16

Regeneration

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16.1 Introduction

Regeneration is found to varying extents throughout the animal kingdom (Goss, 1969). The first recorded discussions of the phenomenon in cockroaches occurred in the 1840s at meetings of the Royal Society of London. At that time, a purely philosophical debate ensued on whether cockroaches could replace lost limbs. The first productive experimentation confirming that leg regeneration does occur in cockroaches did not take place until a half century later (Brindley, 1897). Since then, the phenomenon has been studied in considerable detail, particularly in cockroaches, with the majority of work concentrating on leg regeneration. The reason for this preoccupation with legs is the special nature of the leg regeneration process in cockroaches. As in other insects, many tissues of the cockroach have the capacity to regenerate themselves relatively slowly over the course of a number of larval instars. Cockroach legs, however, will regenerate in an 'all-or-none' fashion in a single instar (O'Farrell and Stock, 1953). This all-or-none response has not been described for insect appendages other than the legs of cockroaches and the wing imaginal discs of Lepidoptera (Pohley, 1965).

   The ability of cockroach legs to regenerate their form and function within a single moulting cycle has made them an attractive object of research in a number of areas including studies of pattern formation (Bohn, 1976; Bryant et al., 1977, 1981), endocrine regulation (Bodenstein, 1959; O'Farrell et al., 1960; Penzlin, 1965; Bu;;iere and Bulliere, 1977b; Kunkel, 1977), neural specificity (Bodenstein, 1957; Young, 1973; Cohen, 1974; Guthrie, 1975), *in vitro* cuticle synthesis (Marks and Leopold, 1971) and endocrine (Marks, 1973a,b) and insecticide (Sowa and Marks, 1975) action.

l6.2 Tissues with regenerative potential

16.2.1 Epidermal structures

While only legs in cockroaches regenerate in an all-or-none fashion, other appendages, such as eyes (Hyde, 1972; Shelton *et al.,* 1977), antennae (Haas, 1955; Pohley, 1959; Drescher, I960; Sdiarer, 1973), and (O'Farrell and Stock, 1956a), and exoskeletal features, such as ecdysial lines (Shelton, 1979) regenerate more or less gradually over a number of moulting cycles depending on the severity of loss. The failure to regenerate completely in a single moulting cycle can be due to the inability of appendages, other than legs, to delay the moulting cycle. Antennal amputation, while it does nor delay individual moulting cycles, has been observed to increase the number of instars which *Peripianeta americana* and other species of cockroach take to reach the adu!t stage (Pohley, 1959; Tshii, 1971).

In order for tissues involving epidermal structures to regenerate, thcy must go through a series of stages which have been described by various authors (Penzlin, 1963 ;Bulliere and Bulliere, 1977b). After an initial wound healing phase, a period of embryological dedifferentiation and re-differentiation occurs under the old cuticle during which the pattern of the lost structure is in some way re-established. Next, a growth phase allows for the growth of the structure to approximate the size of the eventual regenerate Finally, the pharate-regenerated epidermis secretes a cuticle and awaits ecdysis to reveal its form. The timing of the regenerative programme corresponds to the normal cyclical pattern of determination, proliferation and differentiation described for epidermis (Kunkel, 1975a). The initial regression and re-determination of the pattern of a limb can only occur in the intermoult phase of the moult/intermoult cycle. Any mitoses necessary ro reestablish bristles or glands m the regenerate must occur during the interphase period, the normal riming of this type of mitosis for the general epidermis, If regeneration is not initiated during this phase, no regeneration occurs in the current stadium. If a structure is lost during the intermoult phase it can only regenerate in proportion to how much of the intermoult phase is left for pattern re-establishment and differentiative mitoses. If it is a leg that is lost, a mechanism exists for extending the intermoult phase and allowing a functionally complete pattern to be reestablished.

16.2.2 Internal tissues

The extent of regeneration of internal organs other than leg-related tissues is treated briefly in the literature. Of particular intcrcsr to endocrine research is the apparent ability of the prothoracic glands of *P. americana* to regenerate after extirpation (Bodenstein, 1955b, 1956). Neuro-endocrine cell bodies have no capacity to regenerate (Drcscher, I960), however, the neuropile and commisures of the brain show substantial ability to reform after ablation and section experiments. Two categories of neurons have been proposed: Category one neurons of early

embryological origin, which cannot regenerate, and Category two neurons of later origin, which retain a higher growth rate and capacity for regeneration (Guthrie, 1975; Jacobson, 1978).

Leg-related internal tissues including muscle and nervous tissue have been shown to have extensive regenerative potential. Muscle (Cowden and Bodenstein, 1961) and neuronal regeneration (Young, 1973) have also been studied in the absence of leg regeneration. Following nerve and muscle regeneration in the adult also allows the experimenter to isolate the phenomenon from the confines of a moulting cycle.

16.2.3 Regenerative fields

A morphological structure is surrounded by a space called its field. The field is a circumscribed area of tissue from which the original structure can regenerate if at least a portion of the field is left remaining. Regenerative fields have been observed for eye, antenna, cercus and leg of cockroaches. Of particular interest to the general study of fields is the demonstration (Bohn, 1974b, 1976) of a two-part epidermal field for leg regeneration. The leg field includes both sclerites anterior to the coax and a membranous epidermal region posterior to the coxa. Both must be present for regeneration to occur and each contributes a longitudinal half of the eventual regenerate. This supports the intercalation rule of Bryant. (Section l6,3.4d).

A tissue might nor necessarily have to have been a part of a structural field in order to be incorporated into and contribute to its structure, Pronotal cuticle transplanted to the head adjacent to the eye was reported to incorporate into the advancing margin of the compound eye and contribute to facet development (Hyde, 1972). However, this result has not so far been repeatable in other workers hands (Shelton *et al.,* 1977) which might argue that only cells within an eye field, as embryonicly determined, are competent to form eye cells.

16.3 Phenomenology of limb regeneration

16.3.1 Faithfulness of regeneration

(a) *Gross morphology of regenerate*
That the leg regenerate is not a faithful copy of the original cockroach leg is one of the oldest facts to be reported in the literature (Brindley, 1898). The most obvious difference is that the regenerated tarsus has four segments instead of the normal five (Fig. l6.1). This artifact allows a simple tarsal segment count to establish whether a cockroach has ever regenerated a limb. Since regeneration can affect the race of development (O'Farrell and Stock, 19^6b), this artifact can be a valuable aid to identifying and eliminating animals with a history of regeneration from a developmental study.
Differences between the original and regenerated limb of *P. americana* were
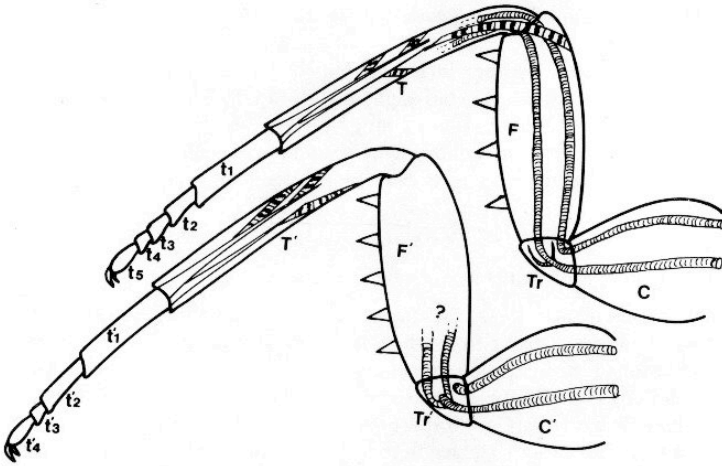
Fig. 16.1 Normal versus regenerated cockroach legs. Composite qualitative differences observed in *P. americana* (Penzlin, 1963) and *B. germanica* (O'Farrell, 1959; Kunkel, 1968). The major differences involve the tracheal supply, an absence of a tarsal muscle inserted in the femur in normal legs, femoral spine number and tarsal segment number. The regenerate exemplifies the form and size attained by at least three instars after the time of autotomy by which time all size regeneration has been completed (Roberts, 1973). The question mark in the regenerated femur indicates a highly variable tracheal branching pattern in the regenerate.

catalogued more completely by Penzlin (1963). He noted differences in internal morphology including differences in musculature and a highly variable tracheal supply to the regenerated femur, tibia and tarsus. The difference in tracheation could reflect the *ad hoc* active role of tissues in directing the distribution of tracheation in other systems (Wigglesowtth, 1959).

(b) *Size of regenerates*

The size of initial regenerates is highly dependent on the time in the moulting cycle at which autotomy occurs (Fig. 16.2a) as well as the time after autotomy at which the delayed ecdysis occurs (Fig, 16-2b) (Kunkel, 1977). The new regenerate, although qualitatively functional, is never equivalent in size ro the contralateral normal leg. However, through subsequent moulting cycles the regenerate rapidly approaches the size of a normal leg (Roberts, 1973). This approach to normality in subsequent moulting cycles includes such details as tibial spine number (Kunkel, 1968). But, the traits mentioned in the previous section (tarsal segment number and tracheal branching pattern) never return to normal. In the regenerated tarsus, the reduction in tarsal segment number is compensated by an increase in the lengths of the first two segments, but primarily tarsal segment two (Roberts, 1973).
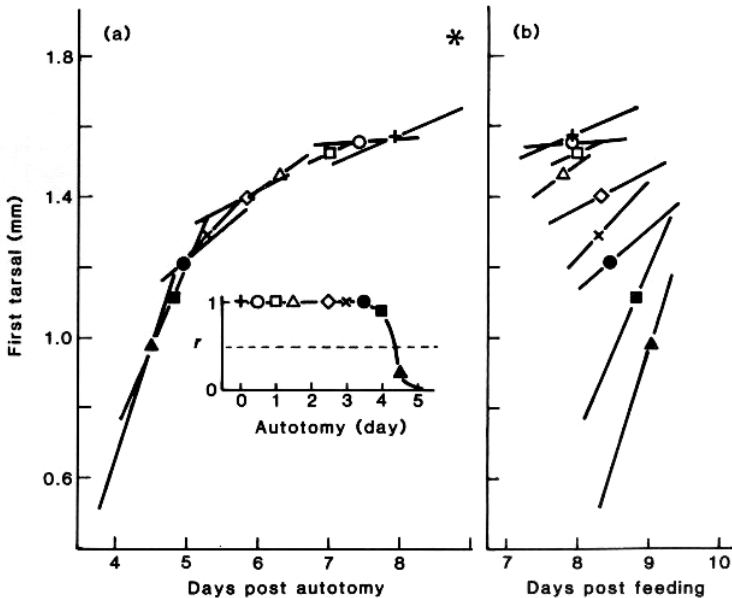
Fig. 16.2 Tarsal size regenerated at the first moult after tarsal autotomy. Size of the first tarsal segment at ecdysis is plotted versus (a) time from feeding to ecdysis and (b) time from autotomy to ecdysis. Groups of 25 animals had a metathoracic tarsus removed at different times after feeding in the VI (metamorphic) instar. An inset to (a) identifies the time of autotomy as well as the proportion regenerating for each group, *r*. The 50% point of this inset curve represents the midpoint of the critical period for regeneration for this stage animal. Each regression line in (a) and (b) passes through the mean size of regenerates as well as the mean time of ecdysis for that group. The regression line spans the times of ecdysis for that group. An asterisk in (a) indicates the length of the normal first tarsal segment (modified from Kunkel, 1977).

After three or four moulting cycles, regenerated segments three and four are indistinguishable in size from segments four and five of the contralateral normal leg. Segment two has also been shown to be the critical tarsal segment that must be lost if a four-segmented regenerate is going to be formed. Amputation at tarsal segment three, four or five results in a normal five-segmented tarsus (Bohn, 1965) (cf. Penzlin, 1963).

Another departure from faithfulness only becomes obvious in the regenerate after a number of instars. The posterior border of the femur is usually spined. While the femur approaches normal size within a few moulting cycles, it consistently has one fourth more spines on its border than a normal leg (Kunkel, 1968). The addition of each spine occurs at a position formerly occupied by a bristle in the previous instar (Kunkel, unpublished). Studying the spacing of spines along the posterior edge of the femur in regenerated versus normal legs provides a one-dimensional system in which to study bristle pattern formation as opposed to the usual two-dimensional models (Lawrence, 1973).

The difference in pattern between normal and regenerate limbs may represent

the effects of different conditions existing in the embryo compared to the regenerating limb during the critical phases when the pattern of the limb is being determined. It is clear that the pattern of bristles and the segment number and length are controlled by gradient phenomena in the epidermal tissue. It is nor clear how these natural aberrations in pattern, observed in regenerating limbs, relate to the experimentally induced alterations in pattern which will be discussed later (Section 16.3.4d).

16.3.2 Moulting delay

(a) *Time of single leg autotomy*
A critical period for leg regeneration was originally described for *Blattella germanica* (O'Farrell and Stock, 1953) but is known to be a general phenomenon in all families of cockroaches, including *P. americana* (Penzlin, 1963). Prior to the critical period an animal will delay its moulting cycle to regenerate an autotomized limb. After the critical period, there is no moulting delay and no regeneration in response to an autotomized limb. Only a healing phase occurs when the moulting cycle is not delayed. This regeneration critical period was shown to occur simultaneously with the brain critical period (Kunkel, 1975a).

(b) *Severity of loss*
The length of delay of the moulting cycle fits the severity of the loss of tissue by autotomy. Thus, within each thoracic segment, loss of a tarsus will delay less than the loss of a femur-tibia-tarsus. Also loss of a prothoracic femur-tibia-tarsus causes less delay than loss of the same segments of the larger metathoracic limb. Loss of two limbs of the same thoracic segment at the same time will cause additional delay compared to loss of one limb (Stock and O'Farrell, 1954).

      These correlations of the amount of delay with amount of tissue to be regenerated were construed by a number of workers to mean that the amount of regenerating tissue was controlling the delay. However, evidence to the contrary suggests rather than the length of delay determines how much regeneration can occur (see Kunkel, 1977 for references and discussion). Exactly what mechanism fashions the length of delay so that it is appropriate for the amount of tissue to be regenerated is not yet understood.

(c) *Autotomy versus amputation*
While leg regeneration will initiate from non-autotomy points, the delay of the moulting cycle depends upon limb loss at either of two autotomy points, the trochanter-femur or the tibia-tarsus joints. Loss at any other joint or mid-segment is rare since the two autotomy points yield so easily to tension. The distinction between autotomy and amputation is most clearly seen in large groups of synchron -ized animals which have a limb either autotomized or amputated shortly before their critical period for regeneration (Fig. 16.3). In such contrasts, the amputated animals do not delay the moulting cycle and, as a result, do not regenerate the lost segment (Kunkcl, 1975a). The same contrast with the limb removed earlier in the
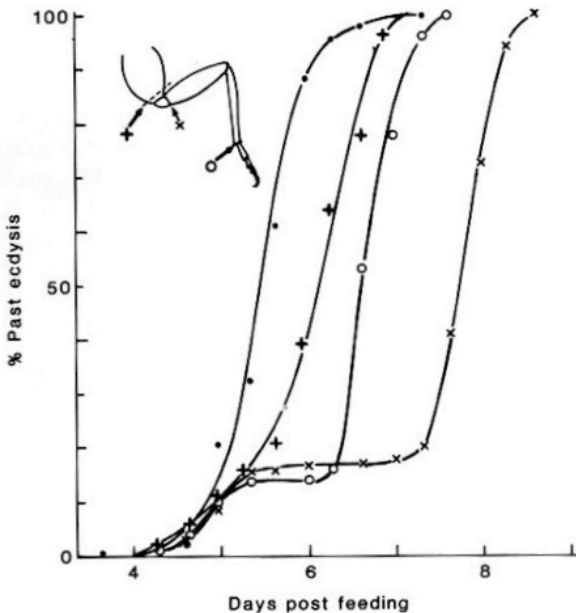
Fig. 16.3 Differences in effect of autotomv and amputation on the cumulative moulting curves of fourth instar *B germanica.* Autotomy of either a tarsus (O) or femur-tibia-tarsus (×) results in a biphasic curve for cumulative ecdyses. Early non-regenerates are separated by a platteau from the delayed regenerating animals. The amputation at the distal end of the coxa (+) produces a largely monophasic curve somewhat delayed compared to the unoperated controls (●) but resulting in no regeneration (modified from Kunkel, 1977).

stadium results in regeneration in both cases without any substantial delay of thc moulting cycle.

(d) *Roles of nerves*
Experiments with feeding-synchronized *P. americana* have shown that innervation of the leg is essential for establishing a delay of moulting. If the nerve trunks innervating the leg are cut, autotomy of the leg close to the critical period for regeneration does not result in regeneration or delay of the moulting cycle. Simultaneous autotomy of a contralateral leg does result in delay and allows time for some of the non-innervated legs to regenerate also (Kunkel, 1977). Furthermore, an intact connection between the brain and the ganglion innervating the regenerating limb in *P. americana* was found necessary for moulting delay and regeneration to be established.

(c) *Role of hormones*
Circumstantial evidence suggests a role of hormones in die delay of the moulting process; however, most of this evidence bears on the effects of hormones on the

regeneration process itself (Section 16.4.2a) rather than on the delay. The size of the corpora allata changes during the regenerative process (O'Farrell *et al.,* I960). Ligating the head shortly after autotomy prevents regeneration but also removes the source of both juvenile hormone (JH) and brain hormone (Penzlin, 1965). Since JH is known to delay brain hormone secretion in other insects (Nijhout and Williams. 1974) and possibly in cockroaches (Kunkel, 1981), the exact route of feedback in delaying moulting is obscure at this point. Bevond this, it is seen that the regeneration critical period is coincident with the brain critical period which suggests that delay of moulting could be caused by a delay in brain hormone secretion (Kunkel, 1975a). Direct measurement of ecdysteroid (ECD) titres in normal and limb-regenerating animals has demonstrated that moulting delay is, paralleled by a delay in the major peak of (ECD) in *Blatta orientalis* (Kunkel, 1977). The delay of moulting could thereby be caused by an insufficient dire of brain hormone to turn on the prothoracic glands secretion of ecdysone.

16.3.3 Synchronization of moulting

(a) *Single leg autotomy*
Species of cockroach from all major families, including *P. americana,* have their moulting cycles synchronized by being forced so regenerate a limb (Kunkel, 1977). Autonomy shuts a moulting gate. Animals which delay their moulting cycle to regenerate an autotomized limb queue up at this gate. When some as yet unidentified regenerative process is completed, the gate is opened and all the animals initiate a moulting cycle in a relatively synchronous fashion.

(b) *Independence of delay, due to multiple autotomies*
Two similar types of evidence suggest that the delays imposed by multiple simultaneous autotomies are independent of one another. The first evidence is the predictable extra delay and synchrony of moulting observed when two legs rather than one are autotomizcd (Fig. 16.4). The extra delay and synchrony derive precisely from the independence of the two delay processes. If a model of a six-legged hypothetical cockroach is considered, the moulting behaviour after autotomy could be easily predicted if all six legs had identical, but independent, delay properties. Autotomy of six legs would have closed six identical and independent gates. Since all gates must be opened for moulting to be initiated, the distribution of moulting initiation then could be predicted from the distribution of the last of six independent and identically distributed events.

However, since pro-, meso- and metathoracic legs have substantially different mean delay times associated with their autotomies, there is no chance for their gate-opening distributions to interact to create a greater delay than an individual leg would cause. In such cases, all the gates for the lesser delay (smaller) limbs will have opened by the time that the frequency distribution of the longer delay gates starts, and thus the delay-of-moulting distribution follows the characteristic of the larger leg. This is precisely che pattern observed when two dissimilar legs are autotomized simultaneously.
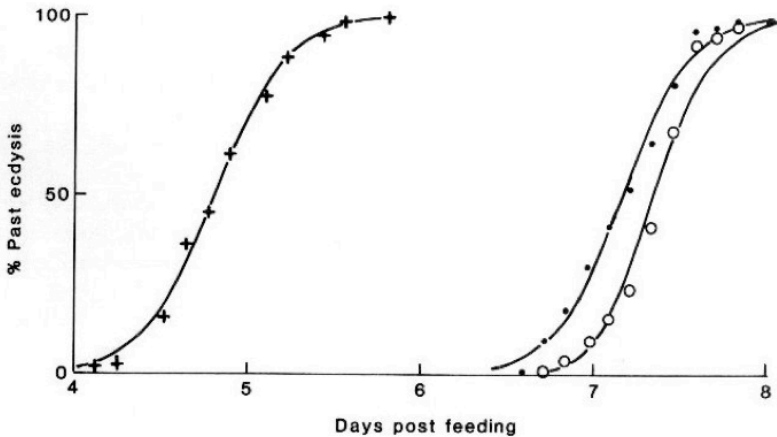
Fig. 16.4 Delay of moulting due to autotomy of 2 versus 1 metathoracic femur-tibia-tarsi. Cumulative percent past ecdysis is plotted against time of ecdysis measured from the time of feeding. The curves for control and single leg regenerates were computed using a logistic curve. The curve for the double leg regenerates was computed by squaring the curve for the single leg regenerates. The data fit the curve according to a Kolmogurov-Smirnov goodness-of-fit test (Kunkel, 1977).

When two identical legs are autotomized sequentially at a sufficiently long interval apart, the last leg to be autotomized controls the timing and synchrony of the delayed moult. Such sequentially autotomized legs can act in concert to extend the intermoult phase considerably because each autotomized limb defines a new, secondary. critical period which is the time before which any subsequent autotomy must occur to further delay the moulting phase and ensure regeneration in the current moulting cycle. Such delays have their obvious limits since the cockroach has but six legs to lose in each instar.

16.3.4 The regenerative process

(a) *Wound healing*
Whether regeneration occurs after an autotomy or amputation, the first response is that of a healing process. This process has been described at the light microscope level in *P. americana* (Bodenstein, 1955b; Penzlin, 1963) and in *Bluberus discoidalis* (Bohn. 1976). Initially, a clot is formed involving haemocytes plugging the opening of the wound. Within a few days, the epidermis extends from its ruptured borders closing the gap spanned by the clot. The closure is an active process. The epidermis is not passively guided by the clot, but must actively pinch through the clot to become continuous. Besides their role in controlling infections attendant to loss of the limb, the haemocytes also play an active role in the healing since epidermal cells are reluctant to grow out from their cuticular sites of

attachment if haemocytes are not present or if the clotting process is inhibited (Bohn, 1976, 1977a). A haemocyte 'conditioning factor' may play a role in the growth behaviour of the epidermis (Bohn, 1977b). This factor has been shown to be a protein and to be antigenic.

(b) *Dedifferentiation*

The regeneration of the metathoracic limb autotomized at the trochanter-femur autotomy plane is the most convenient process to follow since the process occurs largely within the coxal segment, and the progress can be monitored visually through the broad flat ventral face of the metathoracic coxa (Penzlin, 1963). After wound healing, the tissues of the stump undergo a period of dedifferentiation and regression. The epidermis detaches from the cuticle and retreats within the coxa (Penzlin, 1963). The underlying muscles release their attachments to apodemes and also lose substantial portions of their structural detail, including the fibrillar components of their contractile apparatus (Bulliere, 1968). The regression of at least some of the muscles is under the control of the nerves innervating them in *P. americana* (Shapiro. 1976). In autotomized limbs which have also been experimentally axotomized, the muscles of the coxa undergo a more drastic regression. In normally innervated legs, neuromuscular synapses maintain their structure during the regression; and the muscle cells maintain their insertions into epidermal cells, even though the epidermis has released from the apodemes. In the majority of muscle, regression involves loss of mass, including sarcoplasmic reticulum, mitochondria and myofilaments, rather than loss of cells (Shapiro, 1976). This regression involves all the muscles of the coxa, but the distal muscle is involved sooner than the proximal. After losing 40% of their weight, the coxal muscles undergo no further dedifferentiation. This limited and orderly regression is characterized by Shapiro (1976) as the reversal of normal muscle development and is contrasted to the degeneration and eventual autolysis of muscles observed during metamorphosis of hemimetabolous insects (cf. Lockshin and Brauleton, 1974).

 How coxal muscle regression is induced is not known. Although there is a general reduction in activity of the nerves innervating the coxal muscle after autotomy, mini-endplate potentials continue to be recordable during the regression period and muscle spike potentials are recordable from coxal muscles during walking, though in a generally unpredictable pattern compared to the normal phasing of contractions of intact legs. If this innervation of the regressing muscles is removed, severe autolysis of the muscle occurs (Shapiro, 1976).

(c) *Blastema formation*

As in many regenerating systems (Goss, 1969) the form of the regenerate emerges from an apparently undifferentiated cluster of cells, the blastema. The origin of the blastema tissue is of considerable theoretical interest. Does pre-existing coxal muscle dedifferentiate into a pluripotential state, a blastemal cell, from which it can redifferentiate into a different muscle cell type at some other morphological location? Does all the blastemal tissue arise at the wound site or does it come from

some pool of cells held in reserve in another part of the body?

Some of these questions have been answered by transplant experiments between species in which the donor and host cuticular products are clearly distinguishable. It is clear, for instance, that the regenerated epidermis is not clonally derived and that it does originate from tissues adjacent to the wound site (Bohn, 1972). Tracheal regeneration does not originate from an extension of the prior tracheal trunks. Rather it derives from growth of small tracheal branches previously serving local needs (O'Farrell, 1959; Penzlin, 1963). Motor axons regenerate from their central origins (Guthrie, 1975). The only tissue whose origin has not been traced is muscle. During limb regeneration new myotube and muscle differentiation is occurring continuously up to the time of ecdysis (Cowden and Bodenstein, 1961). Initially, these cells, which form the new muscles of the lost limb segments, were thought to be derived from the regressing and dedifferentiating coxal muscle (Penzlin, 1963). However, since current studies at the electron microscope level (Schapiro, 1976) suggest that coxal muscle remains essentially intact, except for an atrophy of their internal machinery, the immediate source of the muscle blastematous tissue is conjectural. Perhaps, like the epidermis, it is derived from a very localized set of cells immediately adjacent to the wound site. The solution of this problem is of general interest since it may shed some light on the general phenomenon of muscle proliferation in insects.

During the blastemal stage the segmental and intrasegmental patterns of the limb are being established. The small distances involved at the blastemal stage may afford the geometry within which the necessary events for pattern formation occur. From the rare occasions when animals seem to have initiated moulting when they had barely passed the blastemal stage, it is obvious to this observer that a primitive segmentation is already set up in the late blastema. Clearly, this stage is the most critical and least understood of the regeneration process. The explanation of the phenomena occurring during blastema development would be an unveiling of the most basic mysteries of developmental biology.

(d) *Epidermal pattern restoration*

The regenerating limb exhibits many of the phenomena associated with pattern formation in vertebrates and other invertebrate systems (Bryant et al., 1977; Palka, 1979). In particular, the segments of the regenerating limb are a set of repeating gradients (Bohn, 1966) similar to the repeating gradients of the abdominal sclerites (Locke, 1959; Lawrence, 1973) (Fig. 16.5). Gradients in adjacent segments of the cockroach limb have been shown to be similar by virtue of compatibility of equiva -lent levels when inter-segmental grafts are performed (Fig. 16.6b) (Bohn, 1966).

Incompatibiliries of tissues in grafts can be explained with reference to two types of gradient operative in each segment of an appendage: an axial gradient and a circumferential gradient (Bohn, 1965, 1966; French, 1976, 1978). The length and longitudinal pattern of each segment is controlled by rhe axial gradients. When the proximal end of a segment (nominal high end of a gradient) is grafted on to the
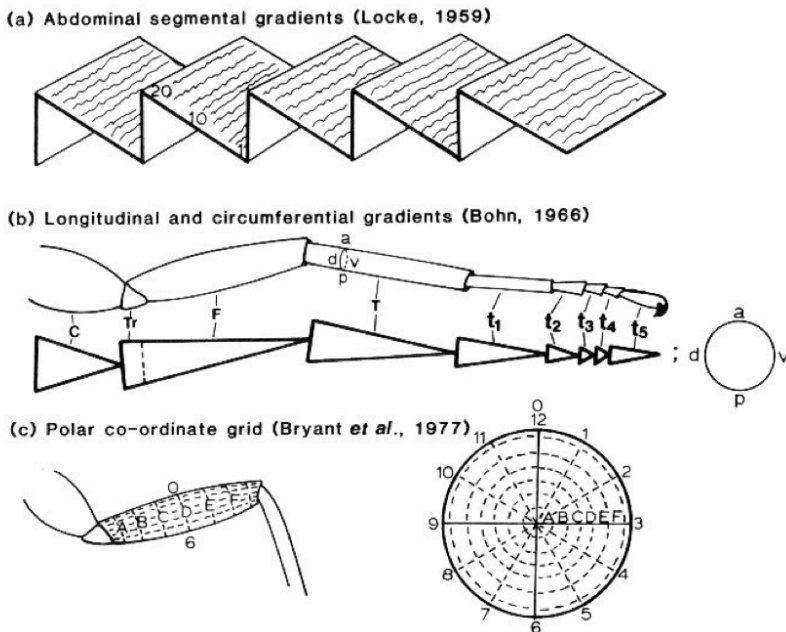
Fig. 16.5 Gradient models of abdominal and leg scgments. (a) The earliest gradient model used to describe the behaviour of the adult transverse tergal ripple pattern of adult *Rhodnius prolixus* when grafts are performed during the previous larval stages. Hypothetical gradient levcls (1-20) in each host segment were required to be in alignment with graft gradient levels in order for the ripple pattern of the adult to be undisturbed. (b) Application of gradient concepts to the cockroach leg. Each leg segment is suggested to contain a gradient (note that the trochanter-femur gradient comprises one gradient). A separate circumferential gradient was postulated to explain results of contralateral grafts. (c) The polar coordinate grid as it is envisioned to apply to each cockroach limb segment. Each point on the surface of a limb segment can be mapped to appoint on the polar co- ordinate grid.

distal end of an identical segment (low end of a gradient) in a host (Fig- l6.6c), an incompatibility of levels of the opposed gradients is set up, which results in the generation of superfluous tissues intervening in gradient level between the two opposed ends. Generation of superfluous tissue can be interpreted as a key to understanding the nature of gradients and how they control tissue growth and pattern.

Epidermal cells grow, divide and orient themselves and their cuticular secretions in relation to the gradient position they rind themselves in. This inferential statement is a direct outcome of the observations on the amounts and orientation of regenerated superfluous tissues that result from experiments such as that described in Fig. 16.61c. The active role of axial gradients in orienting cells was vividly demonstrated by Bohn (1974a) using interspecific grafts of identifiable tissue rotated 90° from the host segmental axis. The orientation of the bristles of the transplant gradually rotated in a step-wise manner in subsequent moulting cycles to eventually assume the orientation of the host epidermal cells adjacent to the graft.
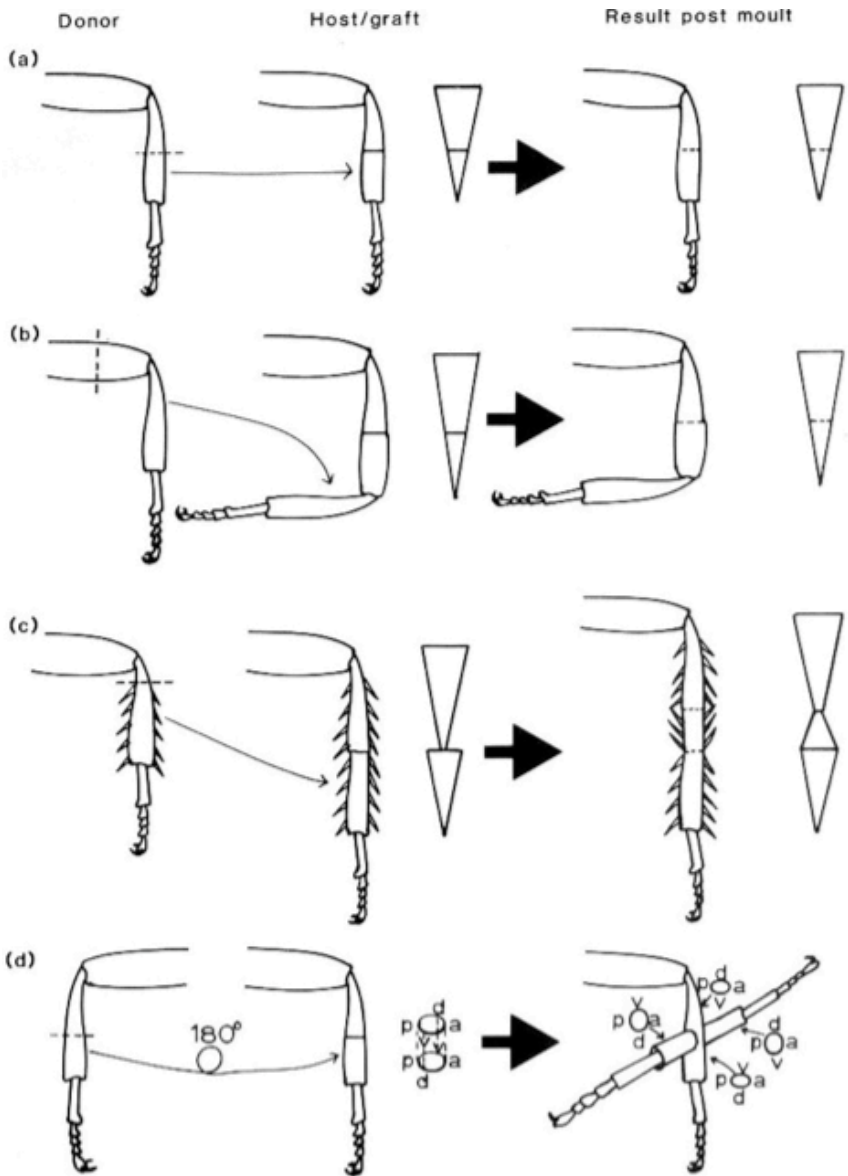
Fig. 16.6 Grafting experiments, (a) Homologous ipsilateral tibial graft (same leg; same segment; same level). (b) Inter-segmental graft (same leg, femur graft on tibial host; same level). (c) Intra-segmental graft (same leg; same segment; proximal level graft on to distal level host tibia). (d) Contralateral tibial graft (contralateral leg; same segment; same linear gradient level but incomplete circumferential gradients) The outcome of each experiment can be predicted by referring to gradient compatibilities. Orientation of the tibial spines is used to predict the nominal direction of the gradient.

While circumferential gradients perpendicular to the axial gradient have not been described in abdominal segments (Locke, 1959; cf. Shelton, 1979). in limbs such gradients control the pattern and increase in girth of limb segments (French, 1978). These circumferential gradients are most graphically demonstrated by grafts of donor limbs to host limbs at a similar axial gradient level, but on a contralateral side of the animal (Fig. 16.6d).   Such grafts result in incompatibilities of the circumferential gradients of apposed tissues which cannot be satisfied by a simple rotation of the tissues relative to one another. The net result is often supernumerary limbs generated at the sites of incompatibility.

A mathematically descriptive and predictive model encompassing both the axial and circumferential gradients has been proposed (French, 1976; Bryani *et al.,* 1977), which is consistent with most of the phenomena so far observed. This model is capable of predicting the outcome of grafting experiments in which incongruities of the apposed gradients result. This extends to predicting the amount of superfluous tissue (Fig. 16.6c), the placement of supernumerary limbs (Fig. 16.6d) and even the handedness of the supernumerary regenerates. This model describes the limb gradients as a polar co-ordinate grid in which the axial gradient is represented by the centripetal rays of a circle (Fig. 16.5c), while the circumferential gradients at each point along the axis of the limb are represented by concentric rings in the model. Any point on the leg segment: can be mapped to a gradient point within the polar co-ordinate grid.

Along with this fine resolution description of the gradients go rules for the behaviour of tissue when the integrity of the system is destroyed by amputation or grafting. When a portion of a limb is ablated, the resulting healing brings together epidermal cells that differ more or less in their gradient values.   This incompatibility stimulates growth to replace the intervening tissue and gradient values. An additional rule states that the shortest route between the two apposed gradient levels will be taken in intercalating a complete pattern.

This model is partially an outgrowth of a more disjointed earlier model of Bohn (1965) in which the poles of the limb circumferential were defined more crudely in terms of dorsal versus ventral and anterior versus posterior borders, plus a distinct longitudinal axial gradient. This earlier model was based almost entirely on cockroach experiments and predicts most of the same results as the finer resolution model of Bryant *et al.* (1977). Neither of the models deals effectively with the transitions from segment to segment of the limb nor with what happens in the terminal segment, which would seem to have some trouble rounding itself off at the tip. Neither approach the fundamental problem of the nature of the gradients involved: Are they gradients of substance or of cell adhesivity or of cell compatibility?

The fundamental contribution of the gradient models is their provision of a mechanism for recognition of wholeness at the tissue level.   When, due to autotomy or amputation or the artificial introduction of a transplant, cells with different gradient level properties come to abut one another, the incompatibilities of gradient level result in a spontaneous correction of the problem. The result for most of the situations an animal will normally encounter (i.e. autotomy or

amputation) is normal regeneration; the result of grafting contralateral legs is supernumerary regeneration. By understanding the artificial situations created by grafting, we may at some later date be able to understand the normal generation and regeneration of pattern.

(e) *Neural specificity*

Another aspect of pattern restoration exhibited by the regenerating limb is the specificity of nerve cell - muscle cell recognition and re-innervation. This topic has been abundantly reviewed in the past decade (Young, 1973; Guthrie, 1975; Anderson *etal*. 1980; Pipa, 1978b). This aspect of regeneration is rarely studied in the normal regenerating limb. As demonstrated by Shapiro (1976), the muscles of the coxa never lose their innervation during coxal regression attendant on limb autotomy. Despite this, the majority of experiments on neural specificity in the cockroach leg concentrate on the specificity of coxal muscle re-innervation after nerve section in adults (Young, 1972; Pearson and Bradley, 1972).

From experiments on artificial nerve section and leg transplantation it has been demonstrated that specific afferent and efferent re-innervarion of coxal muscles occurs (Pearson and Bradley, 1972; Fourtner *et al.,* 1978). Innervation cues are equivalent on right and left sides of the body (Bate. 1976a). Adjacent thoracic segments use the same cues for motor axon recognition of homologous muscles (Young, 1972). The initial finding of the appropriate muscle by a motor neuron is perhaps a trial-and-error search process with inappropriate connections made at first (Young, 1973; Whitington, 1977; Denburg *et al.,* 1977). Subsequently, the appropriate connections are singled our to survive.

If this later suggestion is correct, then more weight is placed on a recognition of appropriateness and inappropriateness of cell junctions. Identifiable muscle fibres of *P. americana* differ from one another in the pattern of their polypeptides (Denburg, 1975, 1978). Whether any of the so-far catalogued differences in *P. americana* are sufficient to serve as a decisive factor m stabilizing an appropriate re-innervation has been questioned (Tyrer and Johnson, 1977). Further study of what happens at initially inappropriate innervation sites is needed (cf. Urvoy, 1970).

16.4 Regulation of regeneration

16.4.1 Roles of nerves

(a) *Direct trophic effects of nerves*

The earliest work on the role of nerves in cockroach leg regeneration (Bodenstein, 1957; Penzlin, 1964) demonstrated that nerves were not essential for regeneration to be initiated nor for the maintenance of the process once Starred. These experiments were done on animals autotomized shortly after ecdysis to allow ample time for the regenerative process to be established before a moulting cycle was initiated. While the epidermal components of regenerates from denervated

legs appear qualitatively complete, they are in general smaller than normally innervated regenerates and are 'hollow', lacking a well-developed musculature (Penzlin, 1964). A general trophic role of nerves seems to consist of maintaining muscle size and integrity (Guthrie. 1967a; Lockshin and Beauleton, 1974). A more specialized trophic role for nerves is an important part of the normal coxal muscle regression which attends autotomy (Shapiro, 1976; Section 16.3. 4d). The effects of nerves on the size of the epidermal components of a regenerate are most likely secondary to neuromuscular trophic effects (though this point is somewhat mute due to rapid regeneration of nerves, cf. Edwards and Palka, 1976).

(b) *Indirect role of nerves*
Without the proper sensory innervation of a limb and without the proper sensory stimulation by autotomy, no delay of the moulting cycle will occur (Section 16.3.2b,c). In this sense, an intact nervous sytcm is essential to a consistent all-or-none regeneration of a leg. A leg lost early in the stadium will regenerate its epidermal form at least, irrespective of whether it is innervated. However, if a leg is lost close to the time of initiation of a moulting cycle and is denervated, an incompletely formed regenerate results, since the moulting cycle is not delayed and die critical phase of morpholaxis is interrupted.

16.4.2 Role of hormones

(a) *In vivo results*

Regeneration is not an entirelv spontaneous activity of the tissue remaning after autotomy or amputation of a structure. This is evident from the observation that adult *P. americana* legs do not go through any of the steps of regeneration other than healing (Bodensicin, 1953c), unless transplanted to or parabiosed to a larval animal. In addition, in larvae of *P. americana,* the steps of the leg regenerative process beyond healing can be prevented by ligating the head from the body within the first two days after autotomy (Penzlin, 1965). While it is clear from *in vivo* experiments that large doses of ECD will terminate the autotomy-induced regenerative process (Bulliere, 1972; Kunkcl, 1975b, 1977) by precipitating a moult cycle, it is unclear from these studies what hormones might be involved in promoting regeneration in any positive sense (O'Farrell *et al.,* 1960; Penzlin, 1965). One suggestion that ecdysteroids might play an enabling role in regeneration derived from the observation that *P. americana* adults would initiate regeneration if a leg was taken off shortly after ecdysis, a time prior to prothoracic gland involution (Bodenstein, 1953c) (see also Section 7.6.13).

 (b) *In vitro effects*
The original suggestion (Marks and Reinecke, 1964) that an organ culture approach to studying limb regeneration phenomena might be fruitful, has paid off handsomely (Marks, 1980). Two dose-dependent functions of ECD in controlling leg regeneration have been demonstrated *in vitro*. Early morphogenetic phases

require low doses of ECD to continue while the later formalization of the regenerate by cuticle production is dependent on higher titres of the same hormones (Marks, 1973b). It is still unclear what specific phases of the morphogenetic process require ECD and whether there are other hormones involved.

16.5 Unified model

16.5.1 Systems involved

The process of leg regeneration requires the involvement and interfacing of three systems in the domain of the leg itself as well as one major system extrinsic to the leg. The three leg systems are: (1) a neuromuscular servo-system involved in walking, (2) an epidermal system involved in cuticle maintenance and moulting and (3) a morphogenetic system involved in pattern generation and restoration. The outside system with which the three leg systems interact is the neuro-endocrine system controlling the moulting process. Each of these systems can be considered separately as a black box with inputs and outputs (Fig. 16.7).

16.5.2 Interactions of the systems

(a) *Neuro-endocrine system*
The inputs of the neuro-endocrine system are feeding, which controls the initiation of the moulting process (Kunkel, 1966), and the delay status input from each of the six independent leg sensory-neuromuscular systems. As long as there are no autotomized legs, the neuro-endocrine system is free to respond to food availability by a rimed production of the mouiring hormone ccdysone. Ecdysone titre is the output of this system. The ecdysone output impinges on two leg systems, the epidermal moulting system and the epidermal morphogenetic system.

(b) *Epidermal moulting system*
This system requires a high titre of ecdysone to undergo its cycle of cuticle deposition. In carrying out its role in the moulting process, this sysrem may cut short any regeneration that is taking place. This behavioural response is a fundamental property of all epidermal tissue.

(c) *Epidermal morphogenetic system*
In the early part of the stadium, the low ecdysone titre stimulates the epidermal morphogenetic system to operate. This self-contained system responds to local needs for new structures such as new glands or sensory bristles by differentiative mitoses of epidermal cells (Kunkel, 1975a). When an autotomy or amputation has occurred, this system responds to the resultant incompatibilities of adjacent epidermal moulting system has not been activated. When moulting is activated, all regenerative activities cease and the epidermal structures so far regenerated go through a cycle of cuticle production. The completeness of regeneration depends

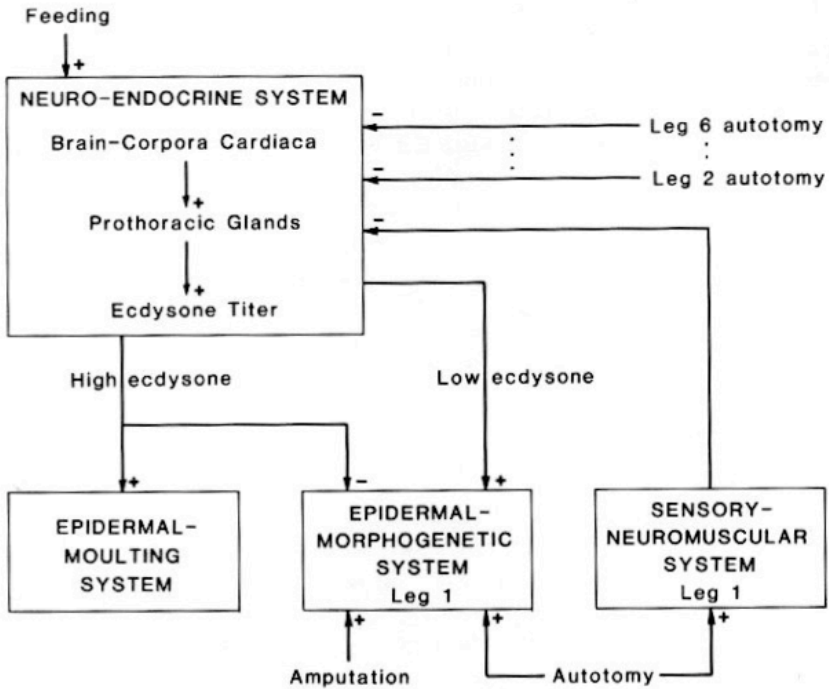on the appropriateness of the delay provided by the sensory neurumuscular system.

Fig, 16.7 Flow diagram of regeneration regulation. Arrows reflect information flow between systems contained in each box. Signs(+ and -) refer to stimulation or inhibition of one system on another.

(d) *Sensory neuromuscular system*
This system is made up of the muscles of the leg, the sensory neurons, motor neurons and interneurons involved in locomotion behaviour. Critically relevant to regeneration is the ability of the nervous system to perceive and react behaviourally to the loss of a limb. The perception of autotomy point is reflected in an immediate change in locomotory pattern (Hughes, 1957; Delcomyn. 197lb). This change in pattern depends on loss of the lirnb at the autotomy point. Artificial limb loss at the femur-tibia joint by amputation does not result in an altered stepping pattern. It is likely that it is the leg sensory system that is involved in the perception of a need for a delay of the moulting cycle, the hemiganglion serving the autotomized limb relaying this information to the brain (Kunkel, 1977; Section 16.3.2d). An earlier hypothesis suggested that the bulk of the regenerating muscle (Cowden and Bodenstein. 1961) or the growing regenerate (O'Farrell *etal.,* 1960) was involved in metabolizing the moulting hormone, as a result delaying moulting.

The perception of loss at the trochanter-femur autotomy plane most likely involves the fields of campaniform sensillae found just pre- and post- the autotomy plane. The pattern of input from these sensillae are likely essential in the instruction of what type of delay is needed. Simple removal of all sensory and motor communication between the hemiganglion and the leg does not result in any delay of moulting whether or not the leg is autotomized. All evidence points to an informed decision within the leg or its hemiganglion as to how much delay to create in order to accomplish the desired amount of regeneration. Exactly how the delay length is meted out is not known. The neuronal regenerative process, initially observed in P. *americana* when the nerve axons to a leg are cut (Cohcn and Jacklet, 1965; Jacklet and Cohen, 1967), is an obvious candidate for supplying the control of delay length. The nature and timing of this process in the neuron cell bodv has been difficult to establish (Young *et al.,* 1970, Denburg and Hood. 1977) and, as a result, it may be hard to correlate the cytologically observed changes with the physiological delay process.

16.6 Conclusion

Clearly, much more work is needed to detail what is happening within the sphere of each of the subsystems involved in regeneration. The phenomenon in cockroaches is a mixture of neuronal, hormonal and developmenral processes which can be orchestrated by the investigator to occur using the cues of feeding and autotomy. Among models of regeneration, the cockroach leg model presents a unique opportunity to study the process from a variety of perspectives. Leg autotomy is also a way of reproducibly perturbing the endocrine system in studies aimed at the physiology of the moulting process.

Acknowledgement