Separation of wound healing from regeneration in the cockroach leg

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SUMMARY

It has been shown that after a critical point in the moult cycle of a cockroach, wound healing can occur but regeneration of pattern does not take place until the following intermoult period. Leg removal after the critical point is used to separate the processes of wound healing and leg regeneration. This permits the study of patterns of cell division resulting from wound healing to be distinguished from those involved in leg regeneration.

During wound healing, cell division occurs in the epidermal cells of approximately the distal half of the trochanter. The cells then return to the resting state until after the next ecdysis. Regeneration starts with cell division occurring in the distal half of the trochanter, and then spreading to include cells of the proximal trochanter and distal coxa. This spread and the following patterns of growth and redifferentiation appear to be the same as for regeneration following leg removal prior to the critical point, with the more distal structures completing early stages of regeneration first.

Scanning electron micrographs of the cuticle of the trochanter after the ecdysis following leg removal support the evidence from the patterns of cell division in suggesting that the distal half of the trochanter is dedifferentiated during wound healing.

INTRODUCTION

It has recently been shown that regeneration of the cockroach limb following autospasy at the preformed breakage plane between the trochanter and the femur, involves considerable respecification of epidermal cells proximal to the breakage plane (Truby, 1983*a,b*). The first stage of regeneration is that of wound closure. The epidermal cells to either side of the wound become activated and detached from the cuticle. Activation has been described previously for wound healing in *Rhodnius prolixus* (Wigglesworth, 1937), and involves enlargement of the cells, their nuclei and nucleoli. The activated epidermal cells migrate across the underside of the clot of haemocytes which forms over the wound, epidermal continuity is restored and normal cell density is brought about by cell division in the epidermis to either side of the wound. After wound closure, separation of the epidermis from the cuticle, activation and cell division spread outwards into more proximal areas of epidermis, to form a blastema. This spread eventually reaches

Key words: Insect leg regeneration, blastema formation, critical point.

the distal end of the coxa. The blastema then grows by cell division throughout its epidermis, to form the new leg. The order of differentiation of joints and muscle insertions and the patterns of cell division suggest that distal structures start to be regenerated before more proximal ones.

As activation and cell division are required for both wound healing and regeneration, it is not possible to say to what extent the observed patterns are a response to wound healing and to what extent they are part of the regeneration process.

About half way through the moult cycle of a cockroach (and of many other arthropods) there is a 'critical point' after which regeneration will not occur until the following moult cycle (O'Farrell & Stock, 1953). However during this time, wound healing can still occur. By removing a leg after the critical point, the processes of wound healing and regeneration can be separated. Wound healing will take place immediately after leg removal, but leg regeneration will not start until after the following ecdysis.

In the experiments described here, the critical point in the second intermoult period of *Periplaneta americana* was found by removing one of the metathoracic legs at the breakage plane on different days after moulting, and finding the day after which leg removal failed to produce regeneration within the same intermoult period. Legs were then removed after this time and their regeneration was studied before and after the following ecdysis. Colchicine was used to show the patterns of cell division in light microscope sections, and scanning electron microscopy was used to show the extent of tissue respecification at the time of ecdysis.

MATERIALS AND METHODS

Stocks of *Periplaneta americana* were kept at 24 °C and fed commercial rat food and water. Oothecae were removed, and checked each day for newly hatched larvae. These were kept until the required number of days after their first moult. Operations were performed after anaesthetizing with CO_2 and consisted of gently pulling the left metathoracic leg to separate it at the preformed breakage plane between the trochanter and femur.

In order to show the distribution of cell divisions at different stages of regeneration, animals were injected 12 h before fixation with a 1 % colchicine solution made up in Clarke's insect saline (Hale, 1965). The parts of the legs to be studied were fixed for 3 h in a glutaraldehyde/paraformaldehyde mixture (Karnovsky, 1965) buffered in a phosphate buffer (Hayat, 1970) at pH 7·4, then dehydrated through an acetone series and embedded in Araldite. Semithin sections (1·5 μm) were cut in the longitudinal plane of the leg, using a Huxley Ultramicrotome with glass knives. Sections were stained with toluidine blue and photographed on a Zeiss Photomicroscope II. Cell densities were calculated as the average number of nuclei in 100 μm lengths of epidermis. To plot the distribution of mitotic figures in sections of colchicine-treated legs, the epidermis of the trochanter and distal coxa was divided into 100 μm lengths, measured from the distal end of the trochanter. The number of figures in each length was counted and an average value was calculated using equivalent lengths of six sections from each of three legs of the same stage.

Material for scanning electron microscopy (SEM) was fixed and dehydrated as for sectioning, critical-point dried in CO_2 and coated with a 1·5 nm layer of gold using a Polaron sputter coater. Pictures were taken on an ISI-60 SEM.
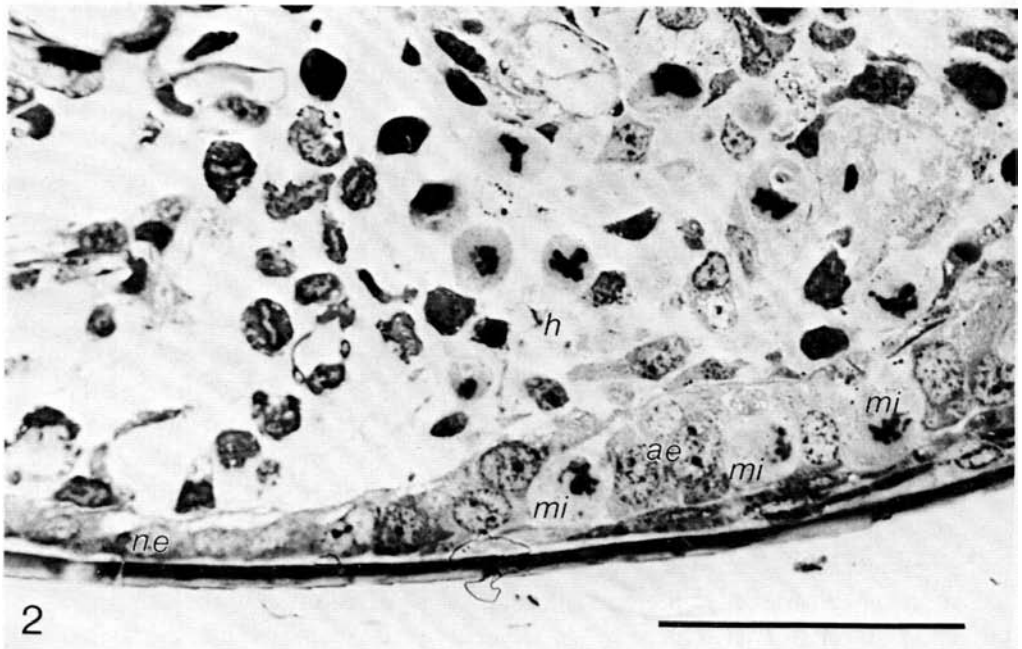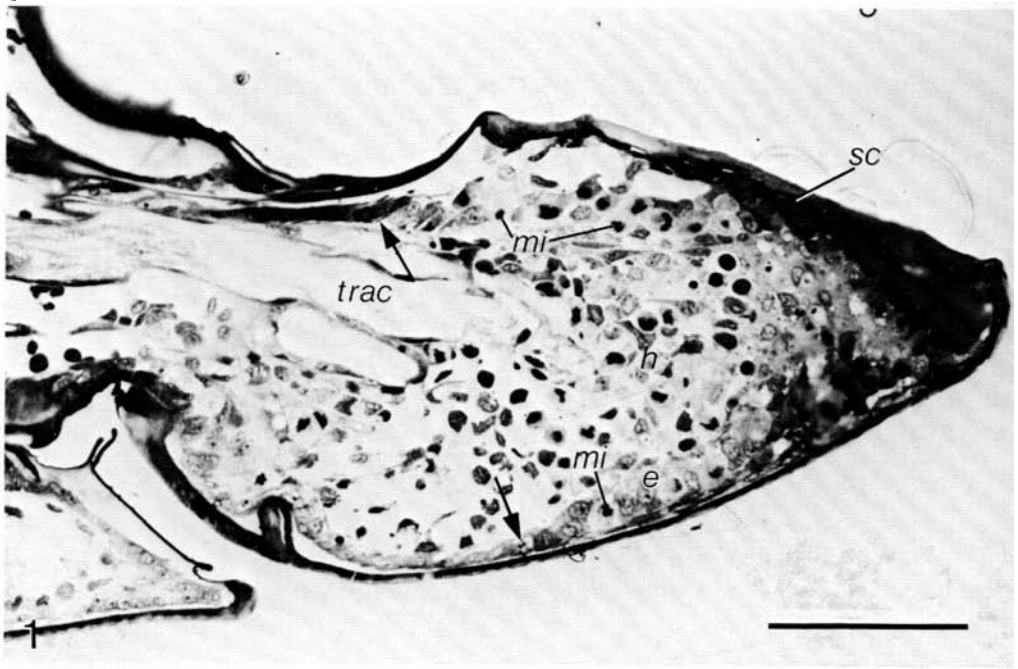
RESULTS

Timing of the critical point

The length of the moult cycle of an unoperated second instar *P. americana* was found to have a mean value of 16 days with a standard deviation of 1·5 days (based on 150 animals). The critical point occurs between the 7th and 12th days of the moult cycle. 5 % of legs removed on the 7th day failed to regenerate in that moult cycle, 25 % on the 8th day, 60 % on the 9th day, 90 % on the 10th day, 97 % on the 11th day and 100 % on the 12th day. In order to study the patterns of cell division in animals that would not regenerate their leg within the same moult cycle as wound healing, the legs were removed on the 10th day of the moult cycle, by which time 90 % of individuals have passed the critical point. To have removed the legs on a later day would have insured that all animals had passed the critical point but in some individuals, this would have left very little time for wound healing to occur before the end of the moult cycle.

The histology of wound healing and regeneration

The early stages of wound healing appear to be the same as those observed during the wound healing that precedes regeneration when a leg is removed prior to the critical point (see Truby, 1983*a*). During the first two days a scab of hardened haemolymph forms over the wound, with a clot of flattened haemocytes below it. Cells immediately to either side of the wound become activated and migrate across the underside of the flattened haemocytes that form the clot. Cell division occurs first in the epidermis to either side of the wound (Fig. 1). In *Rhodnius prolixus* this cell division has been shown to be stimulated by low cell density, caused by cells having migrated away from the area during wound closure (Wigglesworth, 1937). However, whereas following leg removal in the early part of the moult cycle, activation and cell division continue to spread outwards until the cells of the distal end of the coxa become activated, following leg removal after the critical point, activation and cell division spread no further than about half way back along the trochanter. (There is not a sudden change from activated to non-activated epidermis, but a progressive decrease in the extent of activation, extending over several cells (Fig. 2). For these results the limit of the spread of activation was taken as the midpoint between fully activated cells and non-activated cells.) This represents a spread of about 150 µm from the edge of the wound. Cell division then ceases, the epidermis becomes detached from the cuticle and a new cuticle is secreted prior to ecdysis (Figs 3 & 4).

Following ecdysis the epidermis is initially non-activated, and the cell density is the same as for normal epidermis at the start of the moult cycle (profiles of an average of 10 nuclei per 100 µm of epidermis are visible in 1·5 µm sections). Two days after ecdysis, cells in the distal end of the trochanter become reactivated and cell division starts to occur in this region (Fig. 5). Activation, cell division and

Figs 1, 2.

separation of the epidermis from the cuticle then spread out to include the epidermis of the whole of the trochanter and the distal end of the coxa (a distance of about 750 μm or 80 cell diameters) resulting in the formation of a blastema (Fig. 6). Whereas during wound healing, cell division is probably stimulated by low cell density, there is no low cell density preceding blastema formation, which must therefore be stimulated by some other factor. Once formed, the blastema segments and grows, folding back into the space formed by the epidermis of the distal/external region of the coxa becoming separated from the cuticle (Fig. 7). As with regeneration following leg removal before the critical point, the tibia–tarsus and femur–tibia joints, with their associated muscle insertions, are seen first, followed by the coxa–trochanter joint.

Patterns of cell division

Fig. 8 shows the patterns of cell division following leg removal before the critical point (on the 2nd day of the moult cycle) (A) and after the critical point (on the 10th day of the moult cycle) (B) & (C). The patterns of cell division during wound healing (days 2 & 3, (A) & (B)) are similar for leg removal before (A) and after (B) the critical point. Mitotic figures are seen mainly in the epidermis to either side of the wound area. During the equivalent period after ecdysis (days 2 & 3, (C)), there are initially fewer mitotic figures than during wound healing, but they rapidly build up and spread out to cover a wider area by day 3. By day 5 wound healing is complete, cell division has ceased in (B) and the patterns of cell division in (A) & (C) appear to be similar with a fairly high rate near the distal tip, a slightly lower rate in the central region of the blastema and a higher rate towards the proximal region, before decreasing to the base. During growth and redifferentiation of the blastema (days 7–9), (A) and (C) show similar patterns of cell division, with roughly the same level of cell division in each of the femur, tibia and tarsus on days 7 and 8 and the rate decreasing first in the tarsus (day 9).

Changes in the cuticular patterns during wound healing

When a leg is newly regenerated, its bristles are both fewer in number and smaller than on a control leg. For example, in Fig. 9, which shows a normal leg and a leg regenerated from the trochanter–femur joint, there is a reduced bristle pattern on the femur, trochanter and distal margin of the coxa of the regenerated leg as compared with the normal leg, demonstrating that the epidermis forming these structures is involved in the regeneration of the new leg (Truby, 1983*a*,*b*).

Fig. 1. A colchicine-treated leg, 2 days after leg removal on the 10th day of the moult cycle (after the critical point). The wound has healed and mitotic figures are visible in the epidermis of the distal part of the trochanter. *sc*, scab; *e*, epidermis; *mi*, mitotic figures; *h*, haemocytes; *trac*, trachea. Arrows indicate the limits of the activated area. Scale bar equals 100 μm.

Fig. 2. Detail of the limit of activated epidermis in Fig. 1. To show the increased size of the activated cells and their nuclei. *ae*, activated epidermal cells; *ne*, non-activated epidermal cells; *mi*, mitotic figures; *h*, haemocytes. Scale bar equals 50 μm.
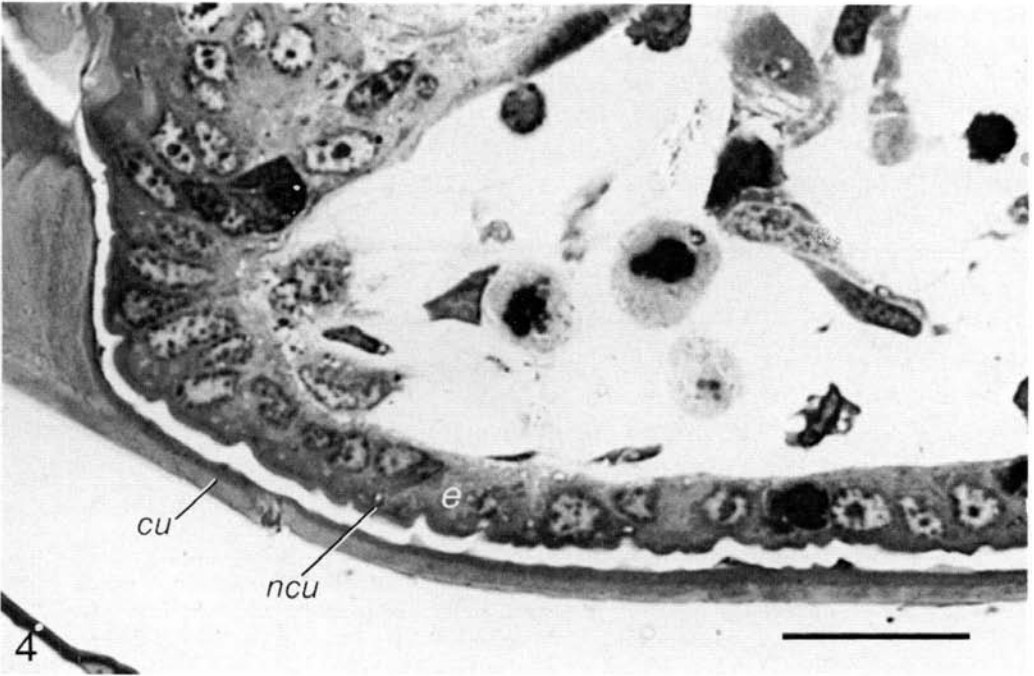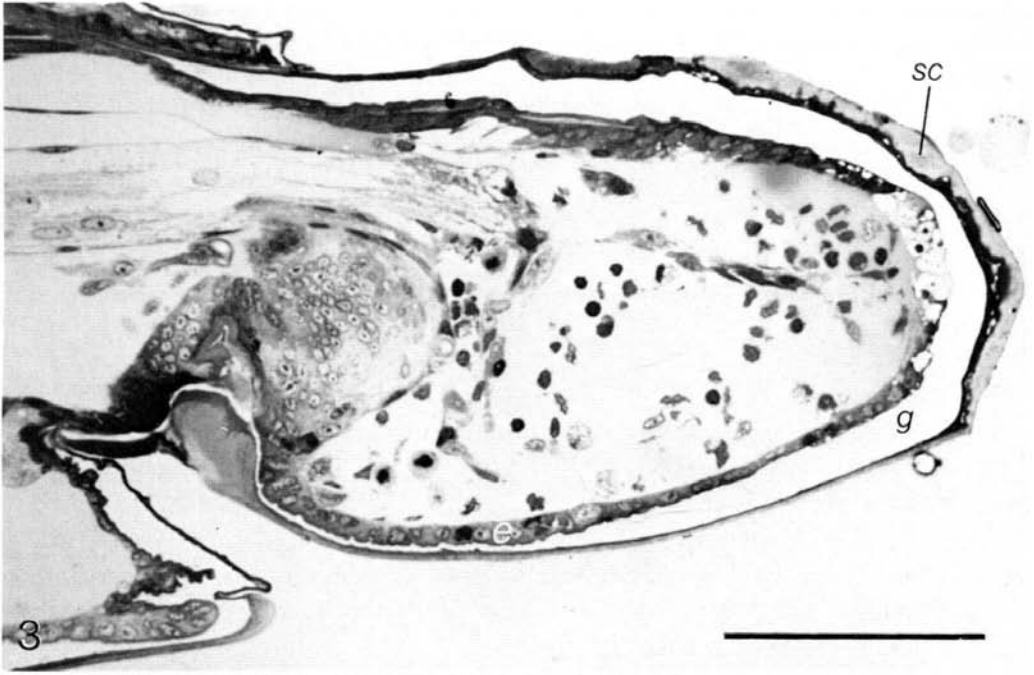
Figs 3, 4.

Fig. 10 shows the distal coxa and trochanter of a leg the distal part of which was removed at the breakage plane after the critical point in the preceding moult cycle. The smooth cuticle over the wound area shows that wound healing has been completed before ecdysis. However the bristles on the internal face of the trochanter and the distal margin of the coxa more closely resemble those of a normal leg than a regenerated one (c.f. Fig. 9), suggesting that dedifferentiation of this region to form a blastema has not occurred.

DISCUSSION

The relationship between wound healing and regeneration

By removing a leg at the breakage plane after the critical point in the moult cycle of the cockroach, it has been possible to separate the processes of wound healing
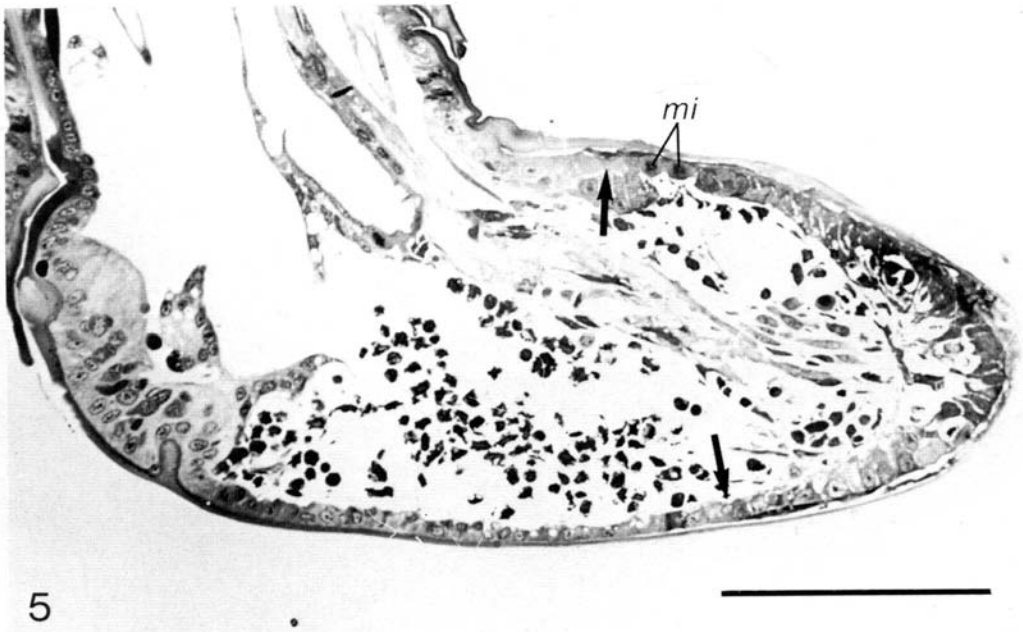

Fig. 5. 2 days after ecdysis following leg removal after the critical point in the previous moult cycle. A few mitotic figures (*mi*) are visible in the epidermis of the distal end of the trochanter. Arrows indicate the limits of the activated area. Scale bar equals 100 μm.

Fig. 3. A colchicine-treated leg, 4 days after leg removal on the 10th day of the moult cycle (after the critical point). No mitotic figures are present and the epidermis is no longer activated. The epidermis has separated from the cuticle and a new cuticle has been secreted. *sc*, scab; *e*, epidermis; *g*, gap between epidermis and cuticle. Scale bar equals 100 μm.

Fig. 4. Detail of Fig. 2, showing the new cuticle. *e*, epidermis; *cu*, old cuticle; *ncu*, new cuticle. Scale bar equals 20 μm.
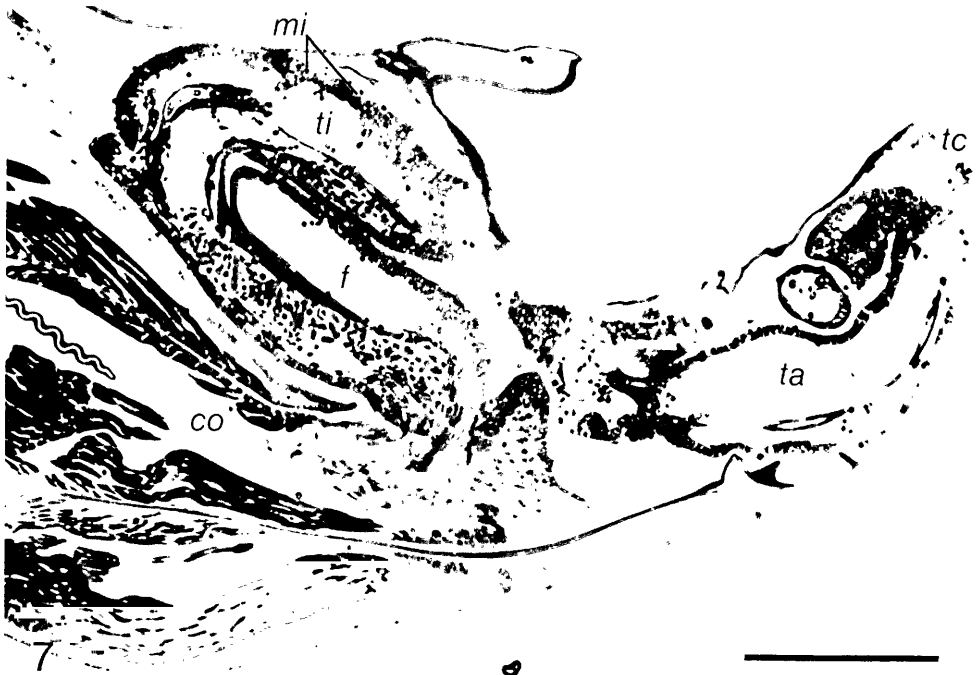
Figs 6, 7.

and regeneration. Wound healing involves activation and division of epidermal cells immediately to either side of the wound area. Regeneration involves a spread of activation and division of epidermal cells from the distal end of the trochanter back to the distal end of the coxa. This extensive process of dedifferentiation is not dependent on being immediately preceded by the spread of activation required for wound healing, as is shown by the epidermal cells returning to the resting state before ecdysis and then becoming reactivated during the subsequent regeneration. Blastema formation must therefore be a response to some stimulus other than the low cell density produced by wound healing. This stimulus is probably related to discontinuities in the normal pattern of the epidermis, generated by removal of the more distal regions of the leg. Such a stimulus has often been suggested in the past as the cause of regeneration and intercalation of new tissues (e.g. Bryant, French & Bryant, 1981; Lewis, 1981; Mittenthal, 1981). However, these results show that the effect of the stimulus is not local intercalation of new cells at the wound surface as has been suggested previously by Bryant *et al.* (1981), but consists of a spreading of activation and cell division over a wider region around the discontinuity as would be predicted by the models of Lewis (1981) and Mittenthal (1981).

The order of pattern differentiation

The patterns of segmentation and of cell division during regeneration are the same whether regeneration occurs in the same intermoult period as leg removal or in the following intermoult period. The fact that cell division both starts and stops first in the distal region of what becomes the regenerated leg and the appearance of the tibia–tarsus and femur–tibia joints before the coxa–trochanter joint, suggest that there is a distoproximal order to the redifferentiation in both cases. This is contrary to the results of O'Farrell & Stock (1954), which suggested a proximo-distal order when the leg is removed before the critical point. However, their conclusion was based on the rare occurrence of incomplete regenerates, in which the distal structures were less developed than the more proximal ones. These were generated by removing the left metathoracic leg of first instar *Blattella germanica* between the 1st and 3rd days of postembryonic life, and removing the right leg between 1 and 3 days later. Their assumption was that they had somehow arrested the regeneration of a few of the right legs part way, and so produced incompletely

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Fig. 6. 5 days after ecdysis following leg removal after the critical point in the previous moult cycle. The blastema extends back into the distal end of the coxa. Cell division is occurring throughout the blastema which is starting to segment. By comparing the position of the joints and muscle insertions with those in later sections it can be shown that the joints that are forming at this stage are the femur–tibia and tibia–tarsus joints. *co*, coxa; *f*, femur; *ti*, tibia; *ta*, tarsus; *mi*, mitotic figures; *mu*, muscle insertions. Scale bar equals 100 μm.

Fig. 7. 9 days after ecdysis following leg removal after the critical point in the previous moult cycle. Most of the main features of the leg have been formed. Cell division is occurring throughout the epidermis of the regenerate. *co*, coxa; *f*, femur; *ti*, tibia; *ta*, tarsus; *tc*, tarsal claws; *mi*, mitotic figures. Scale bar equals 200 μm.
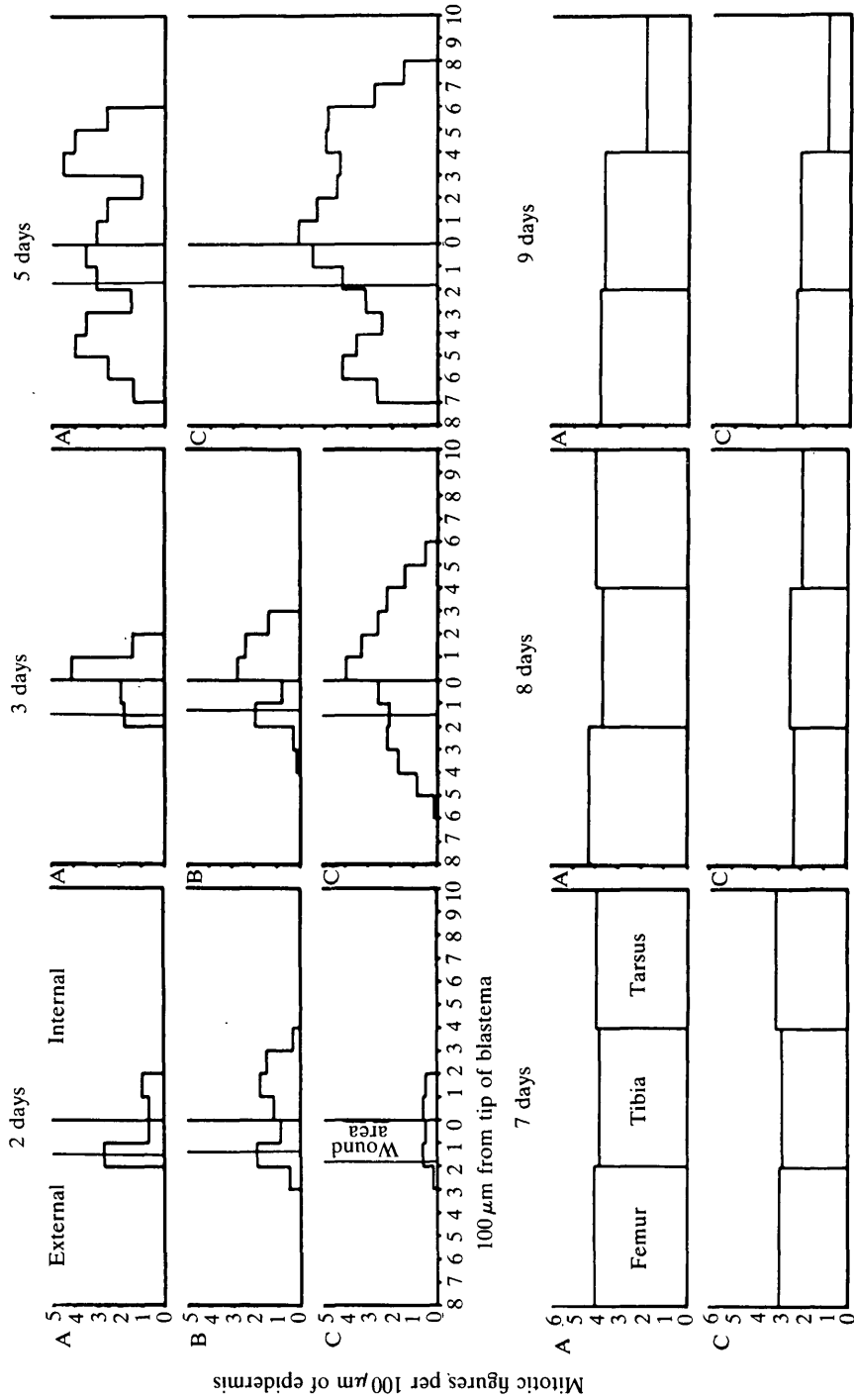
Fig. 8. Patterns of cell division in 1·5 μm longitudinal sections of colchicine-treated legs, following leg removal before (A) and after (B & C) the critical point. (B) shows the process of wound healing prior to ecdysis and (C) shows the process of regeneration after ecdysis. For days 2–5 after leg removal (or ecdysis in (C)) the pattern is shown as the average number of mitotic figures in each 100 μm length of epidermis on either side of the distal end of the wound. After day 5 the regenerating leg becomes segmented and folded, and so for days 7–9 the average number of mitotic figures per 100 μm of epidermis is plotted for each of the femur, tibia and tarsus.
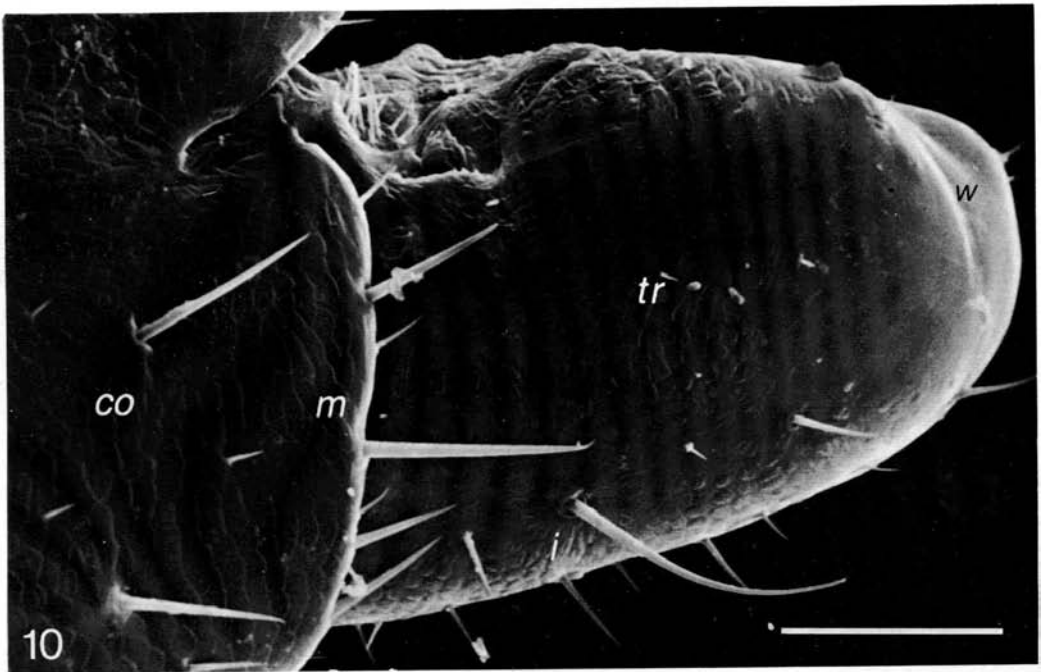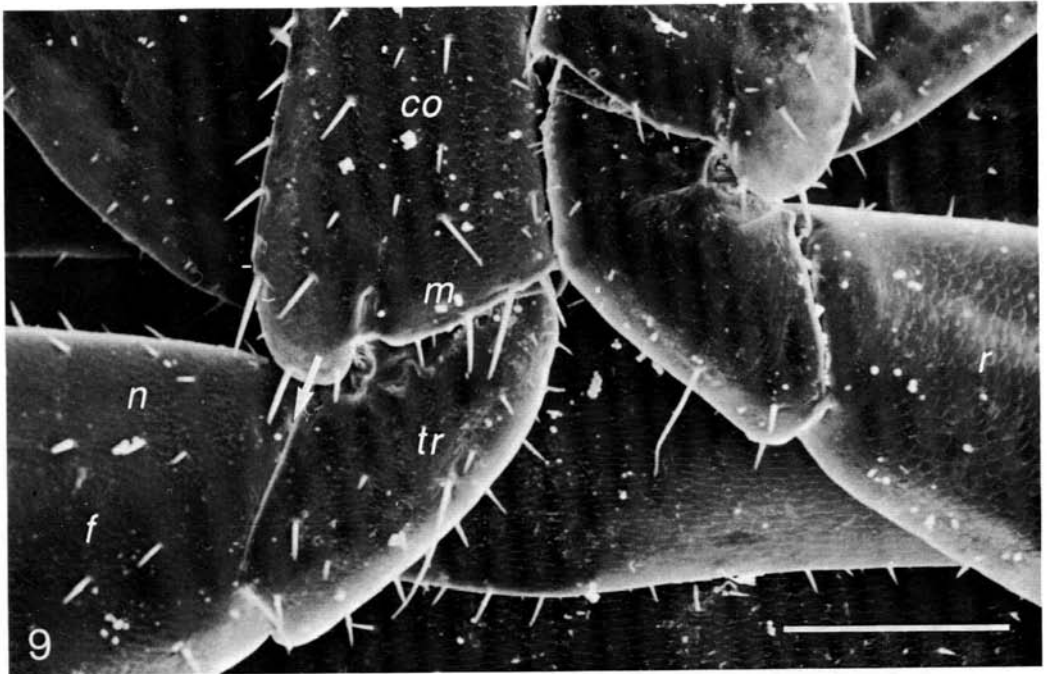
Figs 9, 10. For legends see p. 188.

regenerated legs. From the results shown here and previously (Truby, 1983a) it would appear that such legs must be the result of an abnormal regeneration process, possibly one in which the early cell divisions that occur in what will be the distal end of the blastema are inhibited or are followed by cell death. In both *Rhodnius prolixus* (Lüscher, 1948) and *Oncopeltus fasciatus* (Shaw & Bryant, 1974) the distal end of the leg is regenerated first but the order of the more proximal regions is less clear as they regenerate rather poorly in both these species.

The relationship between the critical point and regeneration

The critical point has been shown to coincide with a peak of ecdysone in the haemolymph (O'Farrell, Stock, Rae & Morgan, 1960; Roberts, Wentworth & Kotzman, 1983). During the embryonic growth of *Blaberus craniifer*, low levels of ecdysone stimulate DNA synthesis and cell division required for growth, whereas high levels of ecdysone inhibit growth and stimulate the DNA synthesis required for differentiative cell division (Bullière & Bullière, 1977). In *Rhodnius prolixus*, normal intermoult growth requires ecdysone for cell activation, but cell division does not occur until the cell density is reduced by stretching of the epidermis during feeding (Wigglesworth, 1963). However, activation and cell division are not dependent on ecdysone during wound healing.

The activation and cell division that occurs during wound healing following cockroach leg removal occurs both before the critical point, when ecdysone levels are low, and after, when ecdysone levels are high. Blastema formation on the other hand, only occurs at low ecdysone levels, but once formed, the blastema will continue to grow even at high ecdysone levels. Blastema formation, once started also appears to inhibit the peak of ecdysone that coincides with the critical point, until the spread of activation is complete (Roberts *et al.* 1983). Blastema formation is thus seen to be the result of discontinuities in the normal epidermal pattern, but is dependent on the correct level of ecdysone.

CONCLUSION

By removing a leg after the critical point in the moult cycle it has been possible to isolate the process of regeneration from that of wound healing. When this is

Fig. 9. This shows the distal coxa (*co*), trochanter (*tr*) and proximal femur (*f*) of the metathoracic legs of an animal that has recently moulted after having regenerated its left metathoracic leg from the trochanter–femur breakage plane. The bristles of the femur, trochanter and the distal margin of the coxa (*m*) are reduced both in number and size on the regenerated leg (*r*) as compared with the normal leg (*n*). The arrow indicates the breakage plane on the control leg. Scale bar equals 100 μm.

Fig. 10. The distal coxa (*co*) and trochanter (*tr*) of a leg the distal part of which was removed at the breakage plane after the critical point in the previous moult cycle. The cuticle in the wound area is relatively featureless but the internal face of the trochanter and the distal margin of the coxa more closely resemble those of a normal leg than a regenerated leg. *co*, coxa; *tr*, trochanter; *w*, wound area; *i*, internal face of the trochanter; *m*, distal margin of the coxa. Scale bar equals 250 μm.

done, regeneration appears to be essentially the same as when it is directly preceded by wound healing. Thus blastema formation during cockroach leg regeneration is not caused directly by wounding, but by discontinuities in the normal pattern of the epidermis, brought about by the removal of the leg.

Use of the technique of separating regeneration from wound healing may also be useful for studying these processes separately in other situations. For example, in studying the patterns of cell division following grafting experiments, it has been found that the cell division required for wound healing largely obscures the early patterns of cell division that are caused by the pattern discontinuities (Truby, 1983*b*). By grafting immediately after the critical point it is hoped that separation of regeneration from wound healing will make these patterns clearer.

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