REGENERATION OF PERIPHERAL NERVES TO SUPERNUMERARY LEGS IN COCKROACHES

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SUMMARY

1. Clusters of legs were grown from metathoracic coxae of the cockroach. Legs of a cluster had different segmental origins, sizes, and orientations.

2. Regenerating metathoracic nerves tended to enter the nearest leg, and to a lesser extent the largest leg, but showed no significant tendency to penetrate metathoracic rather than prothoracic legs, or normally oriented rather than abnormally oriented legs.

3. Movements of legs were evoked by nerve stimulation significantly more often in nearest, largest, and normally oriented legs, but were equally frequent in prothoracic and metathoracic legs.

4. Close proximity of peripheral nerves is not required for the differentiation of the legs, since nerves were visible in the legs only at the later stages of their development, and many of the legs were apparently never innervated.

INTRODUCTION

An important question in developmental neurobiology is: do peripherally located end organs direct the growth of developing sensory and motor nerves? In amphibians, regenerating brachial nerves deviate from their normal pathways to grow toward transplanted limb rudiments, nasal placodes, or optic vesicles (Detwiler, 1920, 1922, 1925; Detwiler & Van Dyke, 1934). Although all these transplanted tissues appear to attract the growing leg nerves, only the limb rudiments, which develop into legs, become functionally innervated (Detwiler & Van Dyke, 1934).

It is not known, however, whether amphibian brachial or lumbar nerves can recognize individual legs, rather than simply growing toward the nearest leg. Both forelimbs and hindlimbs can be innervated by each other's nerves when limbs or spinal segments are interchanged (Székely, 1963; Weiss, 1924; Hughes, 1964). When a growing nerve is offered a choice of limbs in close proximity, motor neurones appear to send functional branches to both the normal and a foreign, supernumerary limb (Weiss, 1924; Hughes, 1964). Neurones innervating the foreign limb often degenerate later (Hughes, 1964), but this may not be due to the limb's embryological origin – other possible factors being its position, wound healing following dissection, or inadequate vascular supply.

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We now report on a similar experiment that we have performed on cockroaches, which offer two distinct advantages over amphibians. First, limbs of different segmental origins can be made to grow as a cluster from a single stump (a cluster consisting of one leg regenerated *in situ*, plus one or two supernumerary legs). Each leg retains morphological characteristics of its segment of origin. Secondly, each limb nerve generally grows as a single trunk, penetrating only one leg of a cluster, rather than branching to all nearby legs, as in amphibians. We could thus test the influence of a leg's segmental origin, as well as several other factors, upon selection of a target by the growing nerves.

A further paper (Westin & Camhi, 1975) considers the detailed innervation of legs of clusters which contain only one nerve, rather than the normal complement of three.

MATERIALS AND METHODS

Pairs of cockroaches (*Periplaneta americana*) of late nymphal instars were selected, one to three days following a moult, for leg transplanting operations. One member of the pair was a donor, contributing part of a prothoracic leg, which was grafted to the base of the contralateral metathoracic leg of the other, host insect. For this operation, both insects were anaesthetized with CO_2, and the legs of the donor and the host were severed transversely through the middle of the coxa. We placed a few grains of an anti-bacterial, anti-tyrosinase agent* in the host's wound, and then inserted the coxal end of the donor's leg. The clotted blood fixed the transplant in place.

Usually within a few days, most of the transplanted leg had withered and dropped off, leaving only the donor's coxa inside the coxa of the host. (Sometimes we transplanted not a whole leg, but an isolated coxa, with the same developmental result.) The cockroaches were kept individually in plastic containers with rat food and water *ad lib*.

At the first, second, or third moult after the transplant operation, clusters of two or three legs appeared at the site of transplantation in about 10% of the operated animals: the time that elapsed from transplantation to the appearance of the clusters was several months to more than a year. In most other animals, single legs having a normal metathoracic appearance regenerated, suggesting that the transplanted tissue may have been lost. Of those insects which developed clusters of legs, many produced two or three small protrusions at the first moult following the transplant, and these were followed by clusters of two or three legs at a subsequent moult.

By varying the transplanting technique, it is possible to produce either a horizontal or a vertical row of limbs (Bart, 1965; Bullière, 1970). If, in transplanting the donor's leg, its proper dorso-ventral orientation is maintained, then its medio-lateral orientation is of necessity reversed (since it is placed contralateral to its position on the donor). In most such situations, one leg develops from the medial surface and another from the lateral surface – the two places where the legs of the donor and of the host juxtapose non-homologous tissues. The third leg develops between the other two (Fig. 1a and Fig. 2). If, however, the donor's leg is rotated 180°, transplanting it with its medio-lateral orientation maintained, then its dorso-ventral orientation is

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* A mixture of phenylthiourea:penicillin:streptomycin (2:1:1) (Williams, 1959).
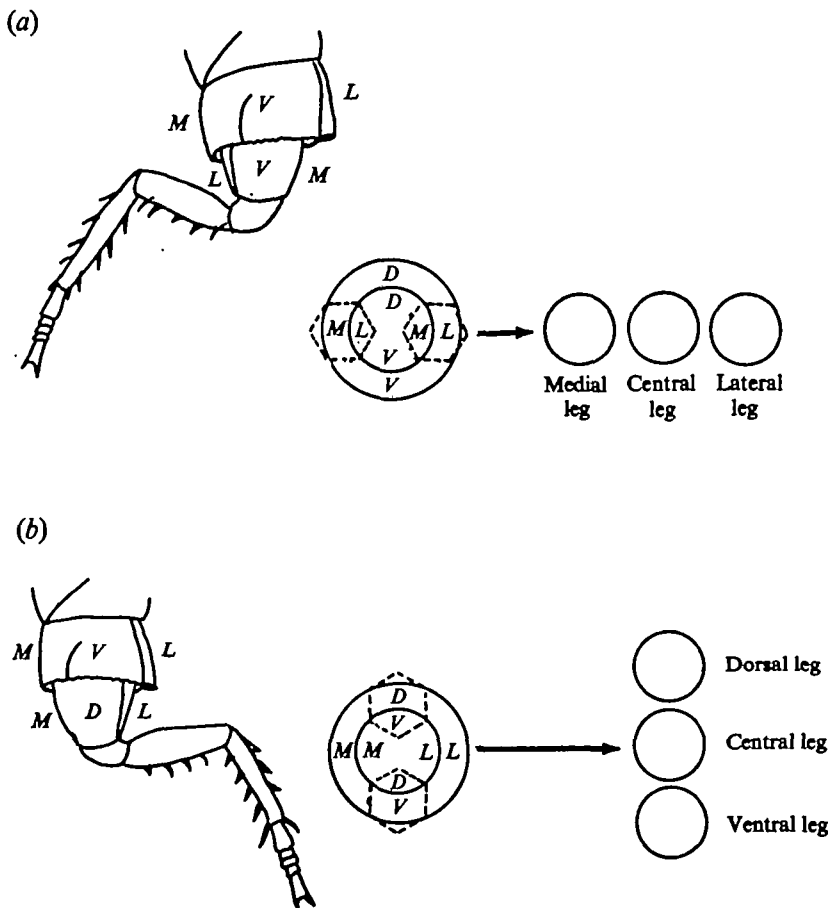
(a)

(b)

Fig. 1. Orientation of transplanted coxae and the clusters of legs they produce. A left host coxa is viewed from the ventral surface with a right donor leg inserted into it. The outer of each concentric pair of circles represents the host's coxal stump in cross section; the inner circle represents the transplanted donor coxa. Orientation of the two coxae: *D*, dorsal; *V*, ventral; *L*, lateral; *M*, medial. (See text for a fuller explanation.) (*a*), Matching dorso-ventral orientation, mismatching medio-lateral orientation, leading to a horizontal row of legs; (*b*), matching medio-lateral orientation, mismatching dorso-ventral orientation, leading to a vertical row of legs.

reversed. In this case, non-homologous tissues of the two legs are juxtaposed in the dorsal and ventral regions, and most resulting leg clusters form a dorso-ventral row (Fig. 1*b*).

Identification of legs of a cluster as prothoracic or metathoracic in origin rested on several criteria. Femurs of all normal legs have two rows of spines. In metathoracic femurs, these rows have about equal numbers of spines, whereas in prothoracic femurs, the anterior row has $3 \cdot 5 - 5$ times as many as the posterior row (Fig. 3*a*). Legs of a cluster could often be categorized by this means (Fig. 3*b*). A second criterion is that a metathoracic leg, since it is regenerating *in situ*, should not be mis-oriented; whereas prothoracic legs may be. A third criterion is that the tibia should be longer than the femur in a metathoracic, but not in a prothoracic leg. Finally, one expects the central leg always to be prothoracic, since it develops from the end of the donor's

coxa (Bullière, 1970). Where two or more criteria each gave a clear identification to the same leg, there were no contradictions. The segment of origin could be determined for all legs in 70 % of the clusters. Ambiguous cases were discarded.

In order to analyse whether a nerve preferentially enters the nearest leg of a cluster, we had to determine which legs were nearest to which nerves. This was accomplished histologically. Metathoracic nerves 3, 5, and 6 occupy the same relative positions in the coxal base of a cluster as in the coxa of a normal metathoracic leg. In most animals with medio-laterally oriented clusters of three legs, nerve 3 is nearest the central leg, nerve 5 nearest the medial leg, and nerve 6 nearest the lateral leg (Fig. 2). While this pattern of proximity may be disturbed in some animals, one should expect no consistent direction to these distortions; so the analysis of proximity is statistically valid.

Additional data are available from those animals which developed only two legs aligned medio-laterally, where nerve 5 is nearer to the medial leg and nerve 6 is nearer to the lateral leg. Since nerve 3 may be nearer to either leg, the proximity of this nerve to each of a pair of legs was not considered. Because the coxa is flattened dorso-ventrally, it is difficult to determine relative proximity in dorso-ventrally aligned clusters of legs, so data from these legs also were not included.

To determine whether nerves preferentially enter the largest leg of a cluster, we arbitrarily chose as an index of size the diameter of the limb just distal to its exit from the common coxa. In about 70 % of the animals, one leg of a cluster was measurably larger by this criterion than the others.

In most animals, at least one of the legs in a cluster was oriented differently from the normal metathoracic leg; however, in only about 50 % of the clusters could all the legs be clearly labelled as either 'normal' or 'backwards'.* Only clusters containing legs oriented both normally and backwards were used in the analysis of the tendency of nerves to enter legs of a given orientation.

To determine which leg of a cluster a given nerve had entered, we used two methods: electrical stimulation of the nerves, and histological examination. For electrical stimulation, all three major leg nerves were cut close to the metathoracic ganglion, and their distal stumps were sucked up one at a time into a suction electrode. Stimuli consisted of negative-going pulses 1–10 msec in duration, given singly or at a frequency of 10 Hz. Voltages were between 0·08 and 8 V. Under a dissecting microscope, we could observe fast twitches and some slow tonic movements of the legs. If two legs moved, we held one of the legs with forceps in order to determine whether each movement was active. Although this method was adequate to show overt movements, we also made ciné films of the leg clusters of five animals during nerve stimulation, using a Bolex H-16 camera and a close-up lens. The films, analysed frame by frame, confirmed the direct observations, and revealed only one possible movement which had not previously been detected.

Histological observations were made on clusters of legs excised and fixed in Dubosq–Bouins, embedded in paraffin, sectioned at 10 μm, and stained with eosin and haematoxylin. The identity of each nerve was based on its size and location in the shared coxa. About 25 % of the nerves could be traced section by section into a

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* A normally oriented leg was defined as one in which the medial and lateral edges match those of the host, and a backwards leg as one with its medial and lateral edges reversed.
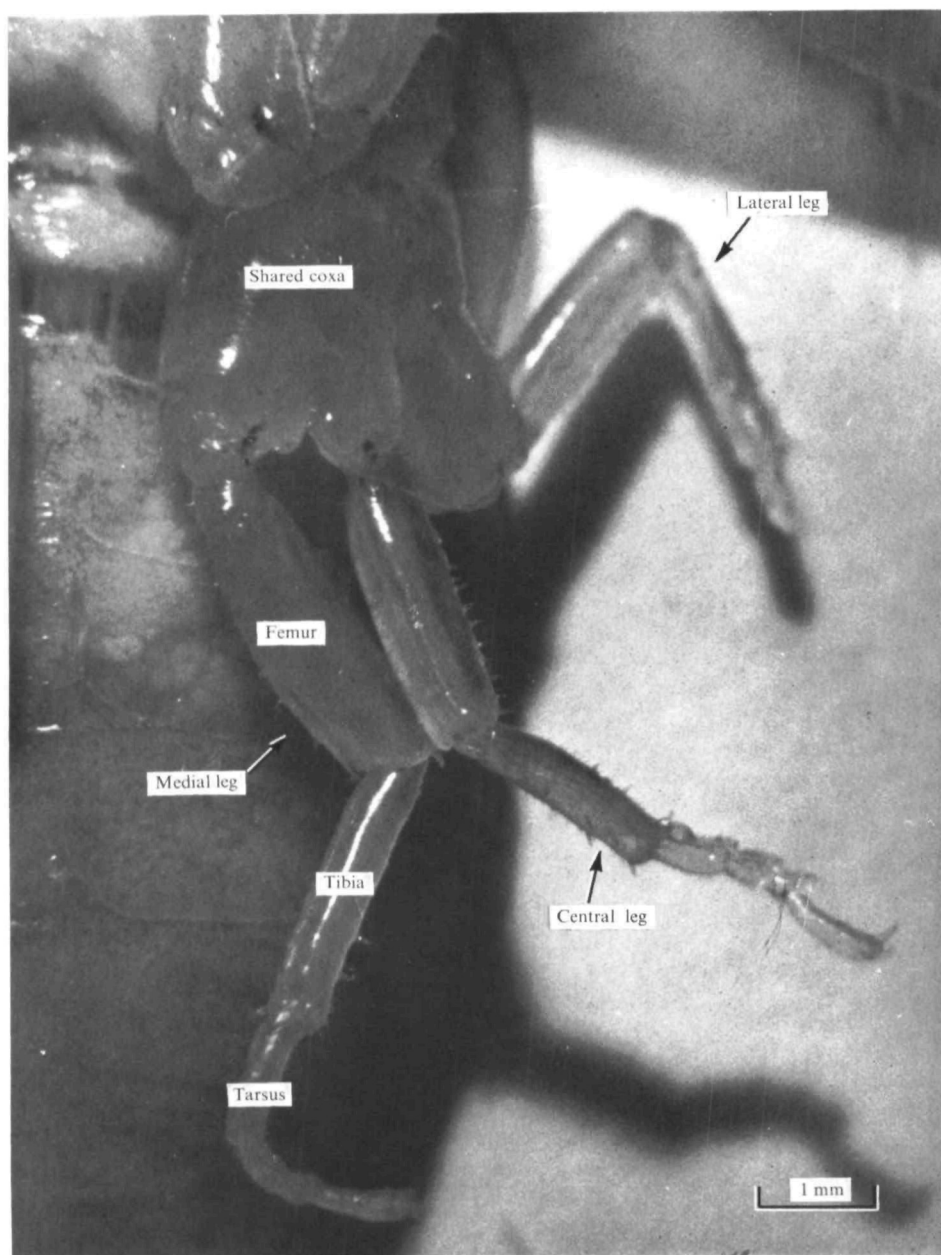
Fig. 2. A medio-laterally aligned cluster of three legs growing from the metathoracic coxa of the host; ventral view.
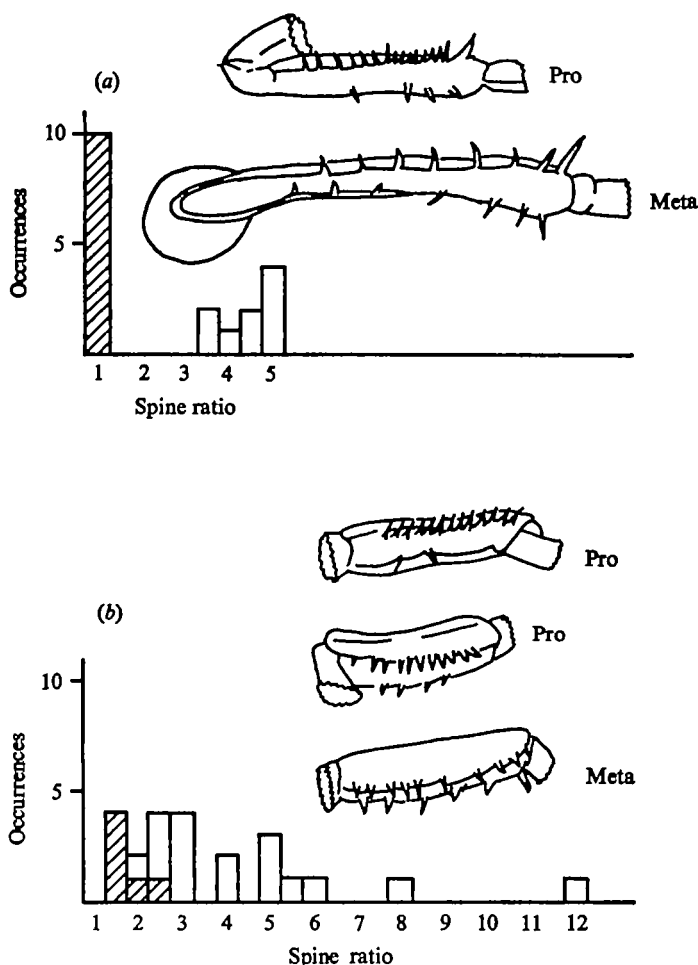
Fig. 3. Spine distribution on the femur. (a) Typical femurs of normal prothoracic (Pro) and metathoracic (Meta) legs. The ratio between the number of spines in the anterior row to that in the posterior row is plotted in the histogram for ten normal prothoracic (open bars) and metathoracic (hatched bars) legs. (b) The femurs of three legs which were members of one cluster. Their segmental origins were determined by their spine patterns as well as other criteria mentioned in the text. The ratio of spines in the two rows is plotted as in (a) for 23 legs from clusters. Cross-hatched bars represent legs identified as metathoracic, and open bars prothoracic by the criteria explained in the text.

leg. The rest appeared to end before entering a separate leg, or else became impossible to follow with certainty. Where successful, this method offers the advantage of showing nerves or nerve branches which have entered legs but have not made functionally detectable connexions.

In order to follow the time course of development and innervation of the clusters of legs, we have also examined sections of 76 host coxae at various times (2 weeks to 2 years) after the transplanting operation, but prior to the emergence of a cluster.
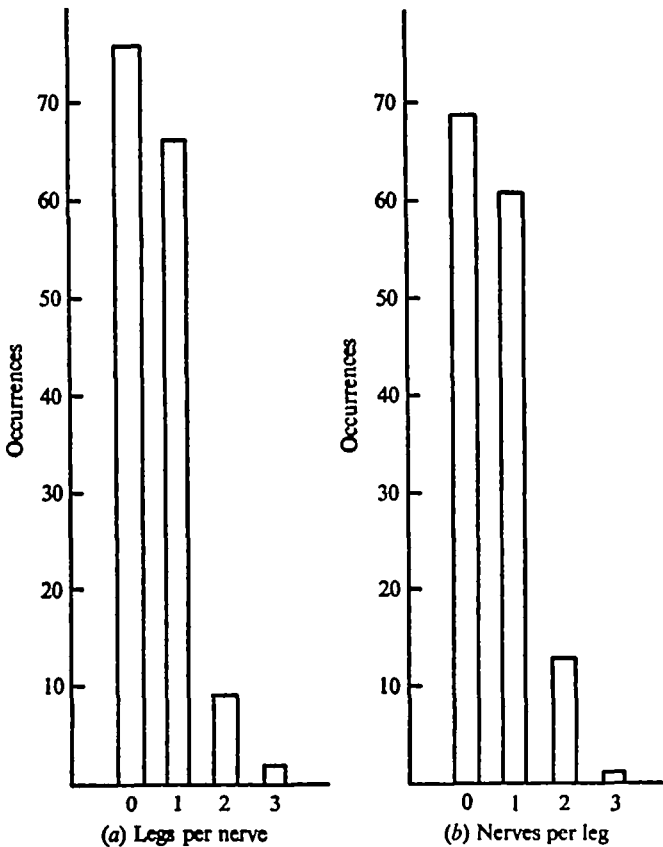
Fig. 4. Innervation of legs forming clusters. (a) Number of legs penetrated by individual nerves. (b) Number of nerves penetrating individual legs.

RESULTS

Of about a thousand animals receiving transplanted legs, 42 which developed clusters of three legs, and 9 which developed clusters of two legs, were used in the analysis. (A further 29 clusters were not used because they were too badly deformed or too fragile.) Almost two-thirds of the clusters were aligned medio-laterally, the rest dorso-ventrally.

In most animals, nerves entering the common coxa of a cluster of legs show a well-defined sheath, and an axonal composition which varied from approximately normal to completely lacking in identifiable axons (especially in more distal sections). Nerve branches at this level were generally no more numerous than in a normal coxa.

Of the 153 nerves examined, 29 could be traced into legs by histological means only, 40 electrical means only, and 8 by both means (all of these last gave the same result for both methods). Thus, 77 nerves could be shown by at least one method to have penetrated at least one leg, and most of these entered only one leg (Fig. 4a). Of the 144 legs examined, 75 contained at least one nerve and most of these contained only one (Fig. 4b).
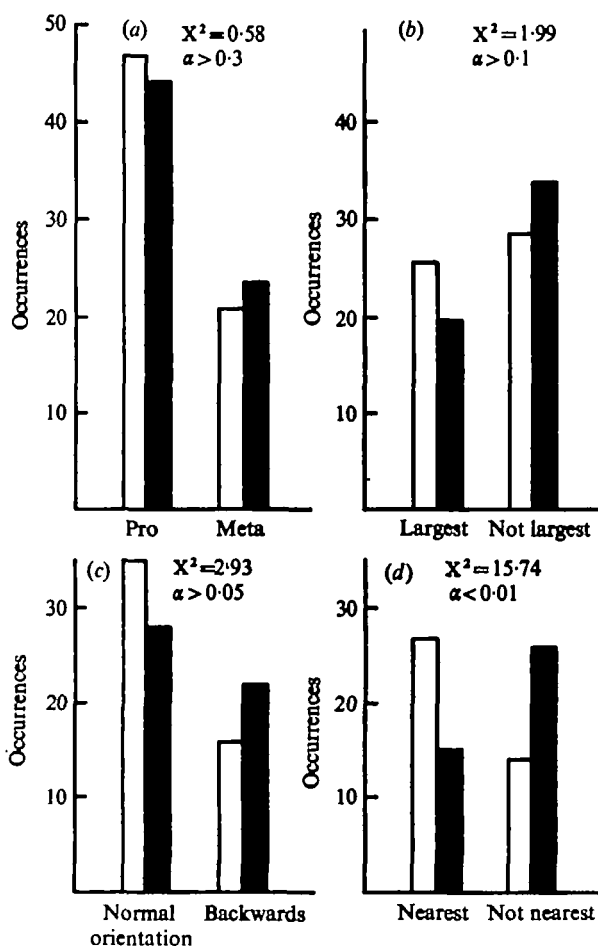
Fig. 5. Frequency of penetration of nerves into particular classes of legs. In each pair of bars: Solid bars, observed frequency of penetration into a specific class of leg; Cross-hatched bars, frequency of penetration of the same class of leg expected by random assortment. (*a*) Legs of different segmental origin. Pro, prothoracic leg; Meta, metathoracic leg. (All nerves were metathoracic.) (*b*) Legs of different size. Largest, leg with the largest diameter at its base; Not largest, leg which was not the largest in a given cluster. (*c*) Legs of different orientation. Normal orientation, leg oriented normally with respect to leg grown *in situ*; Backwards, leg oriented backwards. (*d*) Legs of different relative proximity to nerves. Nearest, leg nearest to a particular nerve; Not nearest, leg which was not nearest to that nerve. The only factor for which observed and expected values differed significantly was proximity. Nerves tended to enter the nearest leg.

Distribution of nerves among the legs of a cluster

One can calculate the frequency with which a given class of nerve (nerve 3 or 5 or 6) would enter a particular class of leg (e.g. the most medial, or the metathoracic, or the largest) if the nerves were choosing legs at random. This can then be compared with the observed penetration frequency.

When viewed in this way, there is no significant difference beween the frequency of innervation expected by random assortment and the frequency with which nerves penetrated a leg of a given *segment of origin, size*, or *orientation*. (Fig. 5 *a, b, c*). Slight
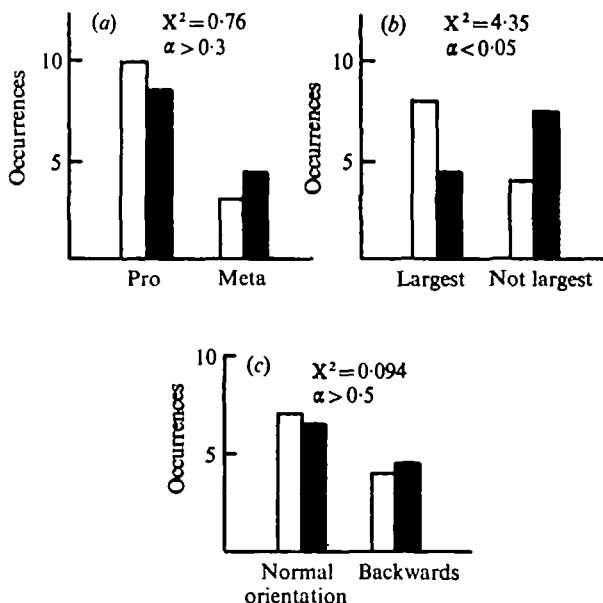
Fig. 6. Frequency of penetration of different classes of legs by nerves not entering their nearest legs. Pairs of bars as in Fig. 4. (a) Legs of different segmental origins. Pro, prothoracic leg; Meta, metathoracic leg. (b) Legs of different sizes. Largest, leg with the largest diameter at its base; Not largest, leg which was not the largest in a cluster. (c) Legs of different orientations. Normal orientation, leg oriented normally with respect to leg grown *in situ*; Backwards, leg oriented backwards. For legs of different size, there was a barely significant difference between observed and expected values – nerves not entering their nearest legs tended to enter the largest leg.

tendencies to enter larger and normally oriented legs were not significant. However, nerves show a statistically significant tendency to enter the *closest* leg (Fig. 5*d*).

It remained possible that *segment of origin, size,* or *orientation* exert weak orienting influences on the nerves, but that these are overshadowed by the strong tendency to enter the *nearest* leg. We therefore compared expected and observed frequencies of penetration for all nerves which did *not* enter the nearest leg. There was still no significant tendency of these nerves to enter a leg based on its *segment of origin* or *orientation* (Fig. 6*c*), but these nerves entered the *largest* leg significantly more often than expected by random assortment (Fig. 6*b*). The size of the remaining sample did not allow a similar analysis of the roles of leg orientation and segment of origin for those nerves that did not enter the nearest or the largest leg. However, any such influences would be very weak.

Finally, data from experiments using electrical stimulation were analysed separately. Detectable movements were found to occur significantly more often not only in the *nearest legs* and in the *largest legs* (Fig. 7*d, b*), but also more often in *normally oriented* legs than in legs oriented backwards (Fig. 7*c*). This might reflect a decrease in the ease with which nerves find muscles and form synapses on them in backwards legs, though the defect could equally well be in the muscle.
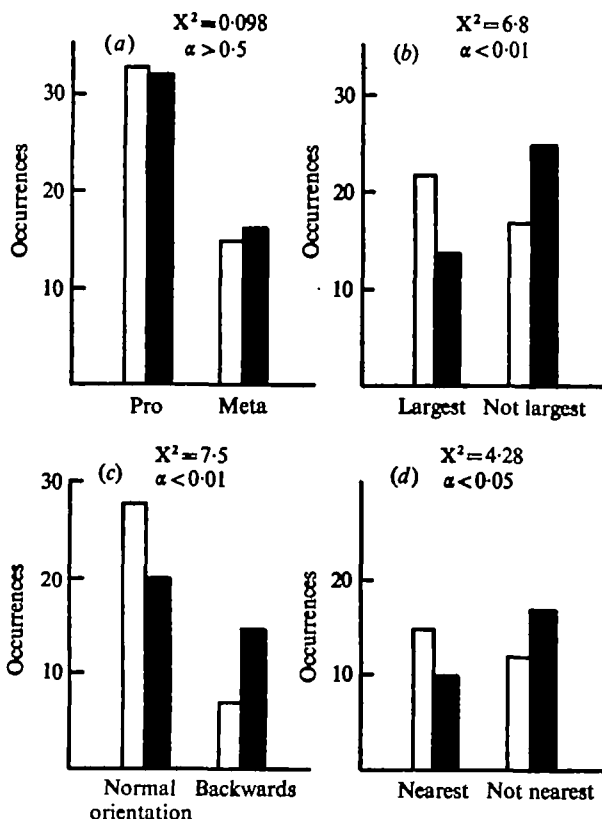
Fig. 7. Frequency of movements of the different classes of legs upon electrical stimulation of single nerves. In each pair of bars: Open bars, the number of times visible contractions were evoked in a specific type of leg; Cross-hatched bars, the number of times a nerve would be expected to innervate the same type of leg by random assortment. (a) Legs of different segmental origins. Pro, prothoracic leg; Meta, metathoracic leg. (b) Legs of different sizes. Largest, the leg with the largest diameter at its base; Not largest, leg which was not the largest. (c) Legs of different orientations. Normal orientation, a leg oriented normally with respect to a leg grown *in situ*; Backwards, a leg oriented backwards. (d) Legs of different relative proximities. Nearest, the leg closest to a particular nerve; Not nearest, a leg which was not the nearest to that nerve. For legs of different size, orientation and proximity, there was a significant difference between observed and expected values. Nerves tended to form detectable functional connexions more often in largest, normally oriented, and nearest legs.

Histology of the developing clusters of legs

We wished to ascertain whether regenerating nerves were making the observed discriminations among the legs at a time when the legs are already formed, or while the leg tissues are still undifferentiated. To do this, we examined developing legs of 76 animals histologically at various times after leg transplantation. Within about 2 weeks after transplantation, degenerating muscles are interspersed with numerous undifferentiated cells within and around the donor's coxa. After 1 or 2 months, no muscle, but only anatomically undifferentiated cells can usually be seen in the host's coxal stump and in the indwelling donor's coxa. These are at first generally dispersed, and then some initial aggregation of cells begins (Fig. 8a). At a later time (3–12 months after transplantation in different animals) most cells are in large aggregations.

Between 3 and 17 months after transplantation, we began to observe differentiation, with irregularly shaped clumps of cells surrounded by an epithelial border (Fig. 8b). Finally (starting at various times between 6 and 20 months), small legs or clusters of legs are apparently folded up inside the cuticle of the host's coxa (Fig. 8c). These usually show very few differentiated muscles, but this is also true of the legs which emerge as members of a cluster.

Nerve 5 of the donor (identified by its size and location) can be seen within the transplanted coxa at early stages only and never in legs which have pronounced cellular aggregations or signs of differentiation (as in Fig. 8b, c). These donor nerves, when visible, are only 200–1000 μm in length. Their diameter always decreases in the more proximal sections, and they disappear without making connexions with nerves of the host.

Peripheral nerves of the host can be seen outside the transplanted tissue in some animals at early stages (as in Fig. 8a), but they probably degenerate partially, or recoil, since at all intermediate stages (as in Fig. 8b), peripheral nerves of the host end at the base of the coxa. In about half the animals the nerves of the host are not visible at all. Nerves were never seen extending from the host into groups of anatomically undifferentiated cells or into clusters of cells displaying initial stages of differentiation. Not until legs are already at an advanced stage of their formation (as in Fig. 8c), can host nerves be seen to penetrate the newly formed tissues. In 14 of 26 animals at this stage, at least one nerve could be traced into a potential leg.

Therefore, within the limits of light microscopy, it appears that at the time that the host's nerves are growing to a potential cluster of legs, the legs are differentiated and their positions are defined, beneath the outermost cuticle of the host. There is no indication that axons of host nerves fuse with those of the donor's coxa (Hoy, 1967, 1969); nor do nerves of the donor's coxa provide observable mechanical pathways for the host's nerves to follow. A further conclusion is that peripherally growing nerves are apparently not required for the formation of legs under the conditions of these experiments.

DISCUSSION

In this paper we have shown that peripheral nerves normally innervating the metathoracic leg of a cockroach tend to innervate the nearest leg when offered a choice of two or three legs growing from one metathoracic coxa. Nerves which do not enter the nearest leg tend to enter the largest leg. In spite of the sensitivity of such a choice situation in detecting guidance cues, we find no evidence that the segment of origin or orientation of the legs influences the direction of a nerve's growth. Also, fewer legs oriented backwards moved in response to nerve stimulation than did legs oriented normally. This may reflect a greater difficulty for nerves in locating muscles in an abnormally oriented leg, though other interpretations, such as those involving malfunctioning muscles, are also acceptable.

It remains possible that a leg, developing as a member of a cluster, may lose some capacity which it normally possesses to inform a nerve of the leg's segment of origin, and direct the nerve accordingly. This seems unlikely, however, since the legs do retain some of their segment-specific morphological characteristics (Fig. 3). Also, in singly regenerated legs, all new tissue appears to develop from cells in the remaining
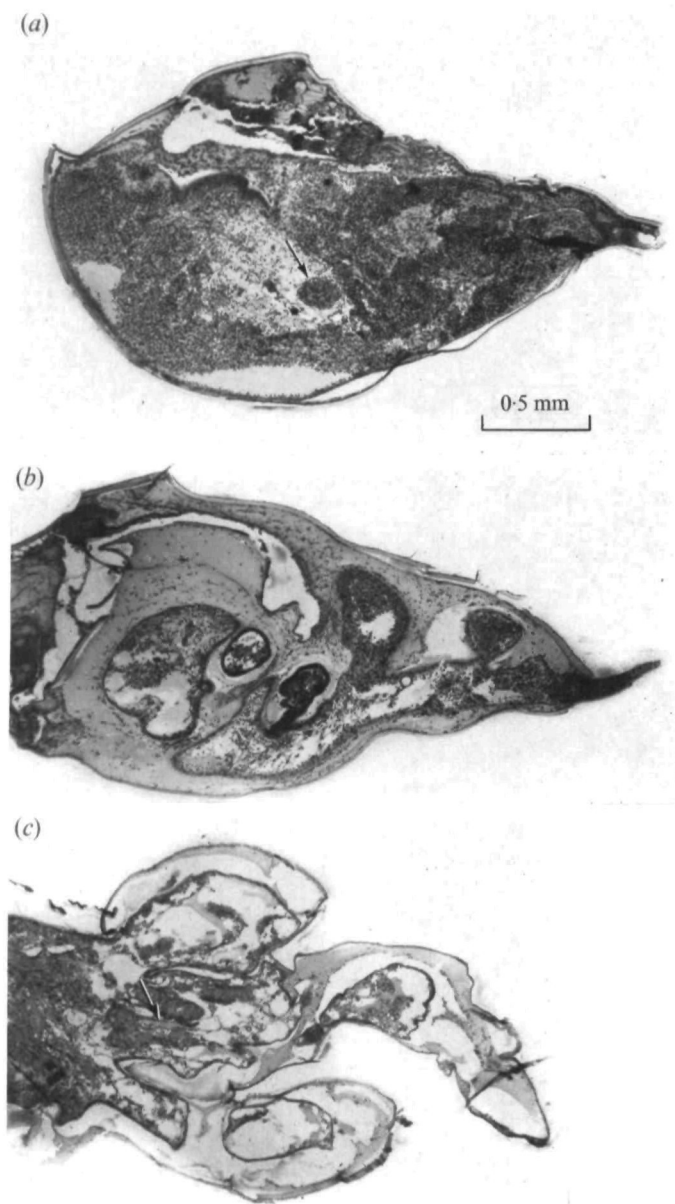
(*a*)

0·5 mm

(*b*)

(*c*)

Fig. 8. Cross-sections of host coxae representing different stages seen between transplanting the donor coxa and emergence of a cluster of legs. (*a*) *An early stage* (in this case, sectioned one month after the operation): host coxa is filled with undifferentiated cells. Some cells are grouped together (arrow). (*b*) *A later stage* (in this case, sectioned five months after the transplanting operation): some evidence of differentiation is seen in this coxa. Cell groups are taking on irregular shapes, and are bounded by an epithelial layer. (*c*) *Final stage of differentiation* (in this case, sectioned one year after the operation): individual leg segments can be seen. Leg(s) are folded up within the coxa. A nerve (arrow) can be seen penetrating the central developing leg.

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leg rather than coming from a nearby region of the body (Bullière, 1971). In clusters of legs, however, some of the legs could be mosaics, having cells of both prothoracic and metathoracic origin (Bohn, 1972).

Our finding that there is no preference of a peripheral nerve for a leg of a given segmental origin agrees with the results of transplanting different ganglia to the region of the metathoracic leg in *Periplaneta*. Nerves from any of the thoracic ganglia can compete with nerves of the host ganglion for innervation of the metathoracic leg (Bodenstein, 1957; Guthrie, 1966; Jacklet & Cohen, 1967). Furthermore, any thoracic ganglion innervates a developing cockroach leg in tissue culture (Levi-Montalcini & Chen, 1971); and no guidance or attraction appears to be exerted by cockroach legs on ganglionic outgrowth (Marks, Reinecke & Leopold, 1968).

The terminations of the host's peripheral nerves are not visible at the site of regeneration at the time that differentiation of legs begins. This contrasts with the situation in amphibians, where a supernumerary limb is induced if one diverts a limb nerve to a surface wound in an abnormal site (Bodemer, 1958). It is clear that in our experiments, the legs were not induced to grow at the nerve terminals, since leg position depends on the orientation of the grafted leg, not on the arrangement of nerves in the coxal stump. In fact induction of supernumerary limbs in insects is apparently due to the juxtaposition of two non-homologous cuticular surfaces (Bart, 1966; Bullière, 1970).

Nerves also appear to be unnecessary for the development of legs of a cluster since leg differentiation precedes the invasion of newly formed tissue by outgrowing nerves, and in almost all clusters of legs, at least one leg received no detectable innervation. Similar findings have been reported in a diversity of insect species (Kopec, 1923; Suster, 1933; Bodenstein, 1955; Penzlin, 1964; Urvoy, 1963). In amphibians, however, a threshold number of either sensory or motor neurones is required at the base of an excised limb in order for regeneration to proceed (Singer, 1943, 1945, 1946, 1952).

Finally, under our experimental conditions, legs regenerated much more slowly than has been reported previously, requiring 3–12 months (or more) instead of 1–2 months (Bodenstein, 1955). The difference may result from the lower ambient temperature at which our insects were kept (20 rather than 25 °C), and from the more complex developmental process required to form three legs. The latter seems important since only 10 % of the insects to which we transplanted a limb developed more than one leg, whereas all legs simply cut off in the middle of the coxa regenerate new legs (Bullière, 1967). Interestingly, nerve regeneration appears to be slowed along with leg development, since the host's nerves do not penetrate the differentiating legs until their formation is at an advanced stage, and do not appear to grow backwards or to coil up. Likewise, in cockroaches following simple leg amputation, the first signs of nerve regeneration occur a minimum of 2–3 weeks after amputation, which is at least a week after the onset of differentiation of the new limb (Guthrie, 1962, 1967; Bodenstein, 1955). Thus, the nerve appears to be informed of the proper time to begin its growth.

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