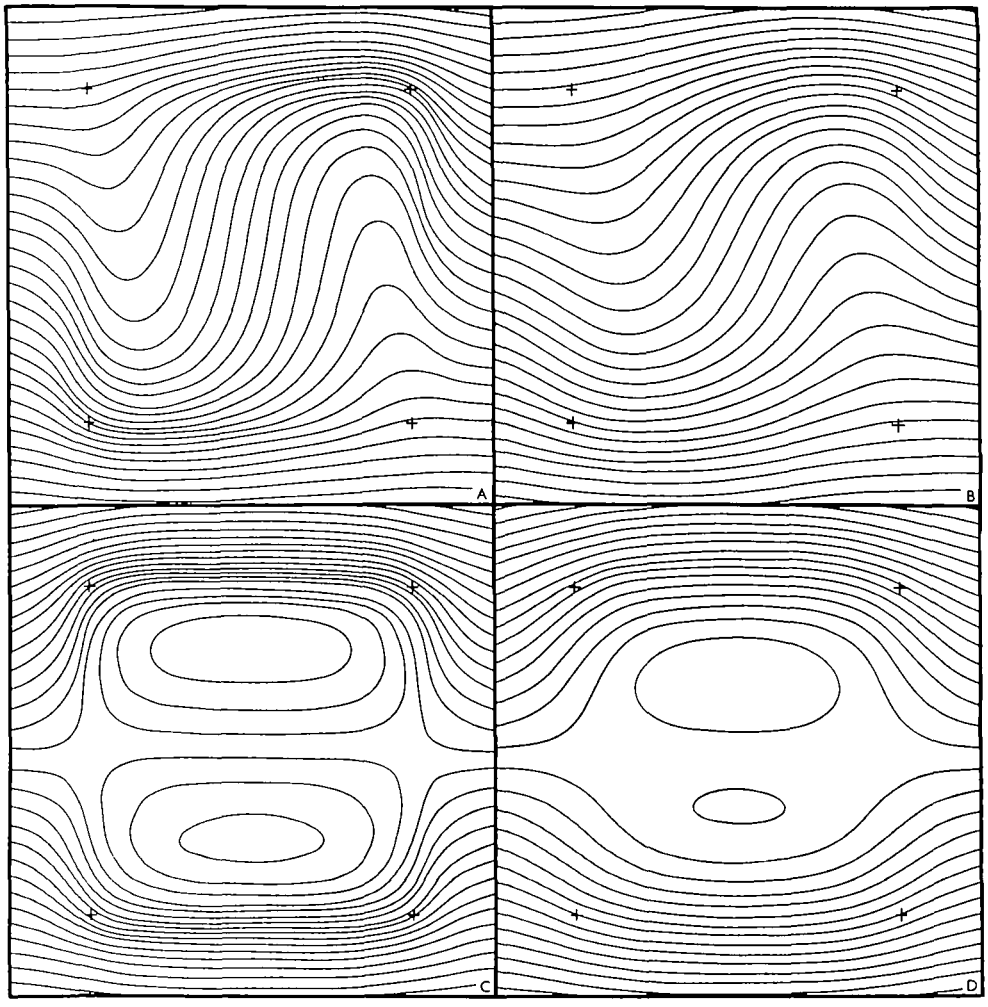

Fig. 8. Results of simulations of Model 2A where the homeostatic ability of the cells (K) is 14 when $C > C_0$ and 9 when $C < C_0$ (see Computer methods).
8A, C, first equilibrium patterns after 90° rotation anticlockwise (8A) and 180° (8C).
8B, D are patterns derived from 8A and 8C by resetting all the cells in the field (see text).
The crosses mark the original area rotated, the gradient is at highest concentration at the top of the page. (Compare with Figs. 20, 21, 23, 24.)

anterior end of the segment be the top of the gradient, where the concentration of the morphogen is high, rotation produces an anterior hollow and a posterior hill. The landscape produced initially has an almost symmetrical contour map. The asymmetry of the later patterns show that the hollow is better maintained than the hill. Any hypothesis designed to explain the asymmetry in these results after 180° rotation must be compatible with the absence of conspicuous asymmetry in the results of the 90°-rotation experiments.

Dr G. Mitchison suggested to us that Model 2 could be adapted (under the assumption made above) to make the cells more efficient sinks than sources, thus being well

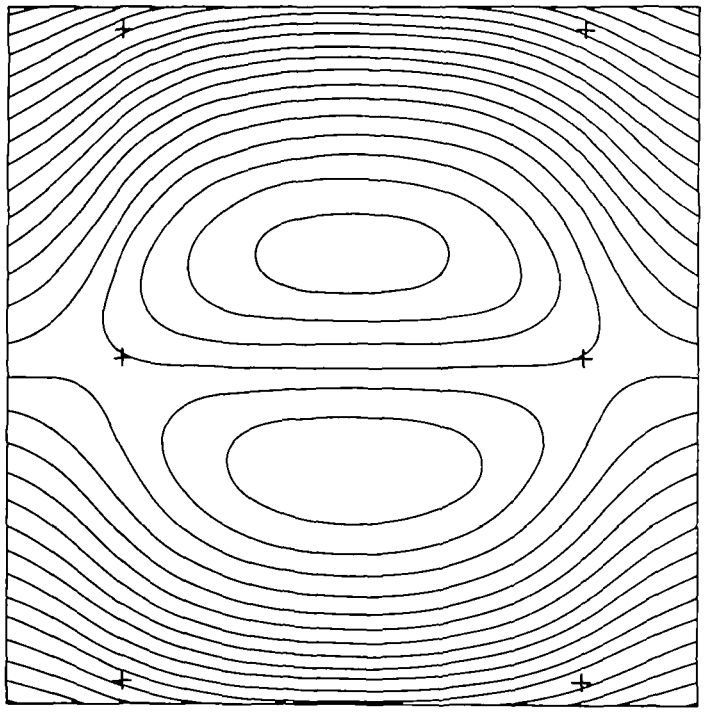

Fig. 9. Simulation of the exchange of 2 adjacent rectangles (bounds marked by the crosses) for Model 2A ($K = 14$ when $C > C_0$; $K = 9$ when $C < C_0$). The first equilibrium pattern has been reset once to give the pattern illustrated. (Compare Fig. 25.)

able to maintain a low concentration if transposed from the bottom to the top of the gradient, while anterior cells from the top of the gradient might not be so able to produce enough morphogen to deform the gradient if moved posteriorly. This feature was, therefore, added to the calculations (Model 2A) and it was found that starting with a pair of homeostatic constants $K_{source} = 9$, $K_{sink} = 14$, we could produce a series of patterns very similar to both the 90° and the 180° results (Fig. 8). It is a feature of the model that the relaxation steps produce more visible asymmetry in the 180°-rotation patterns than in the 90°-rotation patterns. There is one consistent discrepancy between the theory and experiments; following 180°-rotation experiments on 5th-stage larvae some ripples with unexpected orientation appear near the host-graft junction (Fig. 23, arrows). We think this anomaly results from wounding: we noted that ripples tend to turn 90° to run parallel with an antero-posterior cut and it is likely that after a 180° rotation the gradient is too flat in this region to repolarize the cells. The anomaly has disappeared following operations on 4th-stage larvae (Fig. 24).

Transposition experiments. In these experiments on 5th-stage larvae *before feeding* an anterior rectangle from one segment was exchanged with a posterior rectangle from the same or adjacent segment. It was clear that the posterior rectangle had a stronger effect when transposed anteriorly than the anterior moved posteriorly. A typical result is

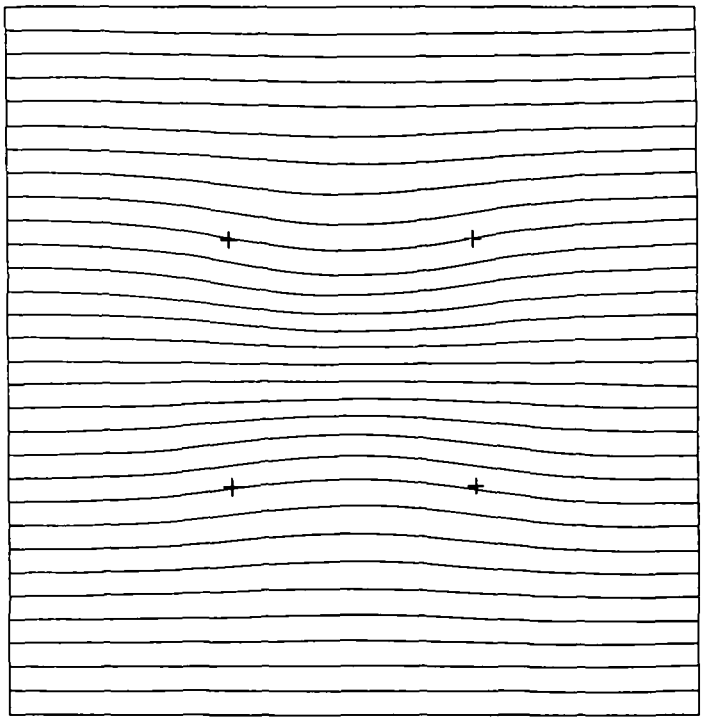

Fig. 10. Simulation of a graft from a 3rd-stage larva implanted on to a 5th-stage larva. Small piece (bounds marked by crosses) from a gradient landscape twice as steep as normal is implanted without change in orientation. The first equilibrium pattern has been reset once to give the pattern illustrated. (Compare Fig. 26.)

shown in Fig. 25 which illustrates further the asymmetry of the gradient, and can be compared with the equivalent simulation for Model 2A (Fig. 9).

Growth of the segment

It is intrinsic to our hypothesis that, as the insect grows, a gradient will be maintained between the 2 fixed margins. The segmental gradient in the 5th-stage larva (segments about 1 mm across) will thus be half as steep as that in the 3rd-stage larva (segments about 0·5 mm across). Transplantation between the 2 instars, without change in orientation, should therefore result in some interaction and change in ripple orientation. Pieces of epidermis from 3rd-stage larvae were transplanted in normal orientation on to 5th-stage larvae, and the result (Fig. 26) compared with the simulation (Fig. 10). The effect is small but the ripples do converge into the graft, as the contours do in the simulation.

Alternative models

Active transport. Are there other models which would fit the results as well as Model 2? In Model 3 we have considered that the gradient is maintained by the active transport of substance from cell to cell (Lawrence, 1966). There are many ways of

formulating this hypothesis; for example, the rate of transport could be either independent of, or dependent on, concentration; the direction of transport could be fixed or always oriented against the local slope of the gradient. Because the cells are packed together approximately in a hexagonal array, each cell does not have an anterior neighbour into which substance could be transported and it becomes difficult to see how transport could be unidirectional. Because of these difficulties, we have made only a preliminary study of Model 3. We have considered a gradient maintained by cells transporting a diffusing substance at a constant rate exactly equal to the back diffusion within the segment and simulated the effects of various operations. At equilibrium after a 90° rotation the calculations show that the angle α of contours in the centre of the rotated piece is always 45°. Since we usually observe angles less than this, either the reading of this kind of gradient must occur before equilibrium or there must be some alteration in pump strength or direction. Our experiments with several models of this type failed to produce patterns remotely like the observed ones so we did not pursue this approach further. The main reason for this failure is that transport models tend to maintain discontinuities of polarity at the host-graft border, whereas the experiments illustrate how discontinuities are soon smoothed away.

Cell migration. Locke (1959) suggested that the patterns could be affected by, or result from, the migration of cells. Some migration does occur during wound healing, but rotation experiments with genetically marked grafts on *Oncopeltus* showed that the graft remains as an intact body of cells, there being no incursions into the host. This hypothesis is also very difficult to relate to some of the experimental results, the most awkward being the cases where polarity is locally altered following interchange of anterior and posterior squares (p. 819). One might argue that the regulation of patterns after 90°-rotation experiments involves the gradual rotation back of the entire graft tissue, as occurs after rotation of the distal portion of a limb (Bohn, 1965*a*, *b*). However, this idea provides no explanation for changes induced in the host tissue nearby. These and other difficulties eliminate any simple model depending on cell migration.

Other classes of model. Some simple models of other types can be eliminated; for example, the cells might receive precise positional information from the organizing margins. One suggestion (Goodwin & Cohen, 1969) is that this information might be in the form of a phase difference between 2 waves propagated with different velocity, but other mechanisms are possible.

After rotation operations our patterns could then be explained by the cells only gradually being able to alter their set positional values towards the correct ones. This simple model can be eliminated by considering the cells of the host neighbouring the graft region after 90° rotation. These cells contribute to the pattern; their polarity is altered in different directions depending on whether the graft nearby is rotated through 90° anticlockwise or clockwise. These effects on the host cells can only be due to a local interaction, as the host cells have not been moved by the operation itself.

DISCUSSION

The effect of cuts and burns

When cuts were made in the antero-posterior axis about 5 days after feeding and the adult cuticle examined it was found that some ripples nearby had turned through 90°. Medio-lateral cuts did not alter the orientation of the ripples. The effects were much more striking when the intersegmental membrane was cut; here the ripple pattern was systematically altered over a large region. Both kinds of cuts when made either before feeding or within the first few days after feeding left almost no trace in the adult.

There are several possible explanations. One is that as the cells migrate in towards the cut (Wigglesworth, 1937), either their anterior or posterior end leads the movement. This will result in a 90° rotation of cells on either side of an antero-posterior cut, but no change in the orientation of cells near a medio-lateral cut. Wigglesworth noted that by about 24 h after a cut in *Rhodnius*, the cells in the sparse zone near the cut had their long axes at right angles to the line of the cut. We have seen this too, and also noted that the crowded cells along the line of the cut itself are oriented parallel to it. This hypothesis does not predict the much more extensive effects of cutting the intersegmental membrane.

Another hypothesis is that the line of the cut represents a relatively free pathway for diffusion of the morphogen, possibly due to an effect of wounding on the cells' permeability, thus reducing the steepness of the gradient in the cut itself. The anterior part (say) of such a cut will become lower than the surrounding gradient, and the posterior part higher. The pattern produced after some diffusion will include contour lines parallel to an antero-posterior cut. Where the segment margin is cut, because of this relatively rapid diffusion in the wound, and the 'dominant' activity of the margins (Piepho, 1955; Lawrence, 1970, and later discussion) there will be a rapid leak between the segments, resulting in a raising of the low concentration of one and the lowering of the high concentration of the other, as is suggested by the ripple patterns (Figs. 14, 27A).

These cutting experiments may indicate when the polarity of the cells is 'read' from the gradient. The simplest hypothesis is that reading occurs at the latest time when cuts have maximum effect, that is about 9 or 10 days prior to ecdysis. This corresponds approximately to a period beginning at the latter part of the mitotic period and ending at the separation of the epidermis from the old cuticle. This alteration in the gradient landscape should be mostly temporary, as it should have no effect on the set level of most of the cells which have divided prior to the time of cutting. To check this prediction, adults showing the effects of cuts through the intersegmental membrane were made to produce a second cuticle (1 dose of 5 μg of ecdysterone) and indeed showed considerable alteration of the ripple pattern towards the normal (Fig. 27). The experiment supports the idea that the cells' polarities are determined by the gradient landscape of concentration, and not by the landscape of set values.

The effect of burns, unlike cuts, is greatest immediately before epicuticle deposition at about 7–8 days prior to ecdysis. It is disturbing that the pattern of converging ripples produced cannot represent any concentration landscape. However, no problem

remains if we simply suppose that the converging ripples are a direct consequence of the cells migrating and carrying with them the partially formed epicuticle. In front of this migrating region the wound cuticle is made by cells which reach there after the time of epicuticular deposition, and behind the converging ripples is epicuticle, made and completed before migration. Burns made at 6·5 days before ecdysis, when the cuticle shows an abrupt transition from normally oriented ripples to wound cuticle, were presumably made at precisely the time of epicuticular deposition, the ripples being completed before migration began (Locke, 1967).

A normal pattern is formed when burns are made around the postulated time for reading the gradient (9 or 10 days before ecdysis). We can offer 2 explanations for such rapid reconstruction of the pattern: first, centripetal migration from a large periphery on to a central point will inevitably cause intermingling and close apposition of cells of different gradient values so that the net effect on the pattern should be small. Secondly, the migrating cells may rapidly acquire the average local level because they are wounded (postulated to increase the permeability of cells to the morphogen) and dividing. To check that the polarity of the healing area is indeed determined locally we rotated a square of cuticle *after feeding* in 5th-stage larvae and then burnt a central area in the graft. We found that the burn had little or no effect on the orientation of the ripples (operation done *after feeding*, $\alpha = 11 \pm 2°$; operations done *after feeding* and then burnt 2–6 days later, $\alpha = 8 \pm 2°$).

The 90°-rotation experiments

It is not clear why 90°-rotation operations done *after feeding* in 5th-stage larvae ($\alpha = 11 \pm 2°$) show less regulation than those done *before feeding* ($\alpha = 26 \pm 1°$), as the main period of cell divisions in the moult cycle follows both classes of operations. Some of the difference is doubtless due to somewhat larger grafts used in insects taken *after feeding*, but exchange of grafts (rotated through 90°) between unfed and fed insects showed that most of the difference is associated with the state of the host, not with the nature or origin of the graft.

It might be thought that the gradient landscape had not time to reach equilibrium following operations *after feeding*, but when adults from such operations were made to moult again without cell divisions, the pattern of the first and second cuticles was almost identical (Table 1).

Two other differences between the classes of experiments are that the insects operated on *before feeding* were given a small meal, and in these insects there was extra time available for wound healing. Both these factors might increase the total amount of cell division following operations made *before feeding*, which is consistent with the increased regulation observed. We have not been able to measure directly the amount of cell division.

The gradient model

Our approach has been to generate a steady-state concentration gradient which, after rotation of square pieces through 90° or 180° attains new equilibrium landscapes of concentration, contour maps of which can be directly compared with the cuticle

patterns produced following 90°- and 180°-rotation experiments in *Rhodnius*. The good fit between computer simulations of Model 2 and these cuticle patterns does not prove that the model accurately describes the situation in the epidermis. There may be other models that would give such a fit; or there may be other features of the segmental gradient which are inconsistent with Model 2.

We believe that we have eliminated the basic form of Model 1 (Stumpf, 1967) and conclude that in addition to the effects of the margins there is a contribution from the other epidermal cells of the segment. One possibility is that these cells actively transport the morphogen up the gradient (Lawrence, 1966). We have explained why this model (Model 3) is difficult to envisage and to simulate, but do not claim that it has been ruled out.

There are several attractive features of Model 2; one is that a cell need only monitor and control its internal concentration of the morphogen, the stability of the gradient as a whole depending on the fixed levels of concentration in the margins, and the periodic feedback in the cells between the margins. Another is that the observed asymmetry arises naturally from the model: the factors which limit the maximum rate of synthesis of a molecule could well be different from those which affect its rate of destruction, and consequently the homeostatic ability of a transposed cell could depend on whether it is acting as a source, or as a sink. The asymmetry of patterns after 180°-rotation experiments can be simulated by assuming that there is such a difference.

The gradient Model 2 asks, however, that the internal concentration of morphogen can be set to different levels. When a cell is moved by experiment to a lower ambient concentration so that the morphogen diffuses out, the cell attempts to restore its original concentration by synthesizing the morphogen: a new steady-state is reached where the actual internal concentration is lower than the set level. We postulate that at some stage in the cell cycle, the level of each cell is reset to this actual internal concentration. In the example outlined above this will result in a reduction in the rate of net synthesis of the morphogen, and a further drop in the local concentration. These effects summed over the operated region can give rise to the patterns observed. Such homeostasis could be achieved by simple enzymic mechanisms, such as with cyclic AMP, where the internal concentration results from a fine balance between the activities of 2 enzymes, one synthetic and one degradative (Robison, Butcher & Sutherland, 1968). Some delicate feedback would be required for the resetting process. It is worth noting that the theory does not demand that the epidermal cell be capable of being set to any level. Calculations have shown that limitation to 1 of only 5 discrete values within the rotated area, and a similar limitation around it, would not alter the results noticeably.

The regulative ability of the cells depends on both the efficiency of synthesis or destruction and the intercellular flux of the morphogen (measured by K). Thus the cells of the margins need not be qualitatively different from the cells of the segment surface; they may only have an increased ability to maintain their own concentrations, and consequently at mitosis are reset to the same level. Although the value of K for the margins and for the centre of the segment are very different, there may be a continuum of cell states between them. The dominant margins thus become the fixed bounds of a

regulative gradient; they can be thought of as the organizers (Spemann, 1938) of the insect segment, as can the hypostome and the basal disk be considered as organizers of the axis of *Hydra* (Webster, 1971). This model of the insect segment is capable of both regulation and stable growth: changes within the segment caused by such perturbations as wounding and experiment are corrected for, while defects in the organizing margins of the segment persist (Sobels, 1952).

Our model may illuminate other observations on insect segmental gradients:

(1) Caveney (1971) has been studying the orientation of the first uni-directional layer of adult *Tenebrio* cuticle. He rotates squares of integument through 90° in larvae of different ages. He reports that regulation of the pattern occurs only when cell divisions intervene between the operation and the time of adult cuticle formation.

(2) Bohn's experiments on intercalary regeneration of the limbs of *Leucophaea* show that the gradient level of the cells within the limb segment is relatively stable. When cells of different gradient level are grafted together, extra growth follows, the amount of tissue formed depending on the amount of gradient difference between host and transplant and its polarity on the sign of the difference (Bohn, 1965*a*,*b*, 1971). It seems that growth regions form near the host-graft border, while the cells beyond remain nearly constant in gradient value. Intermediate gradient values develop by excess growth which continues until the steepness appropriate to that segment is reached. Our model provides a basis for the stability of the gradient in the cells near the host-graft border (a high K value) and for the development of intermediate values associated with mitosis. The stimulus to excess growth by cells of host and transplant near the border might even be due directly to the steepness of the concentration gradient (Lawrence, 1972).

(3) Experiments where segmental margin is transplanted in *Galleria* (Marcus, 1962; Lawrence, 1970) show that the area where the cuticle type is altered is considerably smaller than that where the scale orientation is affected; an observation consistent with the hypothesis that the landscape of concentration determines the polarity while the determined state depends on the set value.

(4) Recent experiments (Piepho, 1970, 1971; Piepho & Hintze-Podufal, 1971) where epidermis is implanted into the body cavity of *Galleria*, suggest that diffusion may occur between closely apposed epithelia. Only the posterior margin of the segment and, to a lesser extent, the cells between the margins, can alter the scale orientation in the responding epidermis. Unlike the situation in transplants into the epithelium (Piepho, 1955) the anterior margin has no effect when implanted into the body cavity. These experiments although subject to some reservations (Lawrence, 1972) suggest that the posterior margin is the source.

Another feature of the model is that the set level is a 'remembered' value of the gradient. Thus, if cells could compare the set level with the actual level, a sharp increase in the difference between them could signal that an organizer had been removed (for example, the head of a hydra) and thus release the regenerative process (compare Wolpert, Hicklin & Hornbruch, 1971).

Gradients may be a common method of establishing positional information. A double gradient generating coordinates may be present in the optic tectum and retina

of vertebrates (Gaze, 1971); rotation of pieces of optic tectum of the goldfish (Sharma & Gaze, 1971) gave results superficially similar to the patterns produced following similar rotation experiments in *Rhodnius*. These two axes of the retina, as in many other organs in vertebrates (e.g. Harrison, 1921) are determined at different times and are independent (Székely, 1954; Jacobson, 1968). Such a separation in time would be necessary if the same morphogen were used in the successive establishment of both axes.

In the insect segment it has been shown that the positional information supplied by the level in the gradient can lead to determination, and it therefore becomes likely that cells in the different regions generated by the initial gradient will respond in diverse ways to the positional information conveyed in subsequent gradients. Thus the same method of generating positional information can be repeatedly used to organize appropriate patterns in different parts of the body (Stern, 1968; Wolpert, 1971).

CONCLUSIONS

Although our results fit reasonably well with the predictions of Model 2, it is impossible at this stage to rule out all other models. For this reason, we now present our conclusions in as general a form as possible.

(1) At least some cells in the insect epidermis display polarity. This is shown by the direction of bristles and hairs, and also by tubercles in the cuticle. Note that this is not just an anisotropy. That is, it must be represented by a line with an arrow ($\rightarrow$), not merely by a line with a double arrow ($\leftrightarrow$).

(2) Our results cannot be explained by polarity alone. This was the major conclusion drawn by Locke (1959) from his pioneer work. The cells 'know' where they are in the antero-posterior direction.

(3) When a cell is moved to a new position, having a different antero-posterior coordinate it 'remembers', at least to some extent, where it has come from.

(4) This memory is partly lost in certain circumstances, although not solely as a result of the passage of time. Our results are compatible with the idea that this information is modified at some stage in the cell cycle.

(5) The positional information possessed by a cell is modified by the position in which it finds itself, due to the influence of neighbouring cells. This influence appears to be, in very general terms, a weighted numerical average of some property of cells in its neighbourhood. Thus, the positional information behaves as if it were a continuous variable; we cannot explain our results by a system of discontinuous unrelated labels. In other words, the positional information is expressed by a gradient. We cannot show that the gradient is linear, but it appears to be monotonic, and (as shown by Locke, 1959) repeats, at least approximately, from segment to segment.

(6) The margins of the segment have special properties, as shown by grafting operations (Piepho, 1955) and the cutting experiments. They appear to have more influence on their neighbours (except across an intact intersegmental boundary) than do the cells in the body of the segment.

(7) The polarity of bristles and tubercles appears to be roughly correlated with the direction of steepest slope of the gradient of positional information.

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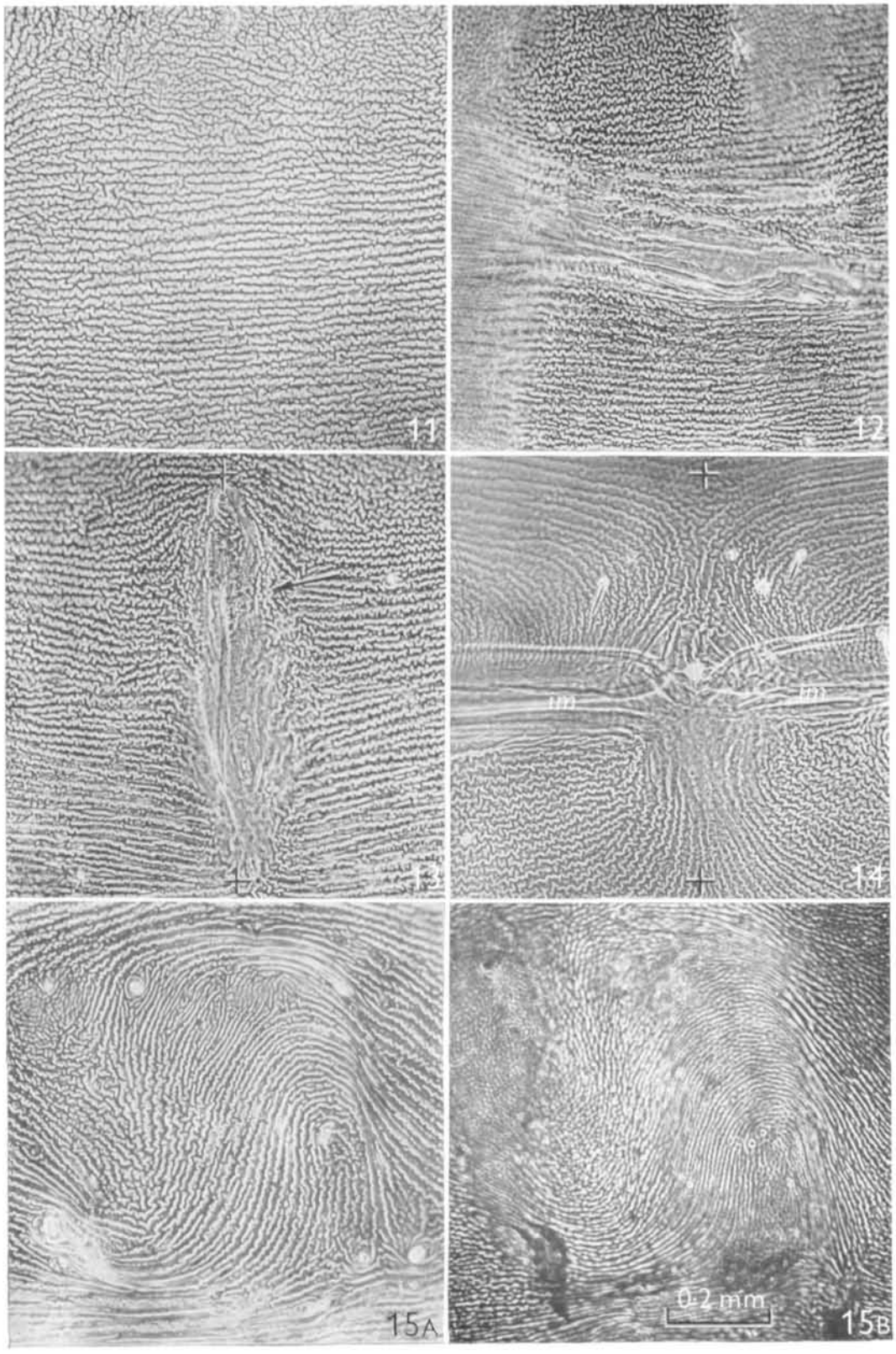


Figs. 11–15. For legend see p. 842.

Figs. 11–15. Phase-contrast photographs of dorsal adult cuticle all to the same scale (× 85) and all oriented so that the anterior is at the top of the page.

Fig. 11. Central part of a normal tergite.

Fig. 12. After medio-lateral cut made 10 days prior to ecdysis.

Fig. 13. After antero-posterior cut made (between 2 crosses) 10 days before ecdysis. Note some ripples have turned through 90° (arrow).

Fig. 14. After antero-posterior cut made (between 2 crosses) through intersegmental membrane (*im*) made 10 days before ecdysis: note extensive pattern of affected ripples.

Fig. 15A. Adult cuticle following 90° rotation operation (anticlockwise) *before feeding* in 5th stage.

Fig. 15B. Supernumerary cuticle from same individual as in Fig. 15A following injection of 5 μg ecdysterone which precipitated moulting without cell division. Note pattern is very similar to Fig. 15A.

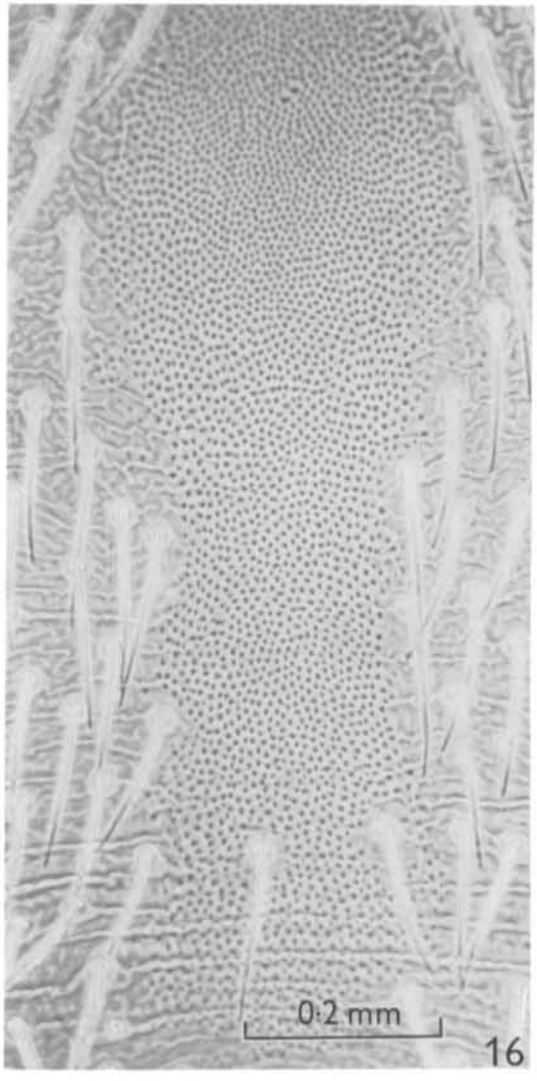
Figs. 16–18. Phase-contrast photographs of mounted adult cuticle oriented with anterior at top of page.

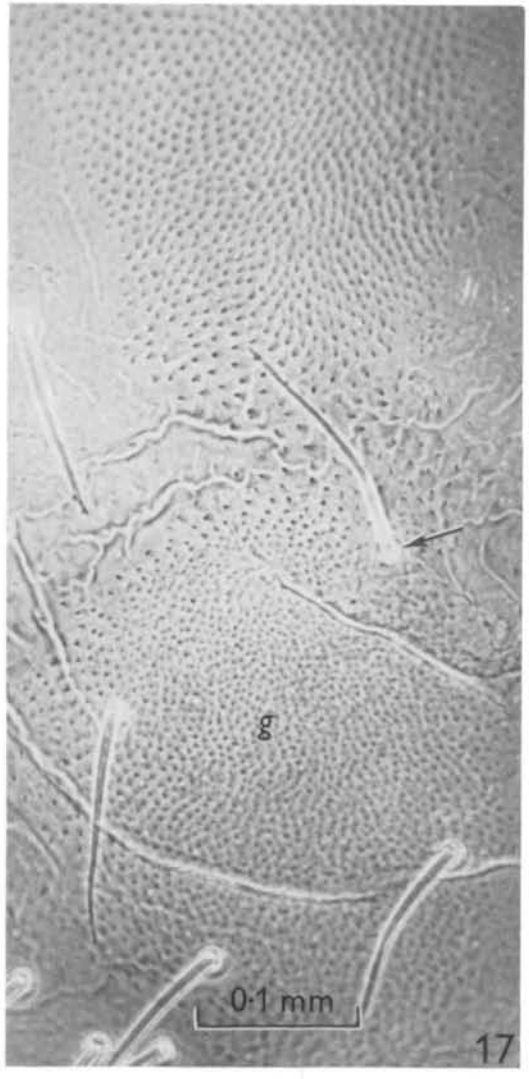
Fig. 16. Ventral midline of unoperated adult. × 130.

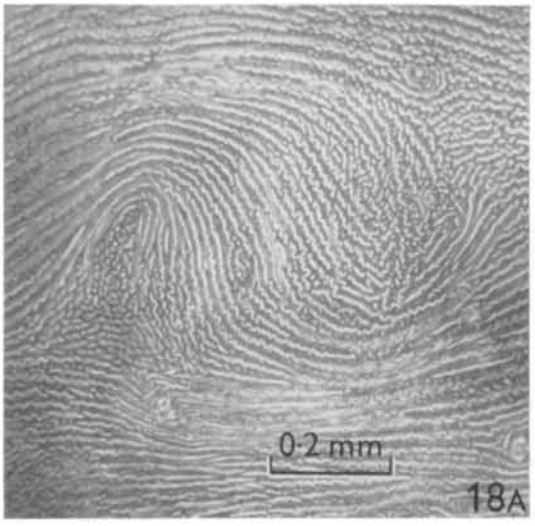
Fig. 17. Detail of ventral midline after transposition experiment (compare graft to posterior site, Fig. 3). Near the host-graft junction the orientation of many tubercles and a single bristle is reversed (arrow). The tubercles on the graft (*g*) are smaller and more dense due to closer packing of the cells. × 215.

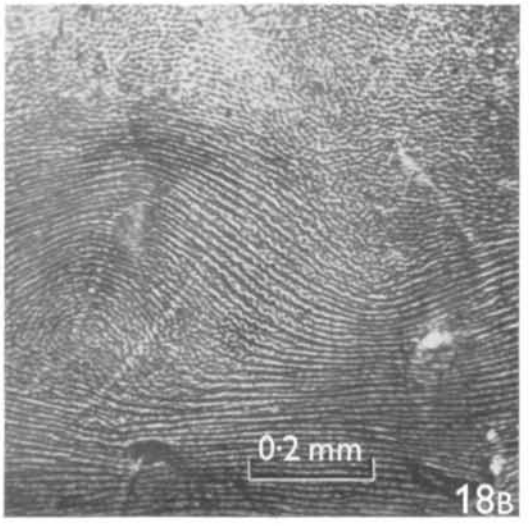
Fig. 18A. Adult dorsal cuticle following 90° rotation (anticlockwise) in 5th-stage *before feeding*. × 85.

Fig. 18B. Supernumerary cuticle from same individual as in Fig. 18A following injection of 3 doses of ecdysterone which induced moulting with cell divisions. Note that pattern is different from Fig. 18A. × 85.


0·2 mm
16


g
0·1 mm
17

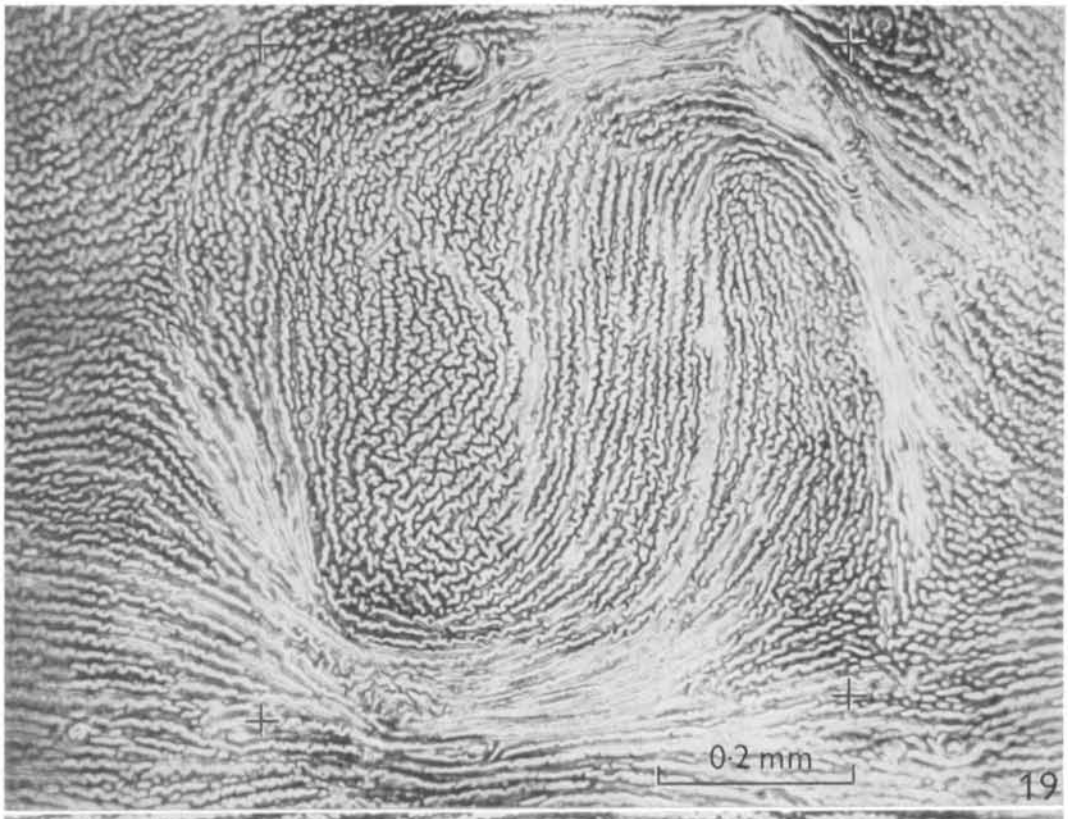

0·2 mm
18A


0·2 mm
18B

Figs. 19, 20. Adult dorsal cuticles mounted with anterior to top of page. × 130.

Fig. 19. Following 90° rotation anticlockwise *after feeding* in 5th-stage larva. Approximate corners of graft indicated by 4 crosses.

Fig. 20. After 90° rotation clockwise *before feeding* in 5th-stage larva (compare Fig. 8A). Approximate corners of graft indicated by 4 crosses.


0·2 mm
19


0·2 mm
20

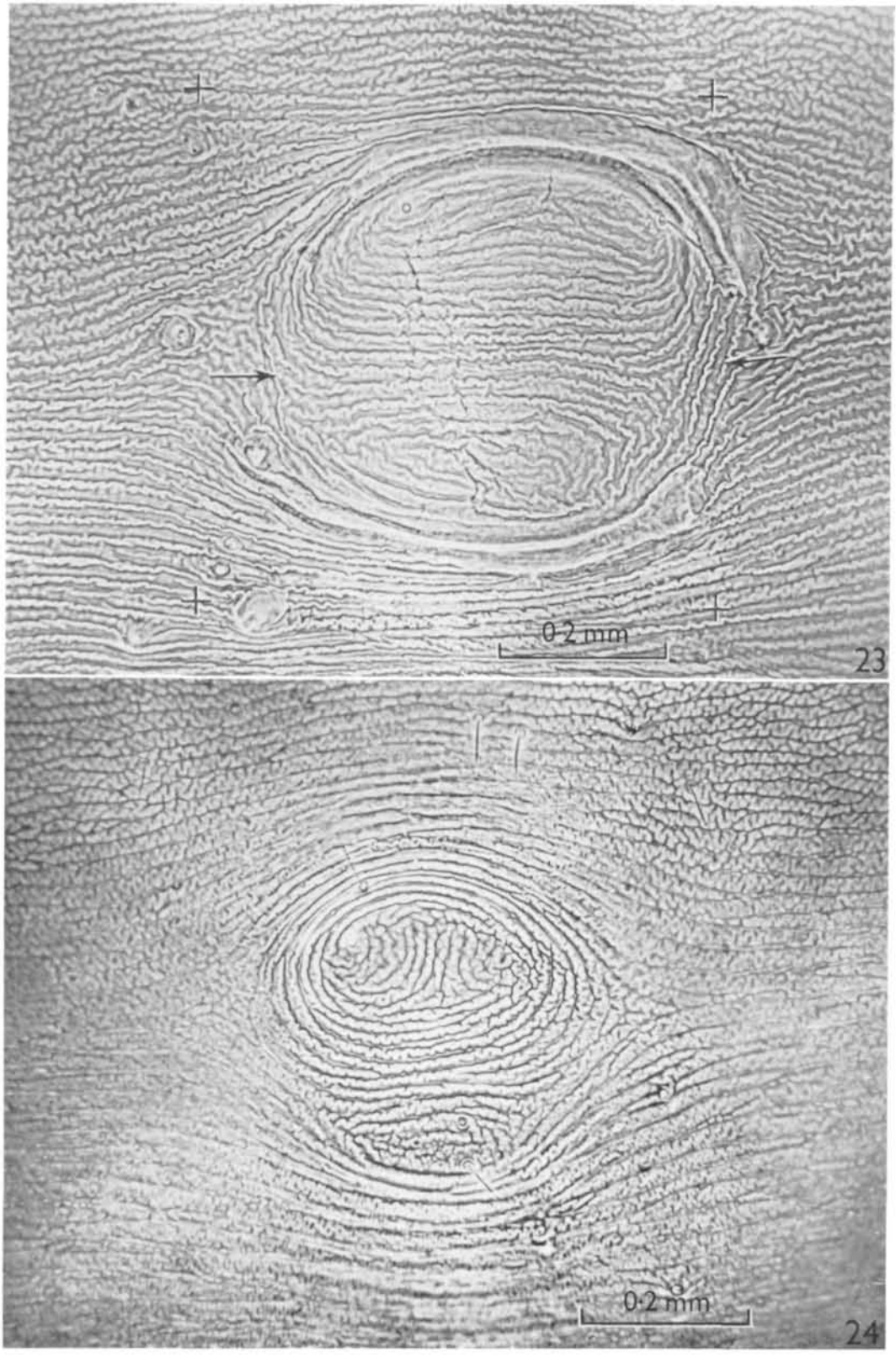
Figs. 21, 22. Adult dorsal cuticles mounted with anterior to top of page. × 130.
Fig. 21. After 90° rotation anticlockwise in 4th larval stage (compare Fig. 8B).
Fig. 22. After 90° rotation anticlockwise in 3rd larval stage.


0·2 mm
21
0·2 mm
22

Figs. 23, 24. Adult dorsal cuticles mounted with anterior to top of page. × 130.

Fig. 23. After 180° rotation in 5th-stage *before feeding*. Note the anomalous ripples (arrows) which have no equivalent in the simulation (Fig. 8C). Approximate corners of graft indicated by 4 crosses.

Fig. 24. After 180° rotation in a 4th-stage larva (compare Fig. 8D).


0·2 mm
23
0·2 mm
24

Figs. 25, 26. Adult cuticles mounted with anterior to top of page.

Fig. 25. After exchange of anterior with an adjacent posterior rectangle in 5th-stage larva *before feeding*. Note anterior disturbance is greater than posterior. Compare Fig. 9. × 180.

Fig. 26. After grafting (without rotation) most of a segment of 3rd-stage larva on to the centre of a 5th-stage segment *before feeding* (compare Fig. 10). Approximate corners of graft indicated by 4 crosses. × 130.

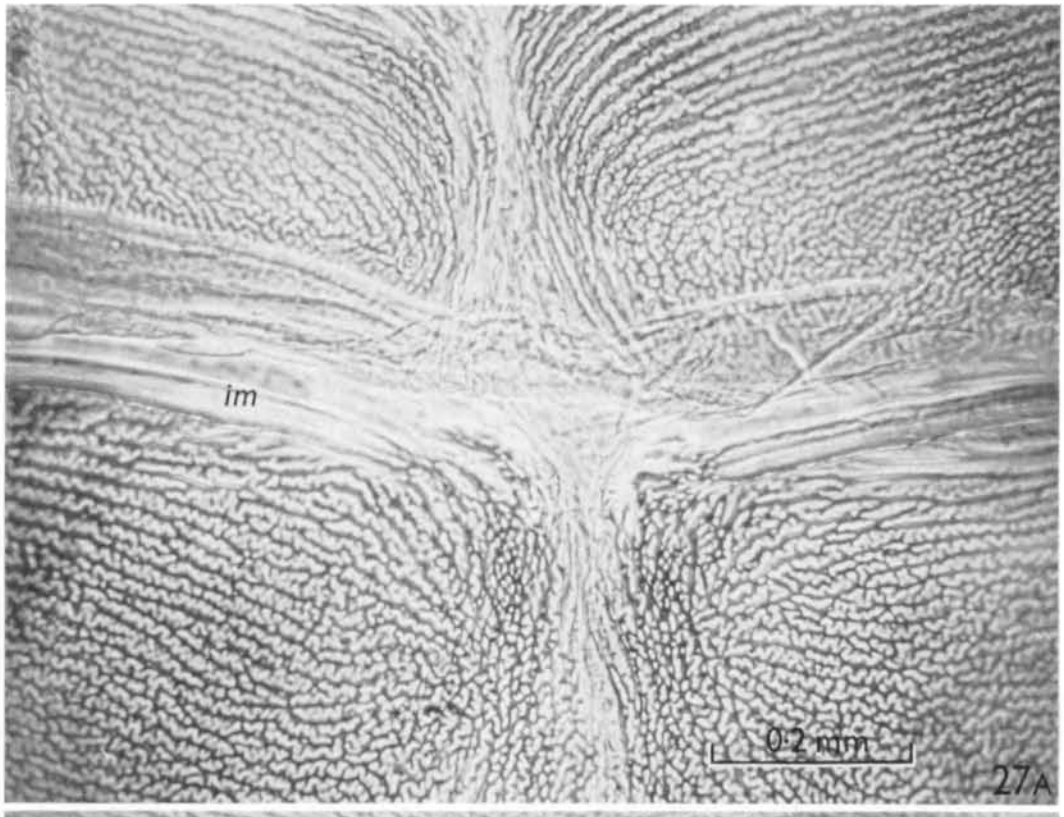

0·2 mm
25

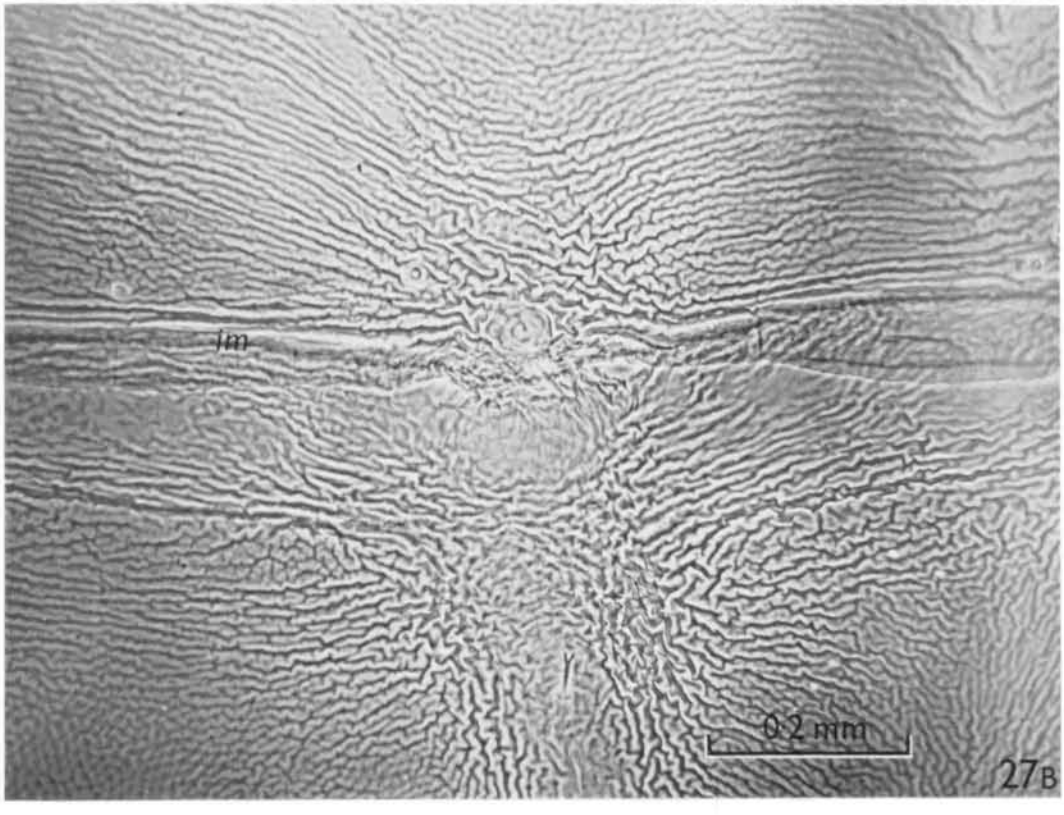

0·2 mm
26

Fig. 27. Adult cuticles mounted with anterior to top of page. × 130.

A, first cuticle following antero-posterior cut made through the intersegmental membrane (*im*). The insect was cut 10 days before ecdysis.

B, supernumerary adult cuticle from same region of individual as Fig. 27 A, following a single injection of 5 μg of ecdysterone, which induced moulting without cell division. Note that the previous pattern of deflected ripples is replaced by ripples in more normal orientation.


im
0·2 mm
27A


im
0·2 mm
27B