Studies on the Innervation of the Embryonic Chick Hind Limb.

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ABSTRACT.

1. A history of the theories of motoneurone development and the formation of the innervation pattern of the limb is given.

2. The effect of three different types of early partial amputation of the chick hind limb bud on the innervation pattern of the limb on day 10 of incubation is investigated. The amputations are:
   i) Transverse amputation
   ii) Amputation of the dorsal muscle mass.
   iii) Amputation of the ventral muscle mass.

3. The morphology and muscle volumes of control and amputated limbs is described.

4. Motoneurone number in the lateral motor column is related to the volume of muscle remaining in the amputated limbs. Loss of motoneurones is in direct proportion to loss of muscle.

5. The position of motoneurones remaining in the rostro-caudal and medio-lateral axes of the lateral motor column is described for all three types of amputation. The innervation pattern of the remaining muscle is normal.

6. The importance of these findings in relation to similar experiments and mechanisms of innervation pattern development is discussed.

DECLARATION

I declare the work described in this thesis and its composition to be entirely my own.

ACKNOWLEDGEMENTS.

I dedicate this thesis to the late Dr. Martin Prestige. I owe him much.

I thank Professor W.E. Watson who took over supervision of my project after Martin's sudden death, and Dr Richard Ribchester and other members of the physiology department who provided encouragement in difficult times. My special thanks also to Dr Alan Lamb and Dr Nigel Laing for their interest despite the distance involved. Finally I thank Clare Glaskin for typing the manuscript, the physiology department secretaries for their help and my friends and relatives who provided support.

PREFACE

This thesis reports a study on the development of the motoneurones that innervate the hind limb muscles of the chick. It forms part of a rapidly expanding field of developmental neurobiology which is aimed at understanding how the structure of the nervous system develops.

The introduction covers the general and historical background of the field up to the time the experiment was conceived in 1979. The aim of the experiment is discussed. The experimental proceedures used are described. Each results chapter has a section discussing the interpretation of the results. The discussion covers the significance of the results in relation to the state of knowledge of the field at the present time. There is an appendix.

Throughout the thesis the stages used to denote the development of the chick embryo are those of Hamburger and Hamilton (1951). The stages used for *Xenopus* tadpoles are those of Nieukoop and Faber (1956).

Abbreviations.

AER- apical ectodermal ridge

BSS- balanced salt solution

DNA- deoxyribonucleic acid

Dorsal muscle- muscle derived from the embryonic dorsal muscle mass.

Dorsal amputation- removal of all or part of the embryonic muscle mass.

Ventral muscle- muscle derived from the embryonic ventral muscle mass.

Ventral amputation- removal of all or part of the embryonic ventral muscle mass.

ZPA- zone of polarizing activity.

74-OP - methylated spirit. H.R.P - horseradish peroxidase.

NGF- nerve growth factor.

3H- thymidine- tritiated thymidine.

CHAPTER I

INTRODUCTION

1.1 The Pattern of Innervation

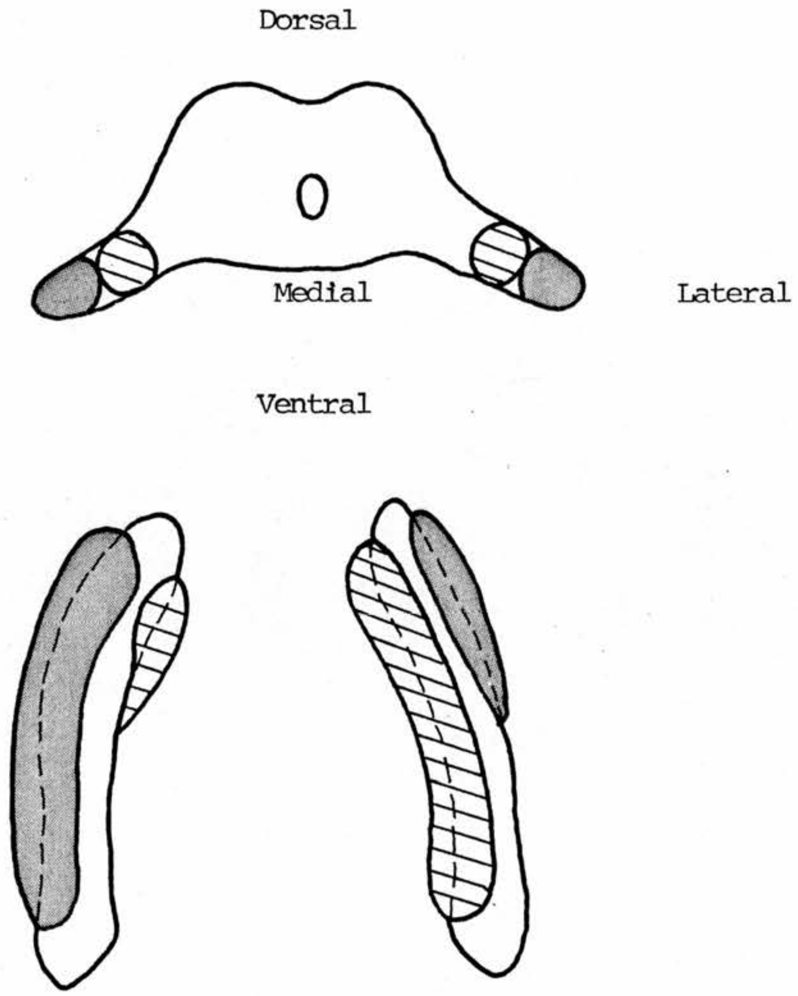
The muscles of the chick hind limb are innervated by the motoneurones of the lumbar lateral motor columns. The lateral motor columns lie in the ventro -lateral part of lumbar spinal cord, one on each side and extend from segments 23 to 30 (Hamburger 1975).

During normal embryonic development, motoneurones innervating any given hind limb muscle, come to lie in a characteristic group or cluster within the lateral motor column. The position of the group is related both to the embryonic muscle mass from which the muscle derives, as well as the position of that muscle in the proximo-distal axis of the limb.

Motoneurones innervating muscles derived from the embryonic ventral muscle mass lie medially with the lateral motor column, motoneurones innervating muscles derived from the embryonic dorsal muscle mass lie laterally (Landmesser 1978a). See fig (1.1)

The relationship between muscle position in the proximo-distal axis of the limb and motoneurone position in the lateral motor column is such that motoneurones innervating thigh muscle tend to lie more rostrally, than those innervating shank muscle which tend to lie more caudally. (Landmesser 1978a). There are exceptions to this however, since motoneurones innervating some thigh muscles lie caudally within the lateral motor column. (Fig (1.2)).

A relationship between motoneurone position in the rostro-caudal axis of the lateral motor column and the embryonic muscle mass (dorsal or ventral) from which the muscle derives, also exists. Motoneurones innervating muscles derived from the dorsal muscle mass tend to lie more rostrally within the lateral motor column than do



Lateral View

Medial View

Figure 1.1

Upper diagram: transverse section of lumbar spinal cord of the chick showing the position of motoneurones innervating muscles derived form the dorsal muscle mass (shaded area) and the position of motoneurones innervating muscles derived from the ventral muscle mass (hatched area).

Lower diagram: the two primitive muscle masses, dorsal (shaded area) and ventral (hatched area) as they appear at stages 27-29 of development. After Romer 1927.

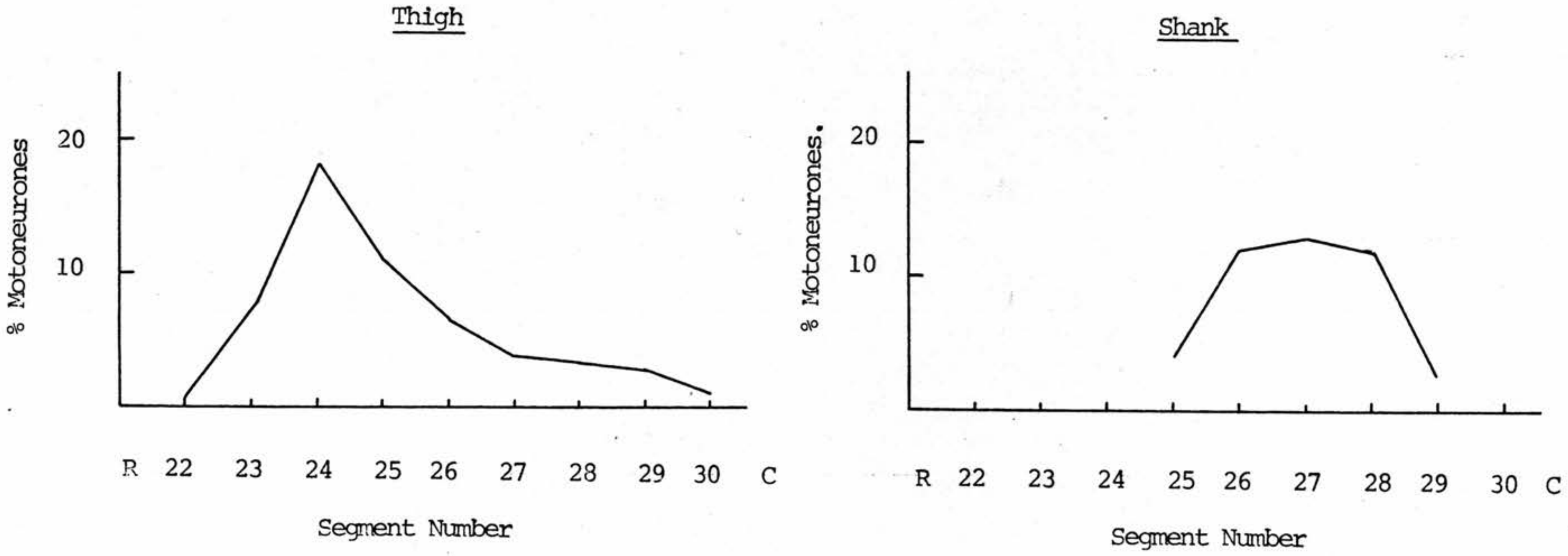


Figure 1.2.

The number of motoneurones per lumbo-sacral segment innervating thigh and shank muscle in the hind limb of the chick on day 10 of incubation. Motoneurone number expressed as as a percentage of total motoneurone number. Data recalculated from Landmesser (1978a). Motoneurone position determined by HRP-uptake labelling of motoneurones. R- rostral. C- caudal.

motoneurones innervating muscles derived from the ventral muscle mass (Fig (1.3)).

Similar relationships between motoneurone position in the spinal cord and muscle position in the limb have been observed in amphibians (Cruce 1974; Prestige 1973; Lamb 1976, 1977) cats (Romanes 1951) and humans (Sharrad 1955).

The adult pattern of connections to the chick hind limb has developed by at least day ten of incubation. (Landmesser 1978b). The developmental events, occuring up to this time, relevant to the aims of this study are shown in Fig (1.4) and can be summarized:

- Motoneurone Production and Lateral Motor Column Formation
- Formation of the Hind Limb and Hind Limb Musculature
- The Establishment of the Pathways followed by Outgrowing Axons into the Limb
- Formation of Neuromuscular Connections
- Motoneurone Death

1.2 Motoneurone Production and Lateral Motor Column Formation

The motoneurones of the chick lateral motor columns are formed by division of neuro-epithelial cells situated in the ventricular layer of the basal plate surrounding the central canal (Hollyday & Hamburger 1977). Using the technique of cumulative labelling autoradiography Hollyday & Hamburger (1977) were able to time the formation of the brachial and lumbar lateral motor columns. Ninety-nine percent of the lumbar motoneurones are formed between stages 17 to 24 of development. Neurogenesis starts slightly earlier in the brachial region - the first motoneurones are formed at stage 15 of development and 99% are formed by stage 24.

Once formed the immature motoneurones migrate away from the

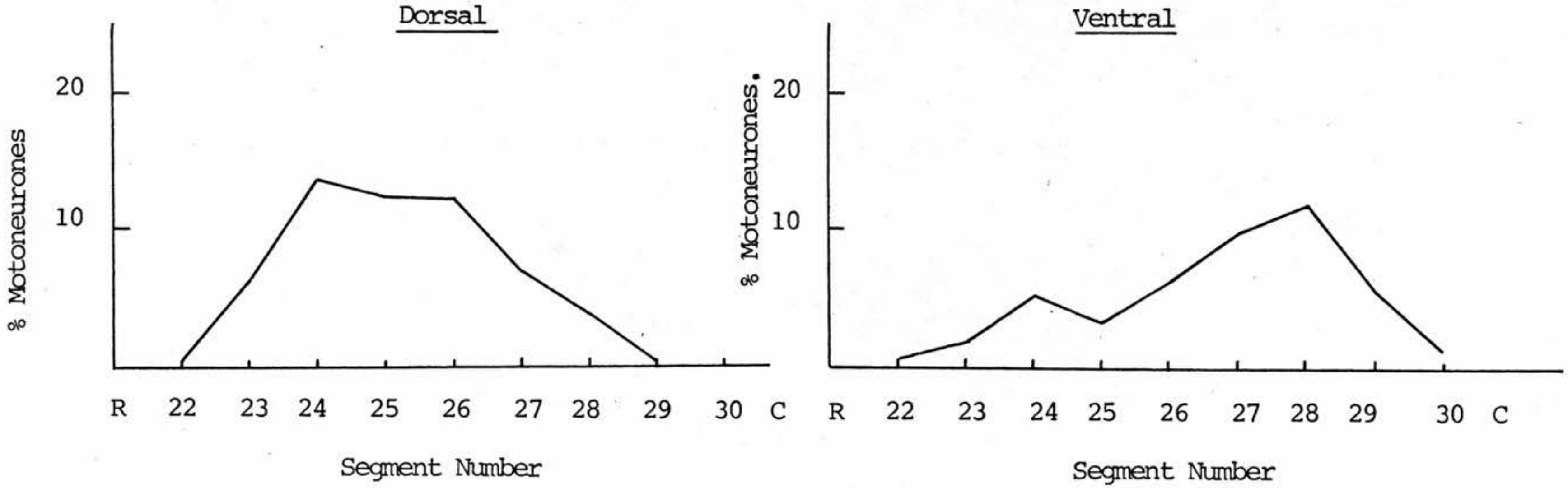


Figure 1.3.

The number of motoneurones per lumbo -sacral segment innervating dorsal and ventral muscle in the hind limb of the chick on day 10 of incubation. Motoneurone number expressed as a percentage of total motoneurone number. Data recalculated from Landmesser (1978a). Motoneurone position determined by HRP-uptake labelling of motoneurones. R- rostral. C- caudal.

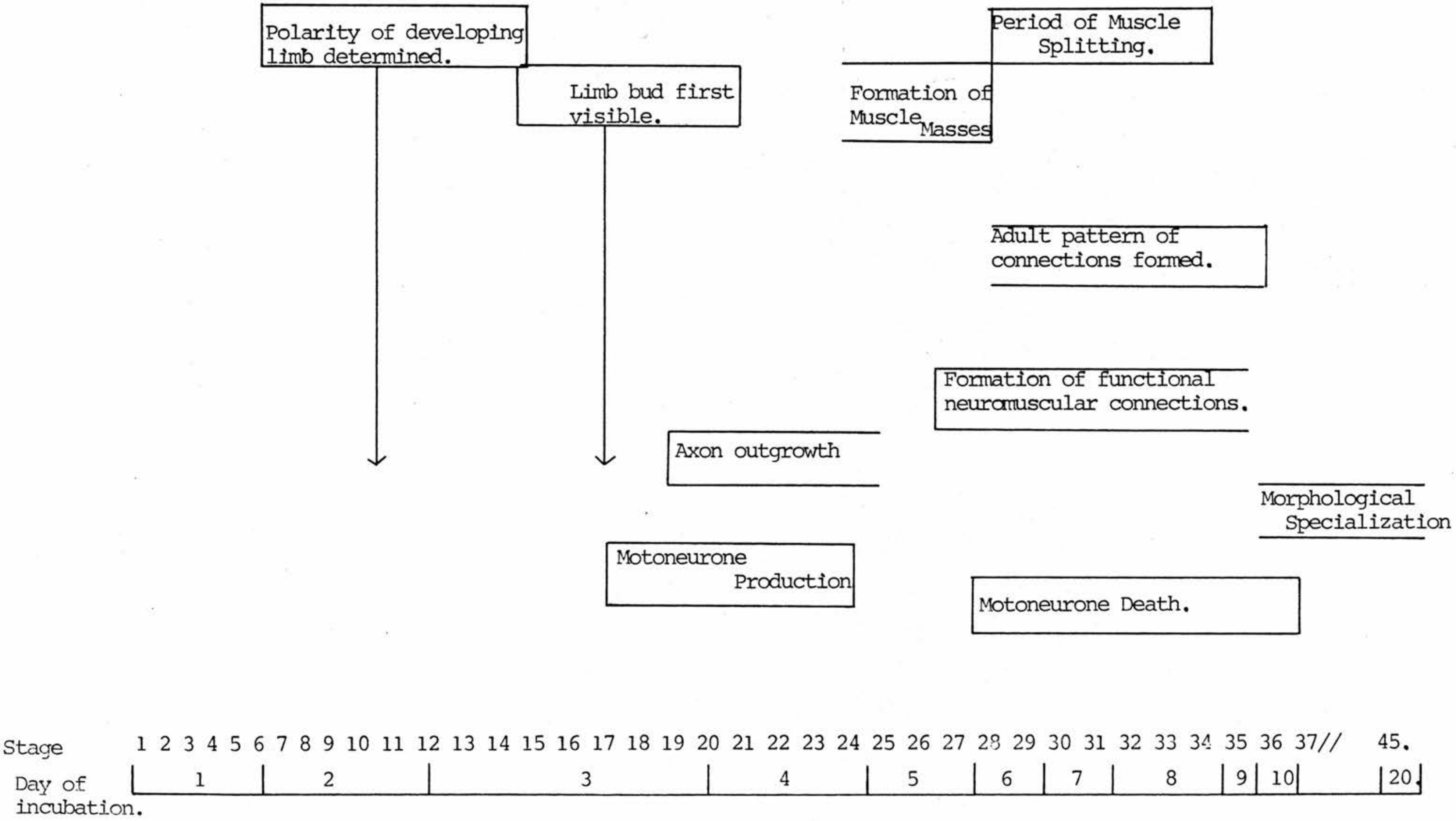


Figure 1.4. The timing of certain events important in the development of the innervation of the the chick hind limb. Closed boxes indicate that the time of commencement and termination of the events is established . Open ended boxes indicate that the time of either the start or termination of events is not known.

central canal and settle in the ventro -lateral part of the spinal cord forming the lateral motor columns. Neurone migration in the mammalian central nervous system may occur along radial ependymal processes (e.g. Rakic 1972). Whether this is also true for chick motoneurones is unknown.

The lateral motor columns are formed in rostro-caudal as well as medio-lateral temporal sequence (Hollyday & Hamburger 1977). This was discovered using the technique of cumulative labelling autoradiography. Motoneurone production commences rostrally and proceeds caudally within the lateral motor columns. In addition at any given rostro-caudal level the 'first born' or 'oldest' motoneurones are laid down medially with the younger cells migrating through the older ones to be laid down on their lateral border. Combining the two gradients the oldest motoneurones in the lateral motor column lie rostrally and medially. The youngest motoneurones lie caudally and laterally. This type of 'layering by birthdate' is common in the nervous system (Prestige 1973).

Similar gradients in the lumbar motoneurone pool of Xenopus were found by Prestige (1973) using the technique of pulse - labelling autoradiography.

The relationship between time of origin from the ventricular layer and position within the medio-lateral axis of the lateral motor column is important because position is also related to the site of axon termination in the dorso-ventral axis of the limb. Motoneurones that innervate muscle derived from the ventral muscle mass lie medially within the lateral motor column, motoneurones that innervate muscles derived from the embryonic dorsal muscle mass lie more laterally (Landmesser 1978a). At any given rostro-caudal level, therefore, 'older' medially positioned motoneurones innervate muscle derived from the ventral muscle mass (ventral muscle); 'younger' more laterally positioned motoneurones innervate muscles derived from the dorsal muscle mass (dorsal muscle).

The time of origin of motoneurones innervating dorsal and ventral muscle has been reported by Hollyday (1978). Without supplying details of the methods used she states:

> "Motoneurones innervating ventral muscle are formed between stages 17-19 of development in segments 23-28, and between stages 19-20 of development in segments 28-30. Motoneurones innervating dorsal muscle are formed between stages 19-24 of development in all segments".

There is therefore an overlap in the time of production of motoneurones innervating ventral and dorsal muscles. Both are formed during stages 19-20 although they are spatially separated - motoneurones that innervate ventral muscle are forming in the caudal segments while motoneurones that innervate dorsal muscle are forming in the rostral segments.

Motoneurone production, migration and initial lateral motor column formation are independant both of the limb musculature and other parts of the spinal cord. Removal of the limb has no effect on motoneurone number until stage 26 of development (Oppenheim et al 1978) by which time motoneurone production is virtually complete (Hollyday & Hamburger 1977). Similarly Bueker (1943) and Hamburger (1946) found motoneurone number to be independant of both ascending and descending fibres, and in an elegant experiment Hamburger and his co-workers (Hamburger et al 1966) showed motoneurone number to be independant of dorsal afferents until day 15 of incubation.

However the production of sensory neurones in the spinal root ganglia is decreased by limb amputation in both the chick (Hamburger & Levi-Montalcini 1949) and <u>Xenopus</u> (Hughes & Tschumi 1958; Prestige 1967a)

Why sensory and motoneurones should differ in this respect is unknown.

1.3 Formation of the Hind Limb

The hind limb bud is first visible at stage 17 of development as a symmetrical crescent-shaped outgrowth adjacent to somites 26-32, (Hamburger & Hamilton 1951), although its axial properties have been determined very much earlier than this by stage 10-11 of development (Hamburger 1938). The limb is laid down in proximo-distal sequence and growth between stages 18 to 33 of development depends on the presence of the apical ectodermal ridge (AER), a region of asymmetrically thickened ectoderm at the apex of the developing bud (Saunders 1948; Saunders & Gasseling 1968). Removal of the AER between these stages results in loss of distal limb parts, the extent of loss being dependant on the developmental stage at which the operation is performed (Saunders 1948; Saunders & Gasseling 1968; Summerbell 1974a).

The mechanisms underlying the formation of pattern in developing structures are not fully understood. Wolpert (1969, 1971) considers pattern formation in terms of cells being 'assigned positional information which effectively gives them their position in a co-ordinate system, and this positional information is then used to determine the cell's molecular or cytodifferentiation'. The AER does not directly specify the underlying mesoderm (Rubin & Saunders 1972). Rather it appears to maintain cells with 400 µm of the tip in an undifferentiated state (Summerbell, Lewis & Wolpert 1973). This region has been termed the 'progress zone' and cells are postulated to attain positional values with respect to the proximo-distal axis, by measuring the length of time they spend in the zone.

(Summerbell, Lewis & Wolpert 1973; Wolpert, Lewis & Summerbell 1975). Almost all cells in the zone divide (Summerbell, Lewis & Wolpert 1973) and therefore cells are continuously leaving the zone. As soon as they leave the change in positional value is assumed to cease. Cells that leave the zone first will produce proximal limb structures, whereas those that leave later produce more distal limb structures.

Although its role in normal development has been questioned (Saunders 1977) there is a considerable amount of evidence to suggest that the anterior-posterior axis of the limb is under the influence of the zone of polarising activity (ZPA). The ZPA is situated at the posterior margin of the limb bud near its junction with the body wall. If an additional ZPA is grafted to more anterior levels supernumerary limb structures form with mirror-image symmetry about the long axis (Summerbell 1974a; Tickle et al 1975; Summerbell & Tickle 1977)

Less is known about the specification of the limb in the dorso-ventral axis. It appears to be under the control of the ectoderm (Pautou 1977; McCabe et al 1974) although the mechanism is unknown.

A more detailed discussion of the role of the AER and ZPA in limb morphogenesis is outwith the scope of this thesis.

1.4 Formation of the Limb Musculature

The muscles of the chick limb are derived from somitic mesodermal cells that invade the developing limb bud early in development (Chevallier et al 1977; 1978). Under certain circumstances the somatopleural mesoderm forms muscle (Chevallier et al 1978; McLachlan & Hornbruch 1979). The somitic mesodermal cells form two primitive muscle masses, one dorsal, the other ventral in position, from which all limb muscles are derived (Romer 1927). The muscle masses are morphologically distinct by stage 24 of development (Hamburger 1975).

The pattern of splitting of the primitive muscle masses into the adult pattern of muscles has been described by Romer (1927) and Wortham (1948) for the chick hind limb and by Sullivan (1962) for the chick wing. The process of muscle splitting in the hind limb commences on day 6 of incubation and is complete by day 8. The shank muscles may split slightly later than those in the thigh.

The processes invovled in muscle splitting are not clear. Wortham (1948) suggested that separation is caused by physical migration of nerves into future spaces, pushing the masses apart. However Shellswell (1977) has found that chick forearm muscles split normally even in the absence of nerves, making this suggestion unlikely. An indirect role for nerves, producing differential contraction of the muscle masses was suggested by Sullivan (1966). He found that paralysis of chick embryos with the neuromuscular blocker decamethonium produces an alteration of muscle splitting in the shoulder region. However muscle splitting occurs normally in the chick hind limb following paralysis with α-bungarotoxin from day 4 of incubation (Laing 1979).

Muscle splitting in the chick forearm and hind limb does not therefore require the presence of nerves although this may not be so for shoulder muscles.

A role for tension in muscle splitting due to differential growth of the skeleton has been proposed by Horder (1978). However muscle splitting in the chick forearm is over at stages 29-30 before tendons have developed and inserted, and muscle splitting occurs normally even if distal insertions are missing following amputation (Shellswell & Wolpert 1977).

Shellswell & Wolpert (1977) have suggested that muscles develop with respect to their position in the limb making use of the three dimensional system of positional information discussed previously. Evidence that this is so comes from the formation of supernumerary limbs. Grafting an additional ZPA to the anterior margin of the host limb produces a supernumerary limb with mirror-image symmetry around the long axis. The muscle pattern is reduplicated (Shellswell & Wolpert 1977) as well as the skeletal pattern (Tickle et al 1975). Preliminary results reported by Shellswell & Wolpert (1977) show that grafting an extra AER to the dorsal surface of an early limb bud produces an outgrowth that is 'composed of two symmetrical dorsal halves as judged by the presence of dorsal muscle and tendons on both sides'.

If indeed the development of the primitive muscle masses are influenced by the three-dimensional system of positional information then this could provide a basis for the theories of innervation pattern development that suggest that muscles are different from one another and are somehow 'matched' to certain motoneurones. Whether this is so remains to be determined.

1.5 The Establishment of the Pathways followed by Outgrowing Axons into the Developing Limb

The direction of neurite outgrowth in culture is influenced by the orientation of the substrate (Weiss 1934; Ebendal 1976), the presence of other tissues (Chamley et al 1973; Pollack Liebig 1977) and certain chemicals (Gundersen & Barrett 1979). The role of any or all of these factors in determining the direction of axon outgrowth *in vivo* is unclear.

Weiss (1941) suggested that the direction of axon outgrowth is determined by the configuration of the structures which an axon encounters - passive 'contact guidance'. In a later paper (Weiss 1945) he suggested that axons may be able to distinguish between different structures and thus select which pathway to grow along, perhaps by 'recognising' surface chemistry. The concept of pathway selection was later developed by Sperry (Attardi & Sperry 1963). Sperry suggested that outgrowing axons posses individual chemical labels or identities and are led to their targets by matching with a similar set of labels along a particular pathway. (chemo-specific guidance). Pathway selection has also been considered in terms of axon response to an orientating or polarising influence on the basis of cell body position within the spinal cord. Axons therefore maintain their topographic relationship to one another (Hope et al 1976; Cook 1979). An alternative theory is that axon outgrowth is directed by the production of certain growth factors in target tissues - chemotaxis (Ramon y Cajal 1928).

Whether axons are passively guided in the limb or whether they select different pathways or both is a matter of debate.

There is evidence that suggests the limb plexus and peripheral nerve branching pattern is determined by the morphology of the limb. The plexus pattern in chick wings innervated by cervical or thoracic spinal cord is normal (Wenger 1950; Straznicky 1967) and transplantation of supernumerary limbs just rostral to the host hind limb induces the formation of an 'extra' lumbar plexus from the rostral segmental nerves (Hollyday & Hamburger 1976; Hollyday et al 1977). Reduplication of the chick wing in the proximo-distal axis produces a reduplicated nerve pattern (Lewis 1978) and deletions of limb segments results in a nerve pattern that is normal for the remaining limb parts (Stirling & Summerbell 1977).

These observations suggest that passive contact guidance of axons may be an important factor in determining limb plexus and peripheral nerve branching pattern. Taylor (1943) suggested that alignment of the mesostroma and mesenchymal cells guides pioneer axons through the limb, later arriving axons grow along the pioneer fibres. However an electron-microscope study on the early outgrowth of axons into the hind limb has revealed no such mesenchymal alignment (Ebendal 1977). An alternative possibility is that axon extension occurs along pathways of selective cell adhesion. (Ebendal 1977).

Parallel outgrowth and passive contact guidance of axons has been deemed a sufficient mechanism to account for the pattern of innervation of the vertebrate limb (Horder 1978). Rotation of the chick forelimb through 180° in both the anterior-posterior and dorso-central axes at stage 19 of development results in rostral spinal cord segments innervating targets normally supplied by caudal spinal segments and vice versa. Cobalt chloride tracing of axonal pathways from the spinal nerves showed that axons grow out into the limb in parallel with one-another (Stirling & Summerbell 1979). This observation and the experiments cited above that suggest peripheral nerve branching pattern is determined by the configuration of the limb lend some support to this hypothesis.

However the results of two experiments are difficult to reconcile with this. Rotation of small portions of chick lumbosacral spinal cord through 180° in the rostro-caudal axis at stages 15-16 of development (Lance-Jones & Landmesser 1978a) results in motoneurones innervating muscles appropriate to their old position and not to their new position. To do so axons followed abnormal pathways albeit within the limits of the plexus and major nerve trunks. Similarly rotation of the limb bud through 180° in the dorso-ventral axis (Ferguson 1978) results in motoneurones innervating their 'normal' targets even though these are 180° out of position. In both these experiments the projections were initially 'appropriate', making it unlikely that diffuse axon outgrowth followed by selective loss of 'inappropriate' projections (see section 1.8) could account for the pattern of innervation. They implicate some form of active pathway selection.

There is evidence then for passive contact guidance of axons and pathway selection. Their relative importance in the establishment of the pathways followed by outgrowing axons remains to be determined as does the mechanism(s) of each.

1.6 Formation of Neuromuscular Connections

Axons first pierce the basement membrane surrounding the chick lumbar neural tube at stage 19 of development, and by stage 20 the ventral roots have formed (Ebendal 1977). Axon invasion of the hind limb bud occurs at stage 24 of development (Hamburger 1975). Functional neuromuscular connections have formed as early as stage 26+ in the dorsal muscle mass of the hind limb and by stage 28 in the ventral muscle mass (Landmesser & Morris 1975). Whether this is due to a dorso-ventral sequence in time of axon ingrowth is not known. Functional neuromuscular connections have formed by stage 29 in both muscle masses of the chick forelimb (Pettigrew et al 1979).

Initial neuromuscular connections are morphologically unspecialized (Kikuchi & Ashmore 1976) although their function can be blocked reversibly by tubocurarine (Landmesser & Morris 1975) indicating transmission to be chemical in nature. The first evidence of morphological specialization - a slight thickening of the sarcolemmal membrane, has been observed on day 10 of incubation in the chick hind limb (Kikuchi & Ashmore 1976), and by day 13 there are signs of junctional infolding (Hirano 1967). The junctional unfolding becomes more marked as development proceeds but does not develop fully until after hatching (Hirano 1967). Acetylcholinesterase can be detected biochemically on day 3 of incubation in the hind limb (Giacobini et al 1973; Filogamo Gabell 1967) but junctional acetylcholinesterase does not appear until days 12 to 13 of incubation (Drachman 1963).

The mechanisms involved in controlling the formation of early connections are poorly understood. Early studies of chick myofibres in culture indicated the presence of localized regions of high acetylcholine receptor density (Fischbach & Cohen 1973) which led to speculation that these areas may be the site of preferential axonal innervation. However later studies (Frank & Fischbach 1979) showed this was not the case. Instead ingrowing axons induce new clusters of receptors beneath the site of contact. Synapses also form on muscle in vivo when acetylcholine receptors have been blocked by α-bungarotoxin (Laing & Prestige 1978) curare or cobra toxin (Pitman & Oppenheim 1978). This indicates that the capacity of the acetylcholine receptor to respond normally to acetylcholine is not essential for synapse formation.

There is no obvious pattern to the site of the initial contact along the length of a developing myotube. Later contacts are however limited to the site of the original synapse in a focally innervated muscle as well as to new membrane laid down outside a certain minimum distance in muscles with a distributed innervation (Bennett & Pettigrew 1974a). The refractoriness of the myotube membrane to further synaptic contacts is a permanent change as the process cannot be completely reversed by denervation. Re-innervation takes place at the old post-synaptic site or on new muscle membrane added during the period of denervation (Bennett & Pettigrew 1974b).

Multiple innervation of single end plates in development was first described for the rat diaphragm (Redfern 1970). It has subsequently been found in other muscles e.g. rat hind limb (Brown et al 1976), axolotl limb (McGrath & Bennett 1979), chick wing (Pettigrew et al 1979) and chick hind limb (Jansen - personal communication quoted by Laing 1979). It is however only transitory since in the adult the majority of muscle end plates are each innervated by a single axon.

1.7 Motoneurone Death

Death of a varying proportion of immature neurones is a widespread feature of developing neuronal populations in amphibians (Hughes 1961; Prestige 1965, 1967b) birds (Landmesser & Pilar 1974; Hamburger 1975; Clarke & Cowan 1976) and mammals (Harris - Flanagan 1969; Arees & Astrom 1977).

A reduction in the number of motoneurones in the lateral motor columns of anurans during development was first described by Beaudoin (1955) and subsequently a similar reduction in <u>Xenopus</u> was shown to be due to degeneration of motoneurones (Hughes 1961). In <u>Xenopus</u> some 75% of the total number of motoneurones in the ventral horn degenerate between stages 53/54 to 61 of development (Prestige 1976b) and they do so around the same time as limb movements commence.

A similar correspondence in the timing of the beginning of hind limb movements and motoneurone death occurs in the chick. Some 40% of the total number of motoneurones degenerate and motoneurone number decreases from 17,000 at stages 28-29 of development, days 5.5-6.5 of incubation, to 10,000 at stage 36 of development, day 10 of incubation. Thereafter the numbers remain constant until at least day 5 post hatching (Hamburger 1975). See fig (1.5).

Motoneurone production and death are temporally separated in both the chick and Xenopus. This is an important consideration if motoneurone counts are taken as an indication of the extent of motoneurone degeneration. Chick lumbar motoneurones are produced between stages 17 to 24 of development (Hollyday & Hamburger 1977) and motoneurone degeneration does not commence until stages 28 to 29 of development. However a few degenerating motoneurones have been observed as early as stage 25 (Chu-Wang & Oppenheim 1978) without a change in total motoneurone number. This suggests that motoneurones may still be arriving in the lateral motor columns at this time. The time of migration is unknown.

1.7.1 Amputation

The effect of amputation on motoneurone number was first recorded by Shorey (1909), who noted that limb removal in the chick results in hypoplasia of motoneurones and dorsal root ganglia. Hamburger (1934) amputated the chick wing on day 3 of incubation and observed large numbers of degenerating motoneurones and a reduction in motoneurone number of 40% on the amputated side compared to the control side on days 8 to 9 of incubation. He concluded that while some motoneurones are dependant on contact with the limb for their survival others are not. In later papers, however (Hamburger 1946, 1958), Hamburger concluded that the majority of motoneurones are dependant on contact with the limb for their survival, which was in agreement with the findings of Bueker (1943). By transplanting chick lumbo-sacral

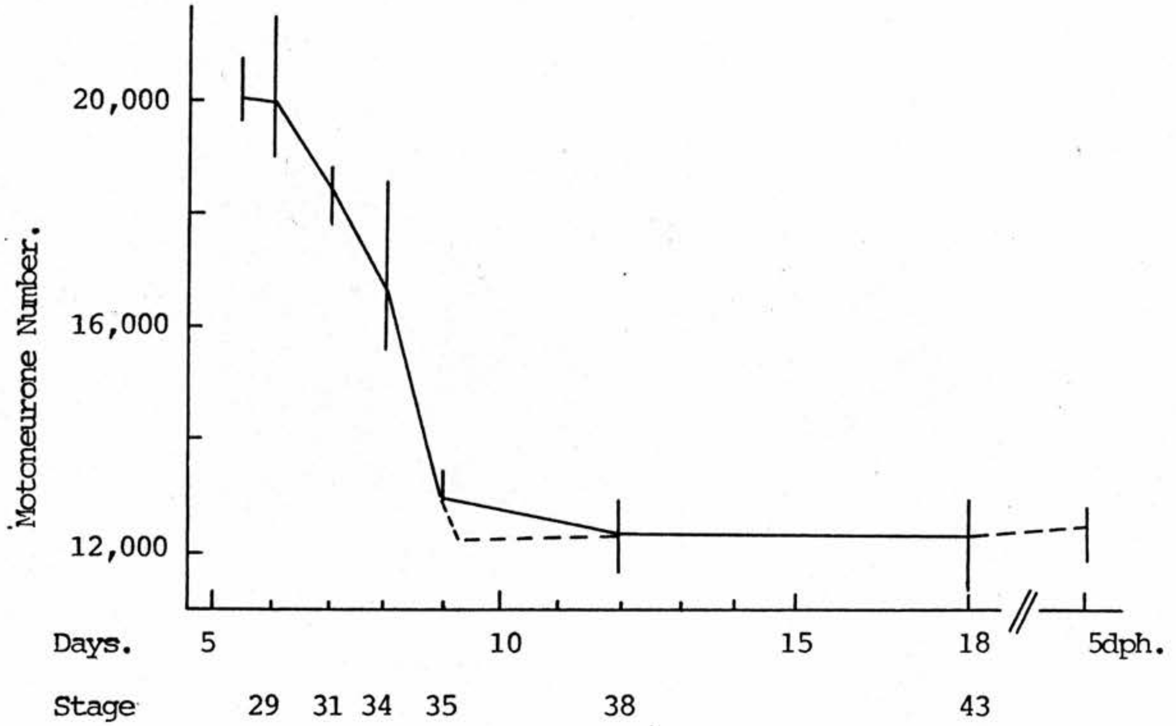


Figure 1.5

Naturally occurring motoneurone loss in the lumbar lateral motor column of the chick, with the range between maximum and minimum counts for each stage (uncorrected counts). After Hamburger 1975.

segments to the body coelom, Bueker found a 90% decrease in motoneurone number. The most likely reason for the 60% of motoneurones remaining after radical amputation recorded by Hamburger in 1934 is that he did not allow the embryos to survive long enough for all motoneurone deaths to occur.

Prestige (1967b) made a detailed study of the effect of amputation at different developmental stages on motoneurone number in Xenopus. Previously it had been shown that early amputation of the hind limb bud has no effect on motoneurone numbers until stages 52-53 of development, when large number of motoneurones degenerate (Hughes & Tschumi 1958). Prestige found that amputation at stages 53-54 of development results in immediate (within 3 to 4 days) loss of motoneurones, whereas amputation at later stages, after stage 57, results in motoneurone loss after a period of delay.

On the basis of these observations Prestige designated three phases of motoneurone development:

Phase I motoneurones are independant of the limb since they are unaffected by its removal - all motoneurones before stages 52-54 of development.

Phase II motoneurones are critcally dependant on the presence of the limb since they degenerate within 3-4 days of amputation. They are present between stages 52 and 58 of development.

Phase III motoneurones are partly independant of the limb but eventually degenerate after an extended peiod of chromatolysis and recovery. Numbers increase from zero at stages 53 until at stages 57/58 they are almost the only type of motoneurone present. See fig. (1.6).

However in a later paper Prestige (1976) doubted the value of distinguishing between phase II and phase III cells since

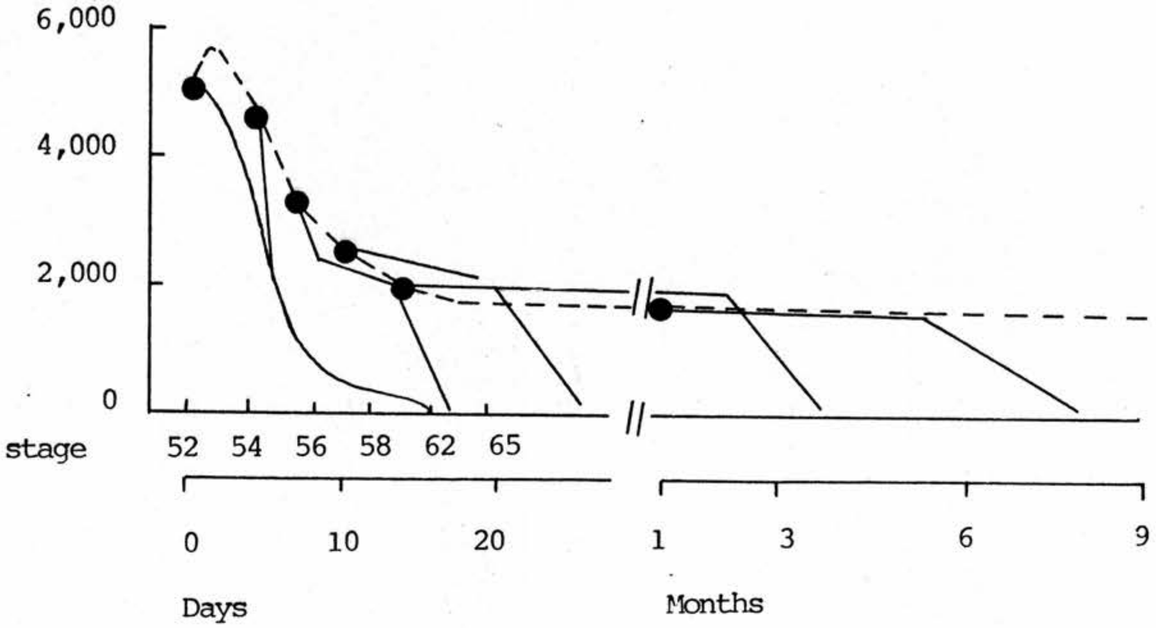


Figure 1.6

The effect of amputation at different stages on motoneurone number in the ventral horn of Xenopus. Number of motoneurones remaining after amputation at the time denoted by the filled circle. Data shows motoneurones are either lost early (Phase I) or late (Phase III) or both.

ultimately both are dependant on contact with the limb for survival. He then designated them simply as 'post phase I' cells.

A similar early independance and then dependance of motoneurone survival on the presence of the limb is observed in the chick (Oppenheim et al 1978). Following amputation of the prospective hind limb bud at stage 16 of development motoneurones develop normally, as judged by ultra structural studies and acetylcholinesterase and cholineacetyltransferase levels, up to stage 26 of development. At this time a few motoneurones have degenerated and by stage 30 of development up to 75% of motoneurones are lost. The remainder degenerate before stage 37 of development.

1.7.2. Why Motoneurones Degenerate

During development large numbers of neurones in chick spinal ganglia at non-limb levels degenerate. The close correspondence in timing between this degeneration and the degeneration observed in spinal ganglia at limb-levels caused by limb amputation, led Hamburger & Levi-Montalcini (1949) to suggest that the neurones at non-limb levels that normally degenerate do so because they fail to contact a peripheral target. Cell death was proposed to be a regulative mechanism relating neurone number to the size of the peripheral target. A similar suggestion was made by Prestige (1967b) to explain naturally occurring motoneurone death in the lumbar ventral horn of Xenopus.

An alternative suggestion was made by Hughes (1968). He proposed that the early innervation pattern of the limb differs from the final adult pattern. The function of motoneurone death is to remove motoneurones that have formed connections 'incompatible' with the final adult pattern. A middle view was taken by Cowan (1973) who suggested that the motoneurones that survive are those that form connections which are both 'appropriate' in both type and number.

Prestige (1976) divided the various hypotheses into two categories : the rejection and redundancy hypotheses. The redundancy hypotheses state that neurone death is due to failure of axons to form connections, there being an excess of fibre endings for the number of available sites. The rejection hypotheses state that neurones degenerate because their axons, having already made connections, are rejected from the sites.

At present it is not known whether the motoneurones that degenerate have formed functioning connections with the limb prior to their death, although almost all axons in both the chick and Xenopus have entered the limb prior to the period of motoneurone death (Prestige & Wilson 1972, 1974; Lamb 1974; Chu-Wang & Oppenheim 1978b).

Lamb (1976, 1977) labelled early projections in the hind limb of Xenopus by injecting HRP into the limb and showed that they were subsequently lost by death of the motoneurones. However HRP is taken up both by transmitting and non-transmitting nerve-terminals and so this only demonstrates that the terminals are in close contact with the limb. There is evidence of early functioning connections in the axolotl hind-limb (McGrath & Bennett 1979) and chick wing (Pettigrew et al 1979) which are subsequently lost. Whether this is due to axon withdrawal or motoneurone death is not known.

Prestige (1976) has provided indirect evidence that at least some motoneurones that degenerate have survived for a short time by virtue of contact with the limb. He amputated one hind-limb in Xenopus, early in development and using the other as a control compared the number of motoneurones between the two sides at different developmental stages. The number of motoneurones present at any developmental stage on the amputated side represents the number of phase I motoneurones - motoneurones that are independant of contact with the limb for their survival. Subtraction of the number of motoneurones on the amputated side from the number on the control side at any developmental stage gives the number of 'post phase I' motoneurones (dependant on contact with the limb for survival) in the control limb at that time. Prestige found an increase in the number of 'post-phase I' motoneurones from stages 52 to 54 of development. From stages 54 to 58 there was a decrease in the number of 'post-phase I' motoneurones. This indicates that at least some 'post-phase I' motoneurones, which, by definition, have survived due to contact with the limb, degenerate. Similar indirect evidence for rejection of connections is found in the chick hind-limb. Amputation not only accentuates but accelerates naturally occurring motoneurone death (Oppenheim et al 1978).

There is however no direct evidence to support either the rejection or the redundancy hypotheses. Further electrophysiological studies may decide the issue.

Trophic Factors

Prestige (1967b) on the basis of amputation studies, postulated the existence of a trophic factor, produced by the limb and required by motoneurones for their survival and maturation. Phase I motoneurones he considered to be independant of the factor, while post-phase I motoneurones, dependant on it for their continued survival and maturation, retrogradely transport it back to their cell bodies via axon terminals. The motoneurones that degenerate during normal development are those that fail to gain adequate amounts of the trophic factor for their survival.

A trophic factor, Nerve Growth Factor (NGF), similar in mode of action to the one proposed for motoneurones has been found to exist for sympathetic and also possibly sensory neurones. The early history of NGF has been well reviewed by Levi-Montalcini (1976) who has played a major role in its characterization.

During normal development of the superior cervical ganglia in the newborn rat there is a decrease in the number of adrenergic neurones. (Hendry & Campbell 1975). This decrease is enhanced by removal of the salivary glands and iris (Dibner et al, 1975), targets for some neurones in the ganglia, indicating that adrenergic neurones, like motoneurones are dependant for survival on the presence of their target. The chromatolytic response and neuronal death in the superior cervical ganglia that follows axotomy in newborn rats can, however, be prevented by administration of NGF (Hendry 1975 a, b) suggesting NGF may mediate the effect of target organ on sympathetic neurone survival. This is further suggested by the fact that testosterone-induced hypertrophy of salivary tubules in neonatal mice, which increases the naturally occurring NGF content, also increase neurone number in the superior cervical ganglia (Black 1978). Further NGF is retrogradely transported by sympathetic neurones from their target organ (Hendry et al 1974). All these results strongly suggest that the availability of NGF may control neurone number in developing sympathetic ganglion.

Despite intensive investigation, there is no direct evidence from in-vivo studies for the existence of a similar trophic factor for cholinergic neurones. There is however, indirect evidence from in-vitro studies. Isolated and dissociated chick ciliary ganglion neurones normally degenerate in culture. This degeneration can be prevented not only by striated muscle myotubes (White & Bennett 1978) but also by adding medium conditioned by skeletal muscle as well as medium conditioned by heart muscle to the cultures (Bennett & Nurcombe 1979). This suggests that a diffusible factor produced both by heart and striated muscle may be responsible for the survival of cholinergic neurones.

However until an effect can be demonstrated in vivo the existence of Prestige's trophic factor will not have been adequately demonstrated.

Afferent Input

Although the amputation studies provide strong support for the idea that motoneurones degenerate either because they fail to contact or fail to maintain their contact with the limb another possibility must also be considered.

This is the role afferent imputs may play in motoneurone death. Afferent connections are implicated in some neurone deaths in the isthmo-optic nucleus of the duck (Sohal 1976) and chick (Clarke & Cowan(1976), and chick vestibular nucleus (Levi-Montalcini 1947). However, there is no evidence for afferent imput controlling motoneurone number in the chick. Isolation of the chick lumbar spinal cord from descending and ascending fibres (Hamburger et al 1966) does not affect motoneurone number until day 15 of incubation, which is well after the normal period of motoneurone death. After this time large numbers of motoneurones degenerate. This suggests that afferent input may not play a role in normal motoneurone death but indicates that it may be required for normal maturation later on. However the possible role of afferent connections in motoneurone death has not been fully explored and remains to be investigated.

1.8 The Role of Motoneurone Death in Innervation Pattern Development

It was Hughes (1968) who first proposed that motoneurone death may play a role in the formation of the innervation pattern. Hughes envisaged axon outgrowth to be diffuse. Motoneurones whose axons form connections compatible with final innervation pattern survive the period of motoneurone death; motoneurones whose axons form connections that are not compatible with the final innervation pattern, ('non-adult' projections), are rejected from the muscle and degenerate. This hypothesis is based on the ideas of Sperry (e.g. 1963) in that it implies some form of pre-determined matching or specificity between motoneurones and muscles.

There are two important predictions from this hypothesis. The first is that the initial innervation pattern differs from the final adult innervation pattern. The second is that the motoneurones that form 'non-adult' projections and which are subsequently lost, degenerate. There is direct evidence that the initial innervation pattern differs from the final innervation pattern in the chick wing (Pettigrew et al 1979), Xenopus hind limb (Lamb 1976) and axolotl hind limb. (McGrath & Bennett 1979). Only in Xenopus has it been shown that loss of the early projections is associated with motoneurone death.

Lamb (1977b) who pioneered the technique of HRP-uptake labelling to study motoneurone projection patterns during development, found that in Xenopus at stage 50 of development motoneurones innervating the antero-lateral thigh lie rostrally and caudally within the spinal cord (Lamb 1976). During stages 51 to 52 this innervation is lost and by stage 54 the antero-lateral thigh receives its innervation from motoneurones lying caudally and laterally.

Similarly the pattern of innervation to the posterior-medial thigh changes during the same period. At stage 50 the innervating motoneurones lie caudally and laterally and by stage 54 they lie rostrally and laterally. Lamb (1977a) demonstrated that this changing pattern of innervation is associated with death of the motoneurones forming the early projections. He injected HRP into both the antero-lateral and posterior-medial thigh at stage 52 and allowed the tadpoles to survive to between stages 51 to 55. The motoneurones forming the early projections became labelled with HRP and were shown to disappear from the spinal cord by stage 54.

However loss of early 'non-adult' projections cannot explain all motoneurone deaths in Xenopus. Lamb (1976) found that the majority of projections to Xenopus hind limb, especially distal limb regions were compatible with the final adult innervation pattern from the start. The situation appears to be similar to that of the chick hind limb. Landmesser & Morris (1975) mapped the innervation pattern of the limb just before and after the period of motoneurone death. By stimulating individual spinal nerves and observing and recording muscle contraction and tension, they found little or no difference in the innervation pattern before or after the period of motoneurone death. Later Landmesser (1978b) extended this work using the technique of HRP uptake labelling of motoneurones and also by stimulating spinal nerves and recording from muscle nerves at different development stages. From as early as stage 27 of development the innervation pattern was almost the same as the final adult innervation pattern. However, as axons first enter the limb at stage 24 (Hamburger 1975) the possibility of early projections that differ from the final adult projections occurring between stages 24-27 of development cannot be ruled out.

Indeed indirect evidence for such projections has come from the work of Laing (1979). Laing prevented motoneurone death in the chick by paralysing the embryos with α-bungaratoxin from day 4 of incubation. He then amputated the right hind limb at the knee in both paralysed and unparalysed embryos and mapped the distribution of motoneurones remaining in the spinal cord on day 10 of incubation. He found that in unparalysed embryos motoneurone loss occurs predominantly from the caudal parts of the spinal cord with little or nc loss from the rostral segments. This is in agreement with distribution of motoneurones that innervate the shank on day 10 of incubation (Landmesser 1978) See fig (1.2). However in paralysed embryos, where normal motoneurone death is prevented amputation results in loss of motoneurones from rostral as well as caudal segments. This suggests that prior to the period of motoneurone death some motoneurones in rostral segments project to shank muscle and that during the period of motoneurone death these projections are lost. A possibility not ruled out in this experiment is that α-bungarotoxin alters the innervation pattern.

The available evidence then for the hind limb of <u>Xenopus</u> and the chick suggests that although a few early 'non-adult' projections do occur, the majority of projections are compatible with the final adult innervation pattern from the start. This suggests that Hughes' hypothesis, that loss of projections incompatible with the final adult pattern results in motoneurone death does not explain the majority of motoneurone deaths and is not a mechanism of major importance in the development of the innervation pattern in <u>Xenopus</u> and chick hind limbs. However this is only true if compatibility with the final adult pattern is considered in terms of projections between different muscles. It may be that matching occurs not between motoneurones and different muscles but between motoneurones and individual fibres within a muscle. In this case Hughes' hypothesis could explain the majority of motoneurone deaths and could also be a mechanism of importance in refining detailed patterns of projections to individual muscles. Whether this is so remains to be investigated.

Early 'non-adult' projections to different muscles have been observed in the chick wing on a scale possibly large enough to explain motoneurone deaths. (Pettigrew et al 1979). Why the chick wing and hind limb should differ in this respect is not clear. However it is not known whether loss of these early projections is associated with their death. A study of motoneurone death in the chick brachial lateral motor columns (Oppenheim & Majors - Willard 1978) shows that the majority of deaths occur much later than the period of loss of 'non-adult' projections. It would seem an unlikely possibility that the innervation pattern of the chick-wing develops by a different mechanism to that of the hind limb.

1.9 Summary

Early in development the immature motoneurones and the cells that give rise to muscles develop independantly of one-another and are separated by a distance that is very large in relation to their size. Nonetheless motoneurones form a predictable ordered pattern of connections with the limb muscles. Axons grow out into the limb under unknown controls. The contribution the direction of axon outgrowth makes to the establishment of the innervation pattern is uncertain although there is some evidence that axons may 'select' between different pathways in the limb. Little is known about the control of the formation of neuromuscular connections and whether this plays a role in the establishment of the innervation pattern, Death of a large percentage of motoneurones that occurs around the time of the formation of neuromuscular connections does not appear to be of major importance in the establishment of the innervation pattern. The significance of motoneurone death remains unknown.

1.10 Experimental Aim

The aim of the experiments was to examine to what extent motoneurones alter their pattern of connections when part of the limb musculature, their normal target, is missing. Three different types of amputation were carried out on day 4 of incubation:

Graded transverse amputation - removal of a varying proportion of distal limb structures.

Graded ventral amputation - removal of a variable proportion of the embryonic ventral muscle mass.

Graded dorsal amputation - removal of a variable proportion of the embryonic dorsal muscle mass.

The innervation of the partly amputated limbs was examined on day 10 of incubation:

(1) The number of motoneurones in the lateral motor column was related to the volume of remaining muscle

(2) Motoneurone position in the rostro-caudal and medio - lateral axes of the lateral motor column was related to the type of amputation - transverse, ventral or dorsal.

The results show that loss of muscle is associated with a proportional loss of motoneurones. The innervation pattern to the remaining muscle is normal in terms of motoneurone position in the rostro-caudal and medio-lateral axes of the lateral motor column.

These results show that motoneurones do not significantly alter their projections to a partly amputated limb. They suggest that motoneurones may be different from one-another and that expression of these differences leads to the formation of the innervation pattern.

CHAPTER 2

MATERIALS AND METHODS

2.1 Experimental Animals

The embryo of the domestic fowl (Gallus Domesticus), strain White Leghorn was used in this study. They were obtained from the Poultry Research Centre, Roslin.

2.2 Care of Experimental Animals

Before being placed in the incubator the position of the yolk and air sac within the eggs was determined by placing the eggs over a strong light source - 'candling'. A square of 1cm was drawn in the middle of the yolk and the position of the air sac marked. The eggs were then swabbed with 70% alcohol and a hole was made through the shell into the air sac using a mounted needle. A Renda 25 dental drill fitted with a carborundum disc was used to drill along one corner of the square, approximately 2mm along each edge, care being taken not to drill through the shell membrane. The resultant hatch was covered in sellotape and the eggs placed in the incubator. All eggs were incubated with their long axis horizontal, the drilled side uppermost, in a Hearson forced-draught incubator at 38°C. The air was humidified by troughs of distilled water.

On the day of operation or injection the eggs were removed from the incubator and placed in a petri dish containing wax in which an impression of an egg had been formed. This served to steady the eggs and allowed greater control over experimental procedures. Using forceps, the sellotape was removed and the shell at the drilled corner was chipped away exposing the shell membranes. A drop of sterile Hanks balanced salt solution (BSS) was placed on the shell membrane and a slit was made with watchmakers forceps.

Air was withdrawn from the airspace using a rubber teat and a false airspace was formed above the embryo. The rest of the shell and shell membranes above the embryo were removed and the operations were performed in the false air space so created. After the operation the hole in the shell was sealed with sellotape and the eggs were returned to the incubator, the drilled side uppermost.

2.3 Operative Techniques

2.3.1. Amputations

All amputations were performed on embryos at day 4 of incubation, between stages 22 to 24 of development. The right hind limb bud was amputated in all embryos, the left serving as a control. This is because after stage 19 of development chick embryos lie on their left side (Hamburger and Hamilton 1951) making amputation of the right limbs much easier than the left.

All operations were performed under a Baush & Lomb zoom dissecting microscope at suitable magnification. The chorion and amnion above the right limb bud were torn using sterilized watchmakers forceps. The amputations were carried out using an electrocautery device. This consisted of a loop of low resistance wire (0.25mm diameter; 4 ohm resistance) through which a current of 1.9amps was passed. The loop, which was attached to a holder, became hot. The limb was amputated by cauterizing the appropriate part of the bud. Three types of amputation were carried out:

(1) Transverse Amputation - removal of distal limb structures
(2) Ventral Amputation - removal of the ventral muscle mass
(3) Dorsal Amputation - removal of the dorsal muscle mass

Transverse amputations were carried out by passing the hot cautery wire transversally over the distal limb tip, fig (2.1a).

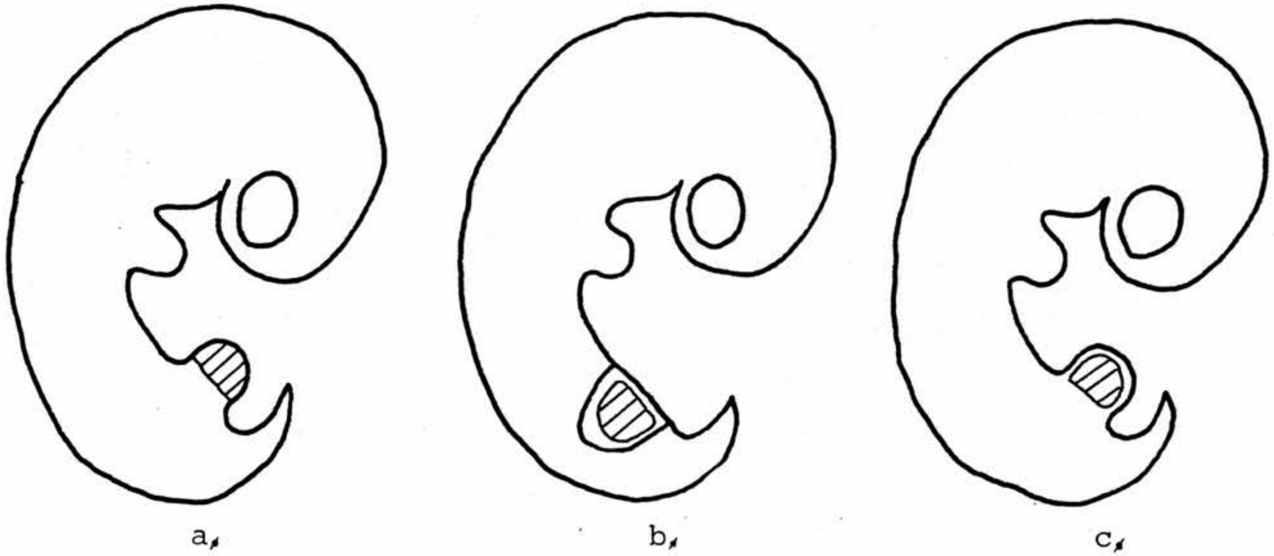


Figure 2.1.

Chick embryos at stage 23 of development. The hatched area represents the part of the limb cauterized. a/. Transverse amputation: the entire limb bud distal to the amputation level is cauterized. b/ Ventral amputation: the limb was held back with forceps to expose the ventral surface. The superficial ventral surface was cauterized.c/ Dorsal amputation: the easily accessible dorsal surface was cauterized.

The extent of removal varied from almost total destruction of the bud to minimal destruction.

Ventral amputations were carried out by passing the cautery wire over the ventral limb surface. The ventral surface is on the underside of the limb and was exposed by holding the limb back with watchmakers forceps. The amputation was carried out and the limb was allowed to slide back into position, fig (2.1b).

Dorsal amputations were carried out by passing the cautery wire over the dorsal aspect of the limb bud. Since the embryo lies on its left hand side the dorsal surface of the limb is uppermost and amputation is relatively easy, fig (2.1c).

Differing amounts of dorsal or ventral muscle were removed in each embryo, ranging from complete to slight removal. Great care was taken during these amputations not to cauterize the limb bud near the distal tip. This is because destruction of the distal tip produces a limb with a transverse amputation.

Following the operation 0.05ml of sterile BSS was dropped onto the embryo. The hole in the shell was sealed with sellotape and the egg returned to the incubator.

2.3.2. Injections of Tritiated Thymidine

The eggs were removed from the incubator and opened in the usual manner. The embryo's development was then carefully staged. Any embryo whose development was later than stage 19 was not used. Any embryo who had not reached stage 19 of development was resealed with sellotape and returned to the incubator for further development. If the embryo was at stage 19 of development 10μCi[3H] - thymidine (methyl - [3H] - thymidine, sp. A. 5 Ci (m.mol), in aqueous solution,(Radio-Chemical Centre, Amersham) dissolved in 0.05 ml sterile BSS was dropped onto the embryo. In some cases the chorionic membrane above the embryo was torn with watchmakers forceps before making the injection. The eggs were then resealed and returned to the incubator.

A dose of 10μ Ci of [3H] - thymidine was chosen because it has been shown that this dose labels all neuro-epithelial cells and subsequently produced neurones in chicks at day 3 to 4 of incubation (Langman & Haden 1970). Similarly preliminary experiments showed that 10μ Ci [3H] - thymidine when given at stage 14/15 of development labels the entire lumbar lateral motor column.

2.4 Histology and Tissue Processing

2.4.1. Fixation

Embryos were fixed on day 10 of incubation. The opening in the shell was enlarged with forceps and the embryonic membranes were torn. The embryo was lifted out of the egg, placed in avian ringer, decapitated, eviscerated and skinned. The vertebral column was transected rostral to the lumbar enlargement and the upper part of the body discarded.

To flatten out the embryo and thus aid in sectioning, the lumbar spinal cord and hind limbs were laid flat on a piece of card and fixed overnight in Carnoy.

2.4.2. Dehydration

Since Carnoy is a dehydrating fixative, embryos were taken straight to methylated spirit (740P) to rinse out the fixative. They were rinsed in four 1 hour changes of 740P and then transferred to cedarwood oil for two days for clearing.

2.4.3. Embedding

From cedarwood oil the embryos underwent the following procedure:

(1) ½ hour Xylene

(2) 3 x 1½ hour parafin wax - 56-57°C Paraplast at a temperature of 60°C

(3) Embedding - the embryos were always embedded such that the orientation was known.

2.4.4. Sectioning

The lumbar spinal cord and hind limbs were serially sectioned on a Reichart rotary microtome in a standard manner such that the right and left sides of embryos were always known. The majority of embryos were sectioned with the knob setting of the microtome at 8µm thickness. However the spinal cords and parts of the limb associated with it, of those embryos that received 10µ Ci [3H] - thymidine at stage 19 of development, and were to be processed for autoradiography, were sectioned at 5µm thickness, the remaining limb parts at 8µm thickness.

A section thickness of 5µm was chosen for autoradiographs so that even the deepest nucleii which had incorporated [3H] - thymidine would appear 'labelled' on the autoradiograph - relatively few β particles penetrate further than 5µm (Rogers 1973).

The sections were mounted serially on glass slides (76 x 26 mm) which had been soaked overnight in chromic acid, washed for 2 hours in running tap water and 1 hour in distilled water, and subbed in chrome-alum gelatin. The sections were placed, approximately, within an area 4 mm from each side and 10 mm from the bottom of the slide.

2.4.5. Preparation of Autoradiographs

i) Coating slides with Emulsion

Those slides to be coated for autoradiography were dewaxed in the following manner :

2 x 15 mins Xylene
1 x 2 mins Absolute Alcohol
1 x 2 mins 80% Alcohol
1 x 10 mins running tap water
1 x 10 mins 5% ice cold tricholoracetic acid
1 x 1 hour running tap water
Distilled water

The slides were then transferred to the dark room.

In the dark, K_2 emulsion (Ilford Ltd.) was diluted 1:1 with distilled water in a dipping cell and allowed to melt over a water bath at $50^{\circ}C$. Two blank test slides were then placed 'back to back' in a pair and dipped twice in the emulsion. These slides were then separated and examined in front of the safety light (Kodak 'Wratten' series No. 2 Filter). If the emulsion had mixed well with the distilled water and the emulsion evenly coated the test slides, then the experimental slides were dipped. If the emulsion was of uneven thickness on the test slides then the emulsion was agitated gently to aid mixing, and the process was repeated until the test slides showed even emulsion thickness.

The experimental slides were placed 'back to back' in pairs so that the sections faced outwards, and each pair was dipped twice in the emulsion. The slides were then separated from one another, and hung from rods by means of paper clips. After an initial period of drying the slides were transported to a light-proof drying box and left for two hours or until they were completely dry. They were then transferred to storage boxes, which were sealed and stored at $4^{\circ}C$ for 2-3 weeks.

In each batch of slides dipped there were between 4 and 8 control slides. These were slides from an embryo that had not received [3H] - thymidine at stage 19 of development but had undergone identical procedures to those that had. Half of these slides were dipped and stored as described. The remainder were also dipped but were then exposed to white light for 5 seconds. They were then allowed to dry and stored as normal.

ii) Developing autoradiographs

Preliminary experiments showed that an exposure time of between 2-3 weeks at 4°C was adequate for silver grains to form over lateral motor column motoneurone nucleii. Exposure times longer than this resulted in excess of silver grains over the nucleii, obscuring their morphology and making identification of motoneurones difficult.

The autoradiographs were placed in staining racks in the dark and developed according to the following schedule:

1 x 4 mins developer (Kodak D19B) at 16°C
A few seconds wash with distilled water
1 x 4 mins fixative (Amfix) at 16°C
1 x 1 hour in running tap water.

They were then transferred to distilled water ready for staining.

2.4.6 Staining

All sections except autoradiographs were stained with haematoxylin (Harris) and eosin (Humason 1972) according to the following schedule:

2 x 5 mins Xylene

1 x 5 mins 740P

1 x 5 mins 30% alcohol

1 x 5 mins Distilled Water

10 - 15 mins Haematoxylin

Rinse in tap water

Dip in acid alcohol to differentiate

Rinse in tap water

5 mins in saturated lithium carbonate (Blue)

5 mins in tap water

Dip in 4% Eosin

Rinse in tap water

2 mins in saturated potassium alum

Rinse in tap water

2½ mins 30% alcohol

2½ mins 740P

2½ mins absolute alcohol

2 x 2½ mins Xylene

The sections were mounted in DPX (B.D.H.)

Autoradiographs were stained with haematoxylin and Eosin as follows:

6 mins Haematal 8

Rinse in tap water

1 - 2 mins lithium carbonate (blue)

Rinse in tap water

1 - 4 dips Eosin

Rinse in tap water

1 min saturated potassium alum

Rinse in tap water

2½ mins 30% alcohol

2½ mins 90% alcohol

2½ mins 95% alcohol

2½ mins Absolute alcohol

2 x 2½ mins Xylene

The sections were mounted in DPX (BDH)

2.5 Solutions

2.5.1. Operating Solutions

Hanks Balanced Salt Solution (BSS)

1) 100 ml normal strength Hank's modified balanced salt solution
2) 1-2 ml Penicillin/streptomycin (5,000 U/ml : 5,000 g/ml)
3) 2 ml 1M Hepes buffer

(All solutions obtained from Flow Laboratories)

The ph. was adjusted to 7.4 with 1N. NaOH judged by the contained phenol red indicator.

The solution was sterilized by being passed through a 13mm 0.45 µm millipore filter with a type AP20 depth prefilter (10mm in diameter) in a Swinnex plastic autoclave holder for sterilising small volumes. (Millipore UK. Ltd.)

Avian Ringer

Howard's modified Ringer solution (Howard 1953; Spratt 1955)

7.2g Na Cl

0.37g K Cl

0.17g Ca Cl_2 made up to 1 litre of distilled water

Fixative

Carnoy

600 mls Cellosolve (2-etho-oxyethanol) (BDH)

300 mls chloroform (M&B)

100 mls glacial Acetic acid (BDH)

[3H]-thymidine in Balanced Salt Solution

(Methyl - 3H) Thymidine (Radio- Chemical Centre, Amersham) of the following specification was used:

Specific Activity	5.0 Curies/mmol
Radioactive Concentration	1.0 mCi/ml
Total Activity	1.0 mCi/ml

The [3H]-thymidine was diluted 1:4 with sterile Hanks Balanced Salt Solution so that the radioactive concentration was now 200μ Ci/ml. Thus each injection of 0.05ml contains 10μ Ci [3H] thymidine. It was stored at 4°C.

2.5.2 Histological Solutions

Chrome Alum - gelatine

0.1g chrome alum dissolved in 100 mls distilled water

0.5g gelatin dissolved by heating in 50 mls distilled water

An equal volume of chrome alum solution is added to the gelatin solution. The gelatin solution must be fresh every second day.

Harris's Haematoxylin

Haematoxylin (Revector)	1g
Absolute alcohol	10 ml
Aluminium potassium suphate (potash Alum)	20g
Distilled water	200 ml

Mercuric Oxide	0.5g

Haematoxylin is dissolved in alcohol, and the alum in water by gentle heating. The two solutions are mixed together and rapidly brought to the boil. They are then removed from the heat to add the mercuric oxide. (Care is needed because the mixture can be dangerously reactive). The solution is then reheated until it becomes deep purple in colour. The flask is immersed in cold water to stop the reaction.

Haematal 8

a) 15.8g Aluminium Sulphate
   1 litre Distilled water
b) 1.88g Haematein
   500 ml Ethylene Glycol
   1 litre Distilled water

Equal quantities of a) and b)

Eosin

4g eosin per 100ml distilled water

Lithium carbonate

Saturated solution of $LiCo_3$ obtained by maintaining a winchester with undissolved $LiCo_3$ at the bottom.

Potassium alum

Saturated solution potash alum - 114g 1 litre

Acid Alcohol

1 ml concentrated HCl in 100 ml 70% alcohol

2.5.3 Autoradiographic Solutions

Trichloroacetic Acid - 5%

5g of trichloroacetic acid dissolved in 100 ml distilled water.

Nuclear Emulsion

K_2 (Ilford) nuclear emulsion, mixed with equal quantities of distilled water and melted over a water bath maintained at 50°C

Developer

Kodak D19B developer made up with distilled water according to manufacturers instructions, and stored and used at 16°C

Fixative

Amfix, made up with distilled water according to manufacturers instructions and stored and used at 16°C

2.6 Motoneurone Counts

Lateral motor column motoneurones were identified both by their position within the spinal cord and also by their morphology. They lie in a column situated at the ventro -lateral margin of the grey matter, fig (2.2), extending from segments 22 or 23 to 30 (Hamburger 1975). They have large oval or round nucleii that contain 1-3 nucleoli and are surrounded by darkly staining cytoplasm, fig (2.3).

The number of motoneurones in both right and left side lumbar lateral motor columns was estimated using a systematic sampling procedure. The number of motonuerones in every tenth section was counted, magnification (x 600), with the aid of a grid. An estimate of total motoneurone number was calculated from

$$N_t = N_s \times P$$

where

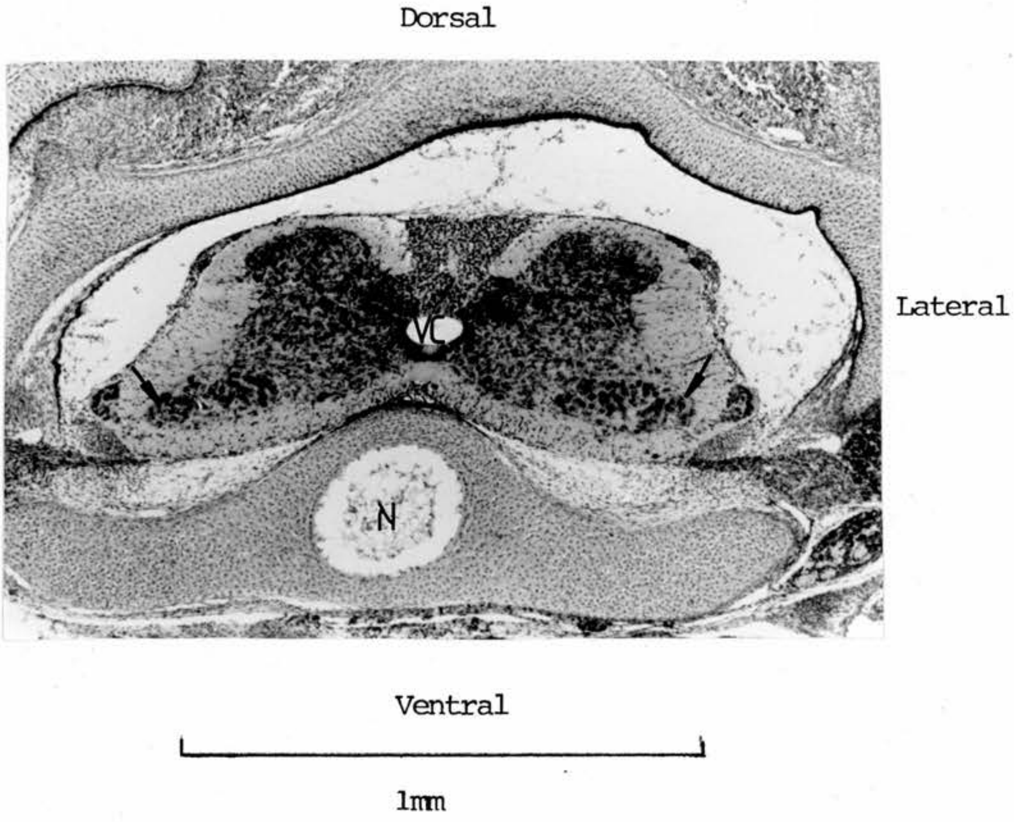


Figure 2.2.

Transverse section of the spinal cord mid -way along the length of the lumbar lateral motor column. The lateral motor columns, marked by arrows, lie in the ventro-lateral part of the spinal cord. N- notochord. VC- ventricular canal.

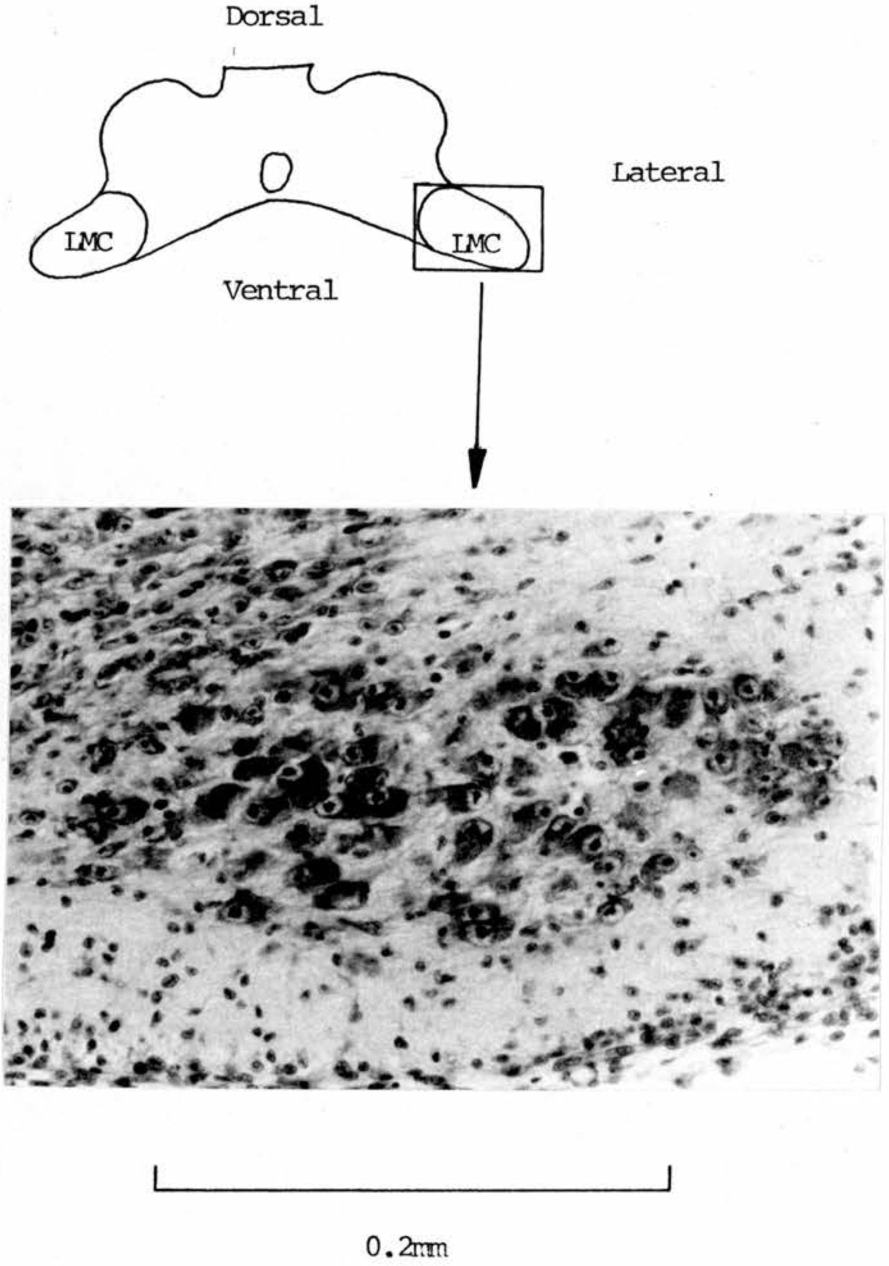


Figure 2.3. Morphology of lateral motor column motoneurones in an embryo at day 10 of incubation. They have large, well defined nucleoli surrounded by darkly staining cytoplasm. LMC- lateral motor column.

N_t is the estimate of the total number of motoneurones in the population.

N_s is the number of motoneurones in the sample (the total from all counted sections).

P is the frequency at which sections are sampled. For these experiments $P = 10$.

The method of systematic sampling assumes that the number of cells in the counted section is an average of the number of cells per section for the 5 preceeding sections and 5 following sections. The most accurate method of estimating the total number of neurones in a given population is to count all neurones in every section. However the extra work does not warrant the small gain in accuracy.

The technique of systematic sampling of periodic sections throughout the lateral motor column was chosen because it has been shown to be more accurate than a random sampling procedure (Konigsmark 1970) and also because the experiments demanded that motoneurone distribution along the rosto-caudal axis of the lateral motor column be known. Further systematic sampling of periodic sections has become the standard method for motoneurone estimations in the chick lateral motor column (e.g. Hamburger 1975; Oppenheim et al 1978; Laing & Prestige 1978).

2.6.1. Errors in Motoneurone Number Estimations

Possible sources of error in estimating motoneurone number include:

(1) The reliability with which lateral motor column motoneurones are identified. Difficulties arise in correctly identifying lateral motor column motoneurones from other large interneurones situated at the ventero-lateral margin of the grey

matter, when very few motoneurones remain per section, as after radical limb amputation. This is because one of the most useful criteria for reliably identifying lateral motor column motoneurones is the presence of other motoneurones. These difficulties in identification may lead to an over-estimation of low motoneurone number.

(2) The accuracy with which motoneurones are counted. The counting error involved in total motoneurone estimates was small since all recounts were within 3% of the original estimate.

One other source of error that must be mentioned is that due to double counting of motoneurones. This may arise because motoneurones are split in two by the microtome knife and therefore appear in two adjacent sections. They are then counted as two cells instead of one. However motoneurone estimates have not been corrected for double counting. The reason is given in the appendix.

2.7 Muscle Volume Estimations from Camera Lucida Drawings

All muscle volume estimations were made from serial camera lucida drawings 160μ m apart throughout the hind limbs. Drawings were made every 20th section for those embryos sectioned at 8μm thickness; for those sectioned at 5μm thickness drawings were made every 32nd section.

In some embryos, those whose spinal cords were sectioned at 5μm thickness for autoradiography, the most anterior parts of the limb in the same sections as the spinal cord were sectioned at 5μm thickness, the remaining parts of the limb at 8μm thickness. In these embryos initially every 32nd section was drawn. However a problem arose on transition of the sections from 5μm to 8μm thickness as to how to determine which section was 160μm from the last drawn at 5µm thickness. The problem was resolved in the following way:

When the number of 5µm sections remaining was less than 32, the thickness of tissue remaining (y) sectioned at 5µm thickness can be calculated from

$$y = \text{No. of remaining sections} \times 5\mu m$$

The next section to be drawn should therefore be (160 - y) µm in from the start of sections cut at 8µm thickness. The number of sections (b) that this distance is from the start of sections cut at 8µm can be calculated from :

$$b = \frac{(160 - y)}{8}$$

Thus the section that is 160µm away from the previous section drawn may be determined. Occasionally the value for b was not a whole number. In these instances the value of b was rounded up or down to the nearest whole number.

Camera lucida drawings were made using a Wild stereo dissecting microscope and camera lucida attachment at x14 magnification. In every section, each muscle was identified according to its insertion and origin using the nomenclature of Hudson (Hudson et al 1959) and then according to its muscle mass of origin - dorsal or ventral (Romer 1927, Wortham 1948). Shown in fig (2.4) is a photograph of a section of a control limb through the thigh. Each muscle has been named. A camera lucida drawing from this photograph is shown in fig (2.5). The muscles are shaded according to their muscle mass of origin.

The drawings were photocopied and the photocopies cut up into separate muscle areas which were individually weighed. For each embryo a square of known dimensions was also photocopied, cut out, and weighed.

Figure 2.4.

Photograph of a longitudinal section of the thigh through the knee of the left hind limb of an embryo at day 10 of incubation. The muscles have been identified according to their origin and insertion and named according to Hudson (Hudson et al 1959).

Key.

A - adductor longus et brevis
Ac- accessory
B - biceps femoris
Fe-femoritibialis externus
Fi-femoritibialis internus.
Fm- femoritibialis medius
I - ischiofemoralis
Il- iliotibialis
K - knee
O - obturator internus
P - pelvic girdle
Pr- piriformis
S - sartorius
Sn- sciatic nerve.

Figure 2.5.

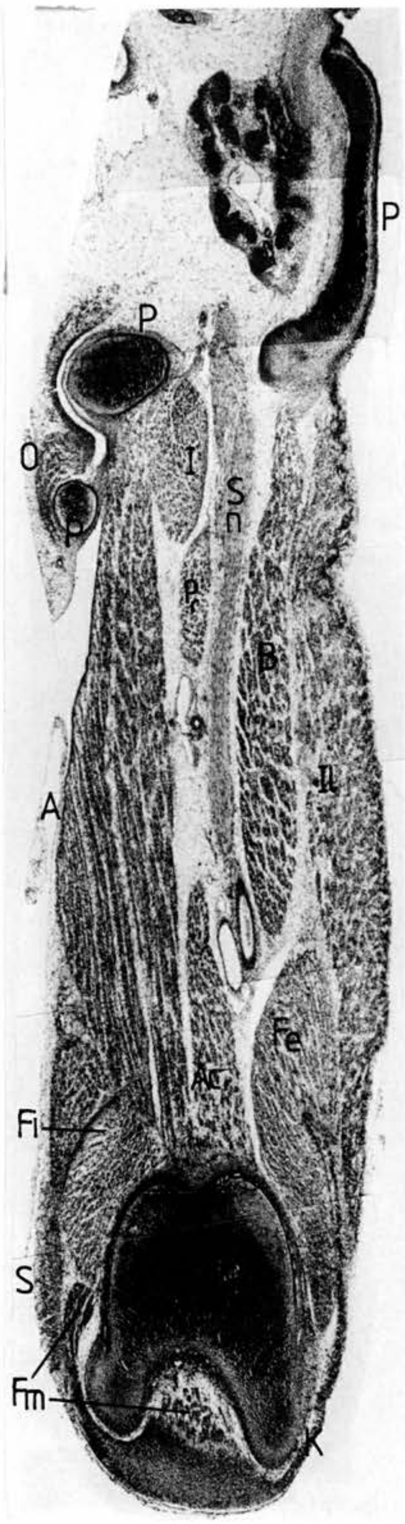
Camera lucida drawing of the photograph shown in Figure 2.4. Each muscle has been identified according to its muscle mass of origin (Romer 1927, Wortham 1948) and shaded appropriately. From these drawings the muscle areas were cut out, separately weighed, and muscle volumes were estimated by the proceedure described in the text.

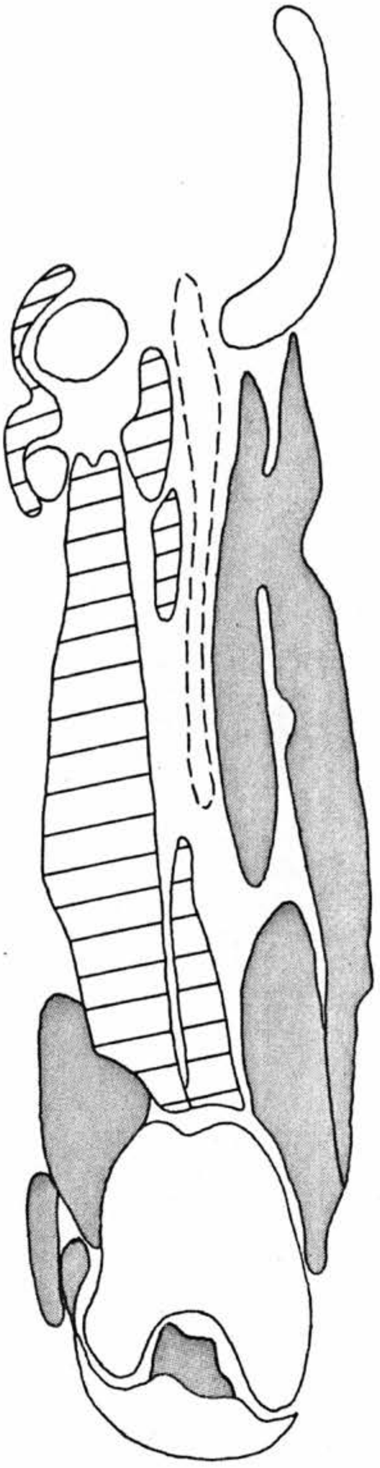
Key.

■ Dorsal muscle
▨ Ventral muscle
O Skeleton
◌ Nerve.

Proximal.

Ventral





Dorsal

Distal

Figure 2.4

Figure 2.5

1mm

The volume of muscle can be calculated from the weight of photocopy paper as follows:

$1mm^2$ muscle is equivalent to χg (14 x 14mm) of photocopy paper.

Therfore the area of muscle represented by the photocopy

$$\text{paper} = \frac{\text{total weight of paper}}{\chi}\ mm^3$$

volume = area x length

then

Muscle volume in mm^3

$$\overset{*}{=} \frac{\text{total wt. of paper x section thickness x sampling frequency}}{\chi \times 1000}$$

The value of χ may be calculated from the weight of the photocopy paper square of known dimensions.

* Wt of paper in g; section thickness in μm.

2.7.1. Errors of the method

The accuracy of the method in estimating the muscle volume depends on several factors. These include:

(1) The accuracy with which drawings are made and muscle areas cut out. Every effort was made to reduce these systematic errors to a minimum. Their magnitude is unlikely to be more than 1%. However an important factor in estimating very low muscle volumes such as occur after radical amputation may be a failure to include scattered muscle fibres in the drawings, because they are not recognised as such. This may then lead to an underestimation of very low muscle volumes.

(2) Constancy of paper thickness. This error was kept to a minimum by using photocopy paper from the same batch for each embryo and by calculating χ from a photocopy square of known dimensions for

each embryo. Its magnitude is again probably of the order of 1%.

(3) The accuracy of section thickness. The section thickness as measured by the knob setting of the mircrotome was checked in the following manner (Konigsmark 1970). The section was placed under a microscope at high power magnification (x 1500) The fine focus of the microscope was then adjusted so that an object on the bottom surface of the section was in focus. The micrometer reading on the microscope was noted. The focus was then adjusted so that an object on the upper surface of the section was in focus and the micrometer reading noted. From the difference in micrometer readings an estimate of section thickness was obtained. The section thickness of embryos sectioned at a knob setting of 8μm was 7.90μm ± 0.55 (SD) (n=40) and of embryos sectioned at 5μm knob setting, 4.92μm ± 0.67 (SD) (n=49). These values are in good agreement with the knob setting estimations of section thickness. Errors in section thickness are important in estimations of absolute muscle volumes and this affects comparisons between animals but not comparisons between different sides of the same embryo.

2.8 Determination of Motoneurone Positon within the Lateral Motor Column

2.8.1 Rostro-Caudal Position

Motoneurone distribution in the rostro-caudal axis of the lateral motor column was determined using rostro-caudal 'tenths'. The length of the lateral motor column was divided into tenths and the total number of motoneurones in each tenth was calculated.

One of the most commonly used methods to gauge motoneurone distribution in the rostro-caudal axis of the lateral motor column is to divide the lateral motor column into segments. The boundary between segments has been arbitarily set by Hamburger (1958) as the section midway between that containing the last axons of one ventral root and that containing the first axons of the next. Landmesser (1978a) uses the section midway between the dorsal root ganglia to mark the segment boundaries.

However the use of segments to divide up the lateral motor column and compare the rostro-caudal distributions of motoneurones between animals suffers from the disadvantage of a large scatter in motoneurone number in any given segment due to differences between pre and post fixed embryos. For instance Laing (1979), expressing the number of motoneurones per segment as a percentage of the total number of motoneurones, found motoneurone number in segment 23 varird from 2.3 to 14.4%. In addition sometimes the lateral motor column started in segment 22, sometimes segment 23. For these reasons the method of using rostro-caudal tenths was adopted. The division into tenths, and not any other fraction was chosen for convenience. The use of tenths is not directly comparable to segments since there are normally eight segments.

Calculation of the number of motoneurones per rostro-caudal tenth is a fairly complicated procedure. This is because of the way in which motoneurones in the lateral motor column were counted. Initially the rough extent of the lateral motor column was determined under low power and the sections were numbered from a point rostral to where the low power scan had indicated the start of the lateral motor column to be. Counting was then started in the middle of the lateral motor column on a section a multiple of 10 from the first numbered section. Counts were made every 10th section to the rostral and caudal ends of the lateral motor column. The lateral motor column thus ended somewhere between the last section counted and the 10th from it which contained no motoneurones - on average this was 5 sections away. This was justified because it was sometimes difficult to be absolutely certain, to one section, where the lateral motor column ended, although it was possible to be certain over a small number of sections.

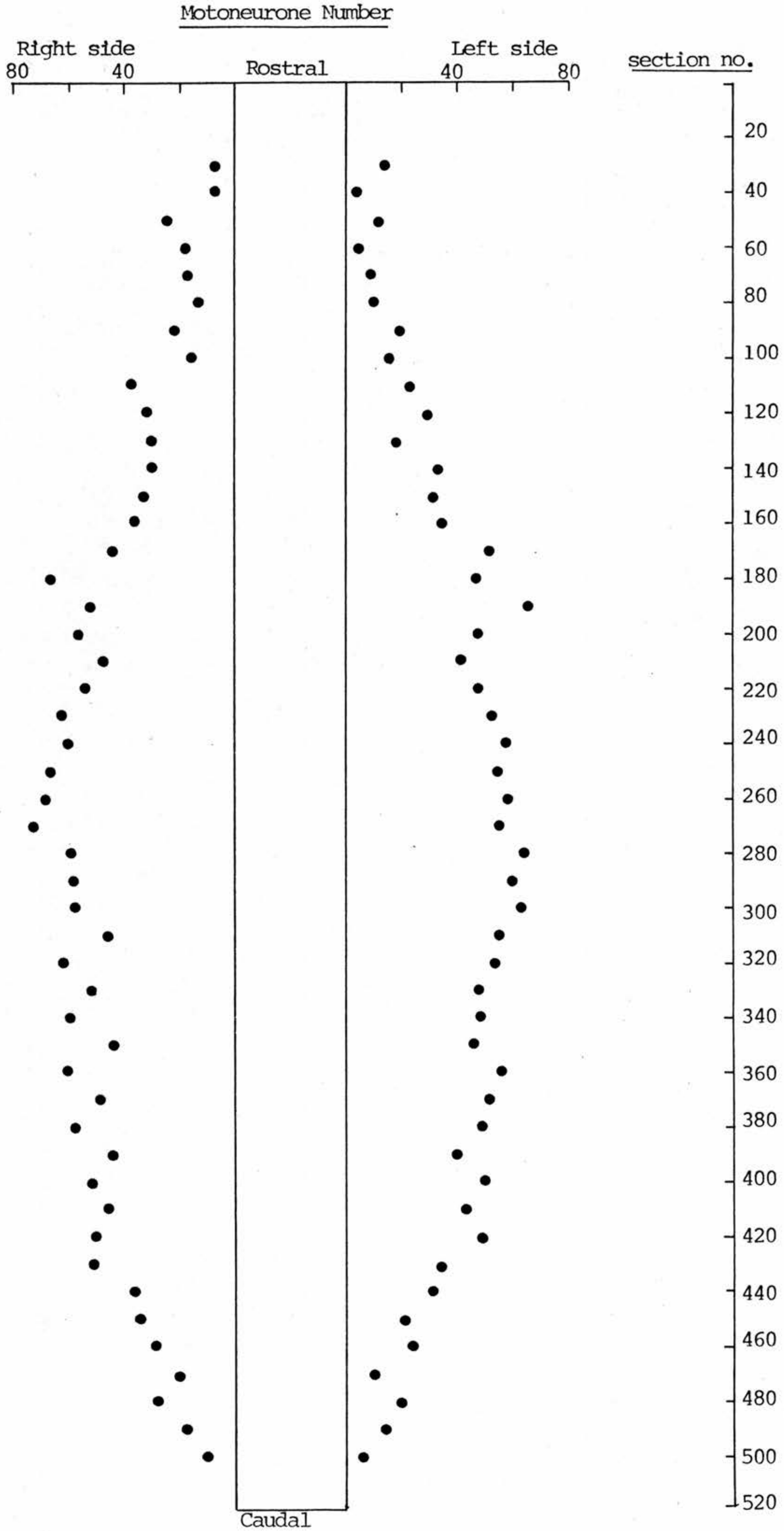
Fig (2.6) shows the rostro-caudal distribution of motoneurones for every 10th section of the lateral motor column of a control embryo, sectioned at 8μm thickness. Each counted section represents an estimate of the average number of motoneurones in the 5 preceeding sections and the 5 following sections. The most rostral counted section to contain motoneurones, section 30 therefore represents sections 25-35. The total number of sections counted in all was 480, and a tenth of the rostro-caudal length of this embryo is 48 sections. The first tenth, or 48 sections, contains the first 4 motoneurone counts plus 0.8 of the 5th count, the next tenth contains 0.2 of the 5th motoneurone count, the next 4 motoneurone counts plus 0.6 of the 10th motoneurone count, and so on until the last section is counted.

2.8.2 Medio-Lateral Position

Motoneurone position in the medio-lateral axis of the lateral motor column was determined by motoneurone 'birthdate'. Lateral motor column motoneurones are formed between stages 17-23 of development and are laid down in the lateral motor columns in rostro-caudal as well as medio-lateral temporal sequence. At any given rostro-caudal level the 'first-born' motoneurones settle medially within the lateral motor column, 'later-born' motoneurones migrate through these 'first-born' motoneurones to be laid down on their lateral border, and so on until the motor columns are formed (Hollyday and Hamburger 1977). The 'birthdate' of motoneurones can thus be used to determine medio-lateral position within the

Figure 2.6

The rostro-caudal distribution of motoneurones for every 10th section of the lateral motor column of a control embryo sectioned at 8μm thickness. Each counted section represents an estimate of the average number of motoneurones in the 5 preceeding sections and the 5 following sections. Embryo on day 10 Of incubation.


Motoneurone Number
Right side
Left side
80
40
Rostral
40
80
section no.
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
400
420
440
460
480
500
520
Caudal

lateral motor column.

The technique of cumulative labelling autoradiography is used in the chick to determine motoneurone 'birthdate'. This is because [3H] - thymidine which is injected to 'label' dividing cells, remains available throughout the duration of the experiment (Fujita 1964). [3H] - thymidine serves almost exclusively as a precursor for deoxyribonucleic acid (DNA) and is incorporated into cells synthesizing DNA prior to mitosis (Reichard & Estborn 1951). The subsequent behaviour of cells that have incorporated [3H] - thymidine may be traced using the standard techniques of autoradiography (Sidman 1970).

Embryos received an injection of [3H] - thymidine at stage 19 of development. All motoneurone precursors undergoing their final divisions after this time incorporate [3H] - thymidine into their nuclei and appear labelled with silver grains on an autoradiograph. All motoneurone precursors that have undergone their final division before this time will not incorporate [3H] - thymidine and will appear 'unlabelled' on an autoradiograph. This injection schedule therefore leaves 'unlabelled' all motoneurones that have undergone their final division before stage 19 of development. These motoneurones normally occupy a medial position within the lateral motor column and innervate muscles derived from the ventral muscle mass (Hollyday 1978).

2.9 Distinguishing 'unlabelled' from 'labelled' motoneurones on an autoradiograph

During the preparation of an autoradiograph, radioactive tissue is placed in very close contact with a nuclear emulsion. In this experiment the tissue contained [3H] - thymidine which emits β - particles. The β - particles enter the nuclear emulsion and react with silver bromide crystals to form a 'latent-image'. The concept of 'latent-image' formation is based on the theory of Gurney & Mott (1938). The emitted β - particles cause the bromide ion to become bromine. The electron so released travels through the crystal until it reaches a sensitivity spot in the crystal lattice. At this site a silver ion becomes converted into an atom of silver. When a sufficient number of atoms of silver form a 'latent-image' is produced. In the presence of a developing agent, the nucleus of metallic silver catalyses the conversion of the entire crystal to metallic silver. The bromide crystals which have not been reduced to silver are dissolved out by the fixative leaving a pattern of silver grains reproducing the pattern of radioactivity falling on the emulsion. In this way the position of radioactive material in a tissue can be visualized.

In the preparation of autoradiographs two different types of control are necessary. One is to control for 'latent-image' fading, that is the conversion of silver grains back to silver bromide. This often happens in the presence of oxidising agents or high humidity. The other is to control for 'background', that is the formation of silver grains in the emulsion other than by the radioactivity in the tissues. Causes of background silver grains include white light, pressure and chemicals within the tissue - chemography.

The control used in these experiments for 'latent-image' fading was sections of 'non-radioactive' chick spinal cord that had been treated in exactly the same way as the autoradiographs, but had been exposed to white light for 5 seconds after the initial drying period. The presence of 'latent-image' fading in these slides can be detected by comparing the grain density over the tissue compared to that over the slide. If 'latent-image' fading was observed in these slides, that batch of autoradiographs was discarded.

The control for 'background' silver grains used in these experiments was sections of 'non-radioactive' chick lumbar spinal cord that had been treated in exactly the same way as the autoradiographs. The level of 'background' activity on these slides was used as the basis for identifying 'labelled' from 'unlabelled' motoneurones. The following procedure was adopted.

The number of silver grains on the control slides over each of the lateral motor column motoneurones on every 10th section was counted and separately noted. This generally involved counting between 60 to 110 motoneurones, depending on the number of control slides. The mean number and standard deviation of silver grains over the motoneurone nucleus was then calculated. If the level of background activity was high, these autoradiographs were not used.

In most instances it was easy to distinguish between 'labelled' and 'unlabelled' motoneurones. The 'labelled' motoneurones had large numbers of silver grains over their nucleii while the 'unlabelled' motoneurones had none, fig (2.7). Some motoneurones were not so easy to distinguish since they had fewer silver grains over their nucleii. In these cases the silver grains over the motoneurone were counted and compared to the background as calculated from the control slides. If the number of silver grains was greater than the mean plus two standard deviations of the mean, then the motoneurone was counted as 'labelled'. If the number of silver grains was less than the mean plus two standard deviations of the mean it was counted as 'unlabelled'.

Estimates of total and 'unlabelled' motoneurone number were

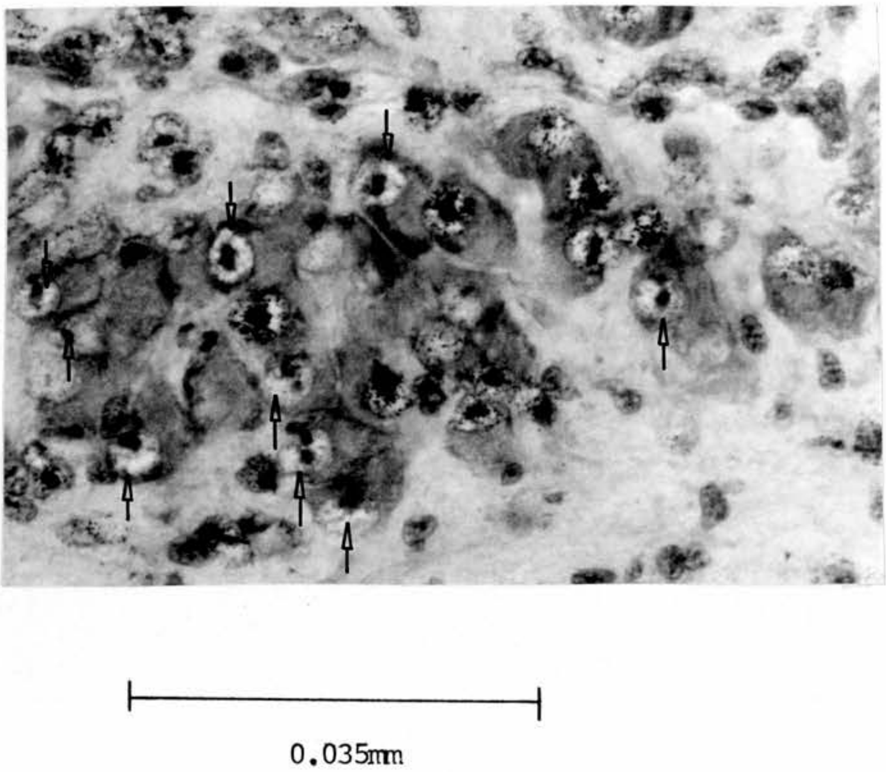


Figure 2.7.

The difference between 'unlabelled' motoneurones and 'labelled' motoneurones. Autoradiograph of a control embryo on day 10 of incubation. 10 Ci 3H-thymidine at stage 19 of development. Arrows mark the 'unlabelled' motoneurones.

obtained using the systematic sampling procedures described previously. (section 2.6).

CHAPTER 3

RESULTS

LIMB MORPHOLOGY AND MUSCLE VOLUMES RESULTS

3.1 Control Limbs

Morphology

A series of camera lucida drawings of sections of a control limb are shown in fig (3.1). Because of the orientation of the limb relative to the transverse axis of the spinal cord during sectioning, the thigh is in longitudinal section, and the shank in transverse section. Photographs of the sections from which two of these drawings were made are shown in figs (3.2) and (3.3).

Fig (3.1) shows that dorsal thigh muscle lies predominantly anterior to the femur although it is situated along the entire anterior posterior axis of the thigh. All ventral thigh muscle lies posterior to the femur. Dorsal shank muscle lies predomininatly on the anterior aspect of the shank, separated from the posteriorally situated ventral muscle by the fibula and tibia. The two muscle types meet on the dorsal and ventral borders of the shank.

Muscle Volumes

Table (3.1) shows a considerable variation in the volumes of dorsal, ventral, thigh and shank muscle in control limbs. In order to allow direct comparison between limbs, muscle volumes were expressed as a percentage of the total muscle volume. The results are shown in fig (3.4).

Reference to fig (3.4) shows that on day 10 of incubation a greater proportion of the control muscle volume is derived from the dorsal than from the ventral muscle mass - 57.6 ± 1.5% (SD) and 42.4 ± 1.5% (SD) respectively. Further the dorsal muscle mass contributes a greater proportion of muscle to the thigh than to

Figure 3.1.

Series of camera lucida drawings through the hind limb of a control embryo on day 10 of incubation.

a/ Longitudinal section through the anterior part of the thigh. Only dorsal muscle is present.

b/ Longitudinal section of the thigh through the femur. Again only dorsal thigh muscle is present.

c/ Longitudinal section of the thigh through the knee posterior to the femur. Both dorsal and ventral thigh muscle is present. A little ventral shank muscle, denoted by the arrow, appears on the anterior border of the knee.

d/ Longitudinal section of the posterior thigh and transverse section of the proximal shank. Both dorsal and ventral thigh muscle are present. Dorsal muscle is present on the anterior border of the shank. Ventral muscle is present on the posterior border.

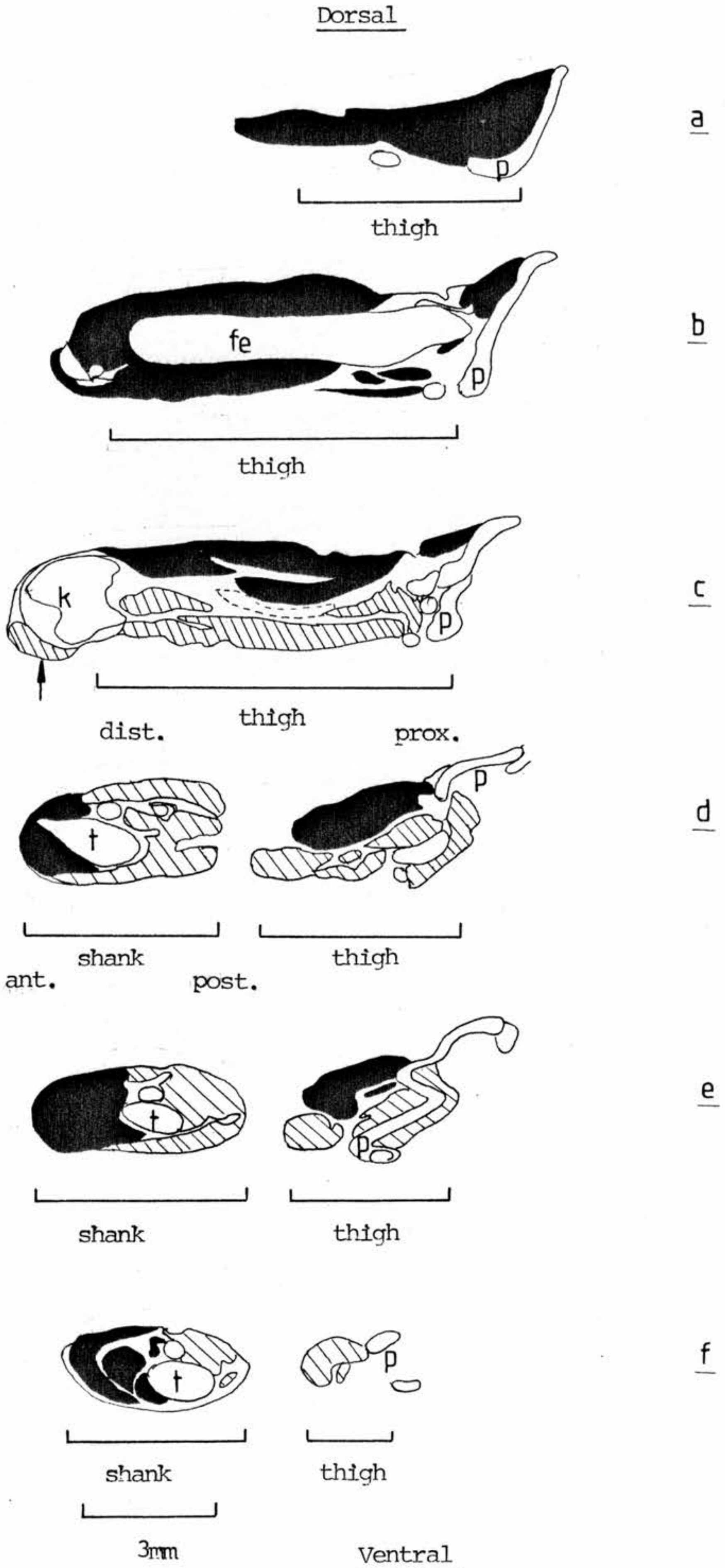
e/ Longitudinal section of the thigh and transverse section of the mid-shank. Again both dorsal and ventral thigh and shank muscle is present.

f/ Longitudinal section of the extreme posterior thigh and transverse section of the distal shank. No dorsal thigh muscle is present.

Key.

■ Dorsal muscle.
▨ Ventral muscle.
⬭ Nerve

p - pelvic girdle
fe - femur.
k - knee
t - tibia.
ant.- anterior.
post.- posterior.
prox.- proximal
dist.- distal.


Dorsal
a
p
thigh
b
fe
p
thigh
c
k
p
thigh
dist.
prox.
d
t
p
shank
thigh
ant.
post.
e
t
p
shank
thigh
f
t
p
shank
thigh
3mm
Ventral

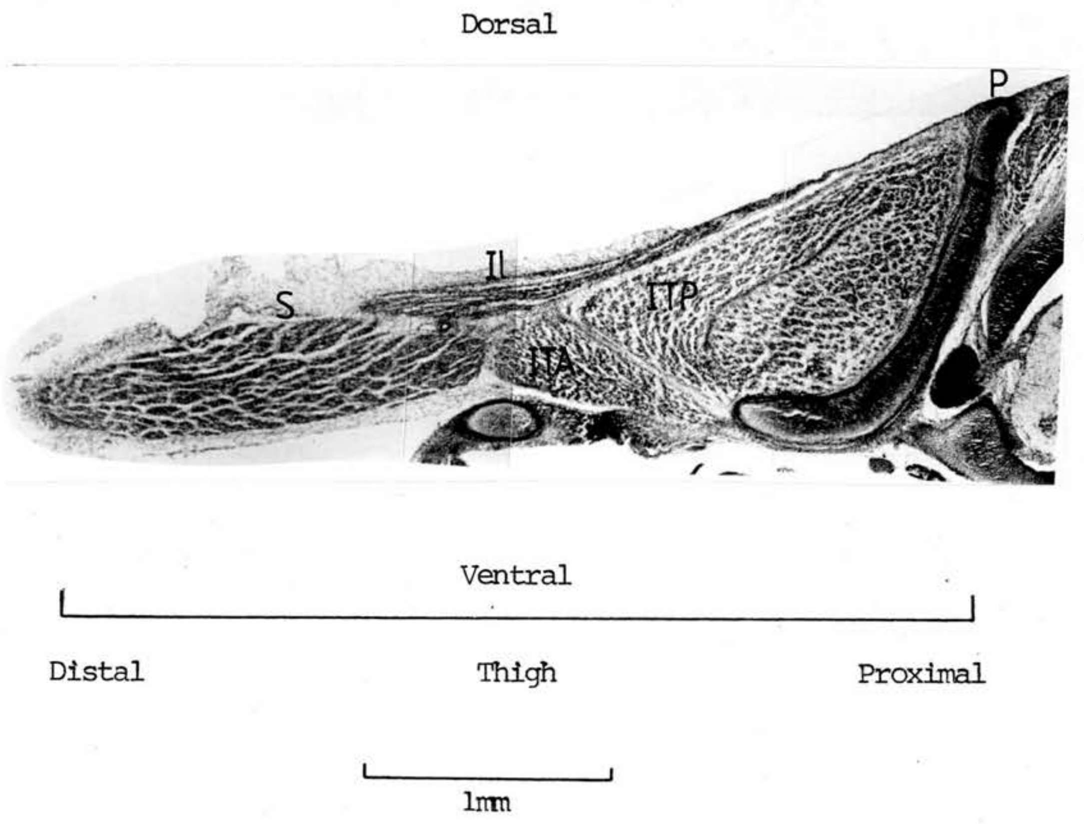


Figure 3.2.
Photograph of the control limb section from which the camera lucida drawing in figure 3.1a was made. All muscle is dorsal thigh.

Key: ITA- iliotrochantericus anterior. ITP- iliotrochantericus posterior. Il- iliotibialis. S- sartorius.

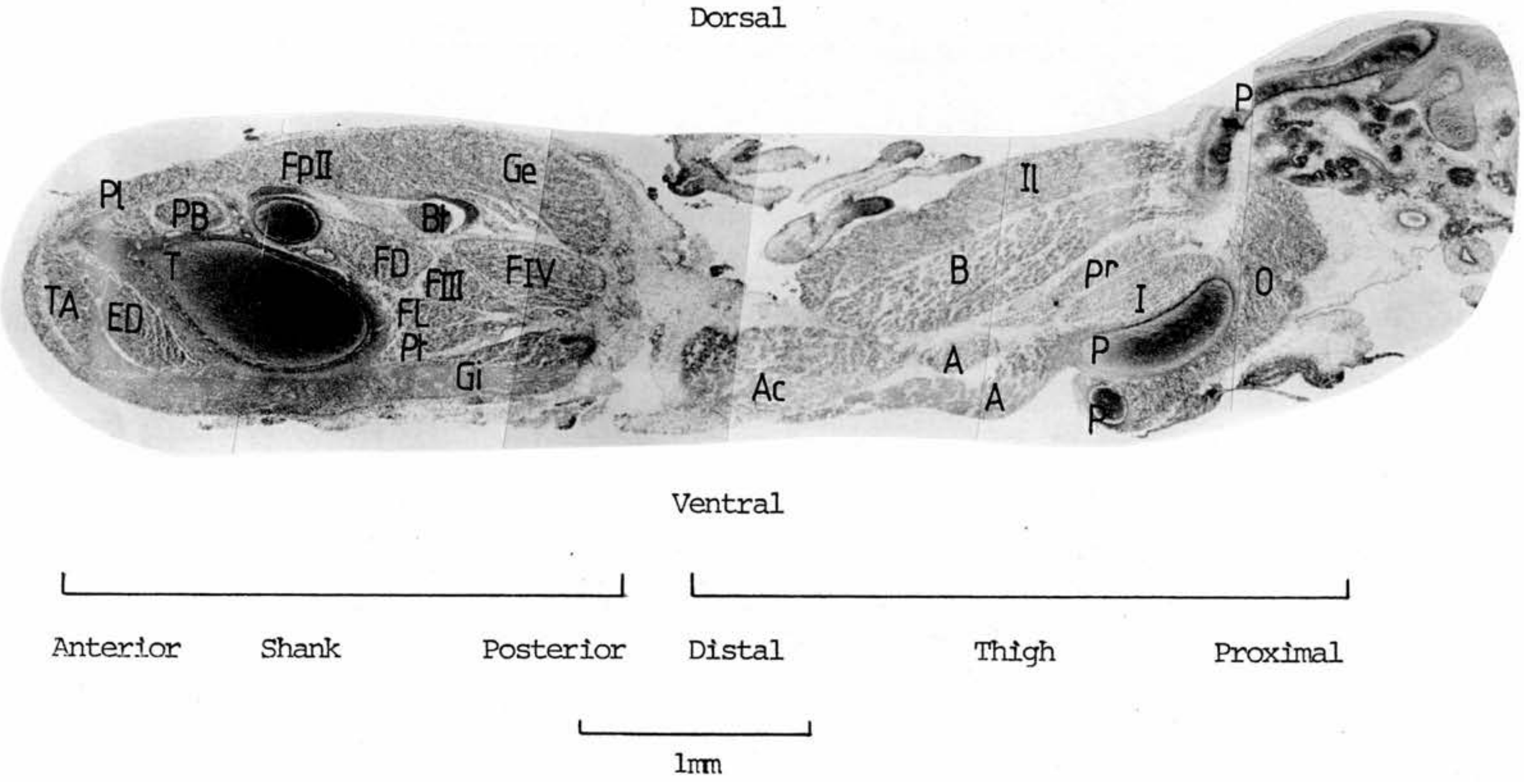


Figure 3.3

Photograph of the control limb section from which the camera lucida drawing in figure 3.1d was made. Dorsal and ventral thigh and shank muscle is present.

Key: A- adductor longus et brevis. B- biceps femoris. Bt- biceps femoris tendon. ED- extensor digitorum longus. FD- flexor digitorum longus. FL- flexus hallucis longus. FIII- flexor perforatus digiti III. FIV- flexor perforatus digiti IV. FpII- flexor perforans et perforatus digiti II. Ge- gastronemius pars externa. Gi gastronemius pars interna. Il- iliotibialis. P- pelvis. PB- peronaeus brevis. Pl peronaeus longus. Pr- piriformis. Pt- plantaris. T- tibia. TA- tibialis anterior.

Table 3.1

Muscle volumes in mm3 in control limbs of embryos at day 10 of incubation.

embryo	total	dorsal	ventral	dorsal thigh	ventral thigh	total thigh	dorsal shank	ventral shank	total shank
A278	16.3	9.4	6.9	7.7	2.8	10.5	1.8	4.0	5.8
P23	16.8	9.8	7.0	7.8	3.1	10.9	2.0	3.9	5.9
P29	17.9	10.1	6.9	8.3	2.9	11.2	1.8	4.0	5.8
P31	13.9	7.5	5.5	6.1	2.2	8.3	1.4	3.3	4.7
P34	15.4	8.7	6.7	7.0	3.0	10.0	1.8	3.7	5.5
P37	18.5	11.1	7.4	9.2	3.6	12.8	1.9	3.8	5.7
P38	17.5	10.2	7.3	8.6	3.4	12.0	1.6	3.9	5.5
P43	13.0	7.1	5.9	5.8	2.6	8.4	1.3	3.3	4.6
P49	17.5	9.8	7.7	8.2	3.6	11.8	1.6	4.1	5.7
P61	20.8	11.4	9.4	9.0	4.6	13.6	2.4	4.8	7.2
P67	17.1	10.0	7.1	8.2	3.3	11.5	1.8	3.8	5.6
P75	17.9	10.2	7.7	8.5	3.5	12.0	1.7	4.2	5.9
P165	20.7	12.0	8.7	9.7	3.8	13.5	2.3	4.9	7.2
P181	21.0	12.0	9.0	9.4	3.2	12.6	2.6	5.8	8.4
P182	18.9	11.1	7.8	9.2	3.6	12.8	1.9	4.2	6.1
P186	19.9	11.6	8.3	9.7	4.0	13.7	1.9	4.3	6.2
P189	21.2	12.6	8.6	10.0	3.8	13.8	2.6	4.8	7.4
P230	20.5	12.2	8.3	9.9	3.9	13.8	2.3	4.4	6.7
P231	25.6	14.3	11.3	11.1	5.3	16.4	3.2	6.0	9.2
P234	17.1	9.8	7.3	8.6	3.6	12.2	1.2	3.7	4.9
Mean	18.3	10.6	7.7	8.6	3.5	12.1	2.0	4.3	6.2
S.D.	2.8	1.7	1.3	1.3	0.7	1.9	0.5	0.7	1.2

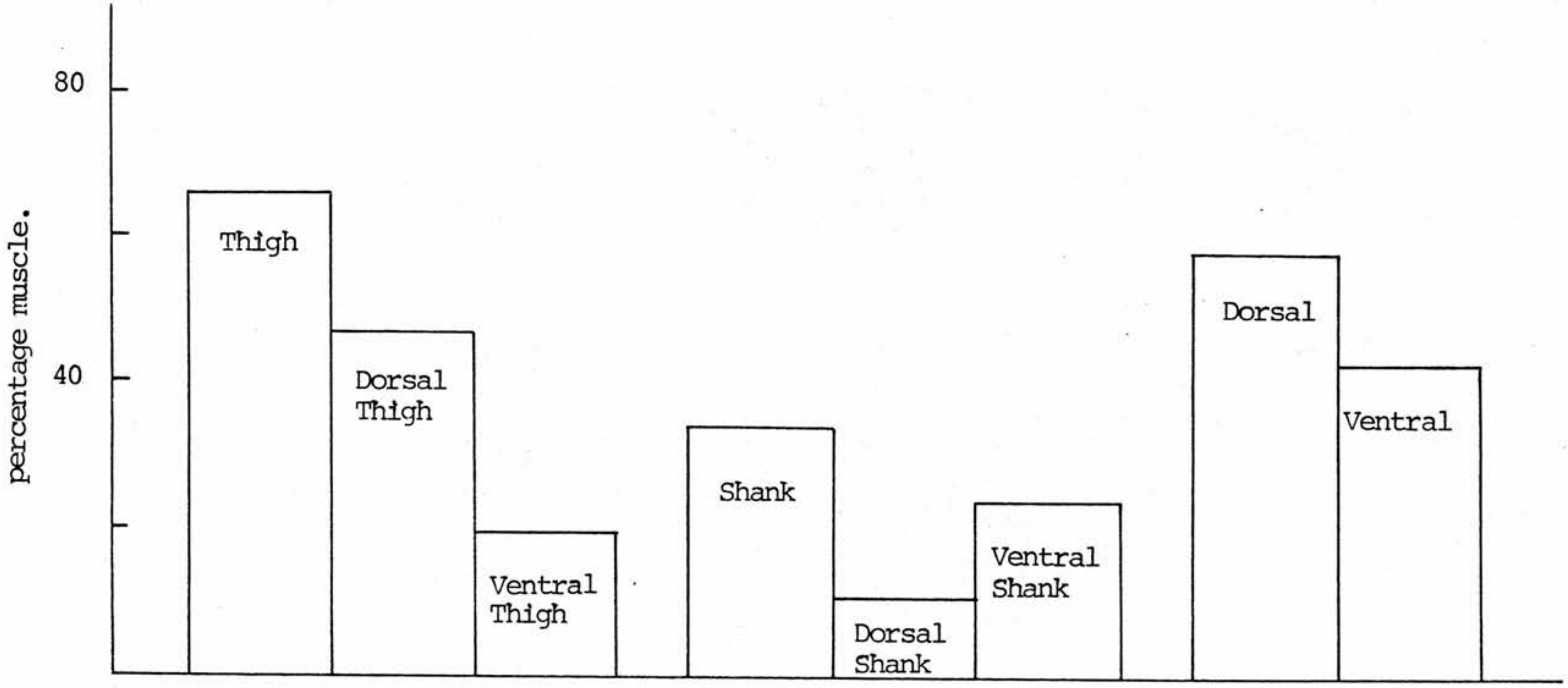


Figure 3.4.

Dorsal and ventral muscle volume in the thigh and shank, total dorsal and ventral muscle volume and total thigh and shank muscle volume in control limbs of embryos on day 10 of incubation. Muscle volumes expressed as a percentage of control limb total muscle volume. n=20. Muscle volume in mm3.

the shank - 47.1 ± 2.0% (SD) and 10.6 ± 1.3% (SD) respectively. The ventral muscle mass contributes approximately equal proportions of muscle to the thigh and shank - 19.1 ± 1.6% (SD) and 23.3 ± 1.6% (SD) respectively.

The thigh is therefore composed of about 47% dorsal muscle and 19% ventral muscle and this comprises about 66% of total muscle volume. The shank is composed of about 11% dorsal muscle and 23% ventral muscle and comprises 34% of the total muscle volume.

3.2.1. Transverse Amputations

Morphology

Embryos with varying degrees of amputation of the right hind limb bud were produced. Shown in fig (3.5) is a photograph of an embryo with amputation of almost the entire limb. A small portion of proximal thigh remains. All limb structures distal to this are missing.

Shown in fig (3.6) is a series of camera lucida drawings, of control and amputated limbs of an embryo with amputatation of the right limb at the knee. Photographs of the sections from which the drawings in fig (3.6 c) were made are shown in figs (3.7) and (3.8). The morphology of the thigh in the amputated limb is similar to that of the control limb. Almost the entire shank is missing

Muscle Volumes

Partial amputation of the shank on day 4 of incubation has no effect on the volume of thigh muscle remaining on day 10 of incubation. The volumes of thigh and shank muscles in control and amputated limbs, are shown in table (3.2), and expressed as a percentage of control muscle volume in Fig (3.9). From fig (3.9) it can be seen that removal of distal muscular, as well as skeletal

1.5cm.

Figure 3.5.

Photograph of an embryo on day 10 of incubation following transverse amputation of the right hind limb bud on day 4 of incubation. Almost the entire limb is missing.

Figure 3.6.

Series of camera lucida drawings of the control and amputated limbs of an embryo on day 10 of incubation following transverse amputation of the right limb on day 4 of incubation. Almost the entire shank is missing.

a/ Longitudinal section through the anterior thigh. Dorsal muscle is present in both limbs.

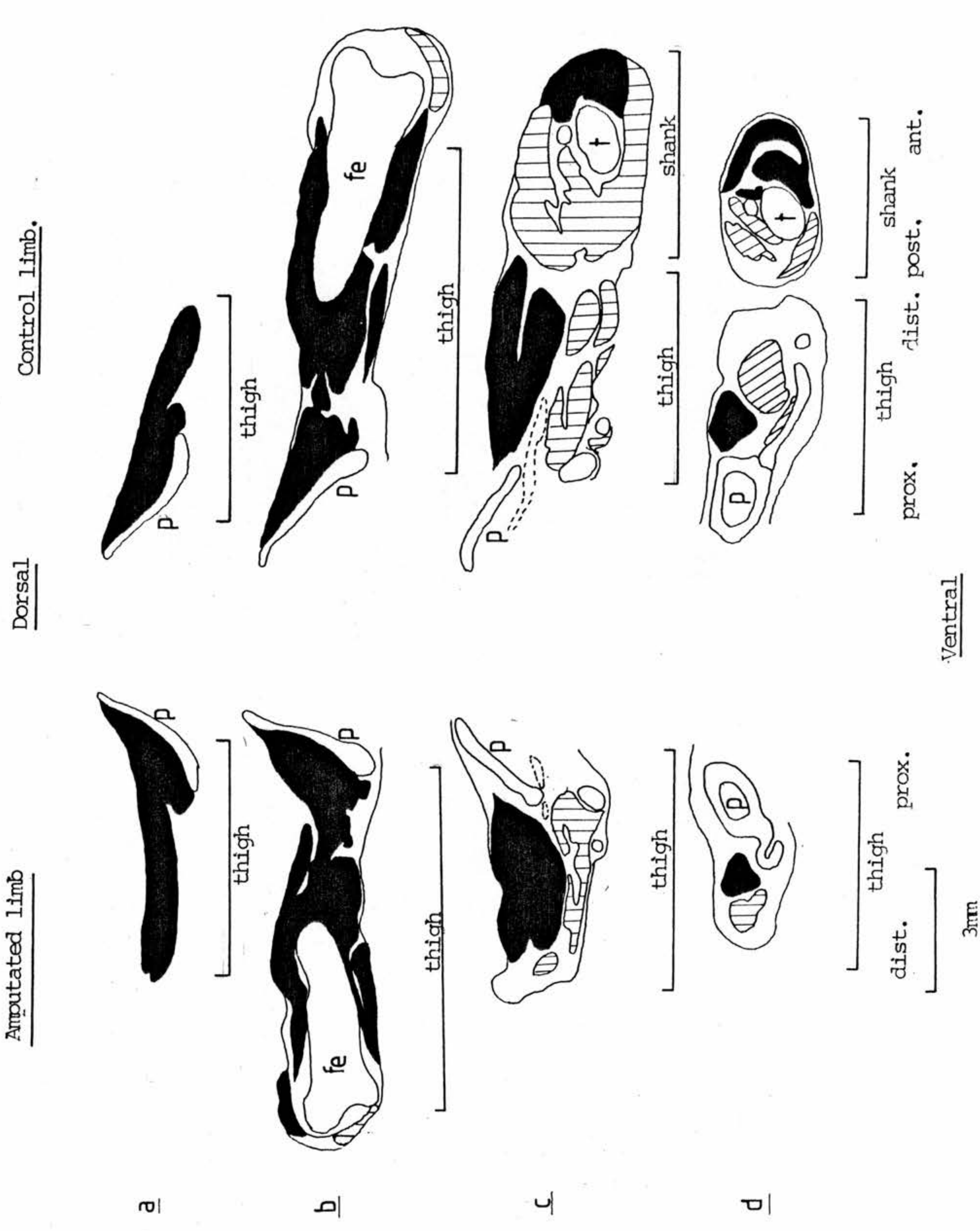
b/ Longitudinal section of the thigh through the femur. All thigh muscle is dorsal. A little ventral shank muscle is on the anterior border of the knee in both limbs.

c/ Longitudinal section of the posterior thigh and transverse section of the proximal shank. The shank is missing on the amputated side.

d/ Longitudinal section of the extreme posterior thigh and transverse section of the distal shank. Again the shank is missing on the amputated side.

Key

■	Dorsal muscle	p- pelvic girdle	t- tibia.	prox.- proximal
▨	Ventral muscle	fe- femur	ant.- anterior	dist.- distal.
◌	Nerve	k- knee	post.- posterior	


Amputated limb
Dorsal
Control limb.
Ventral
a
b
c
d
p
thigh
fe
shank
t
prox.
dist.
post.
ant.
3mm

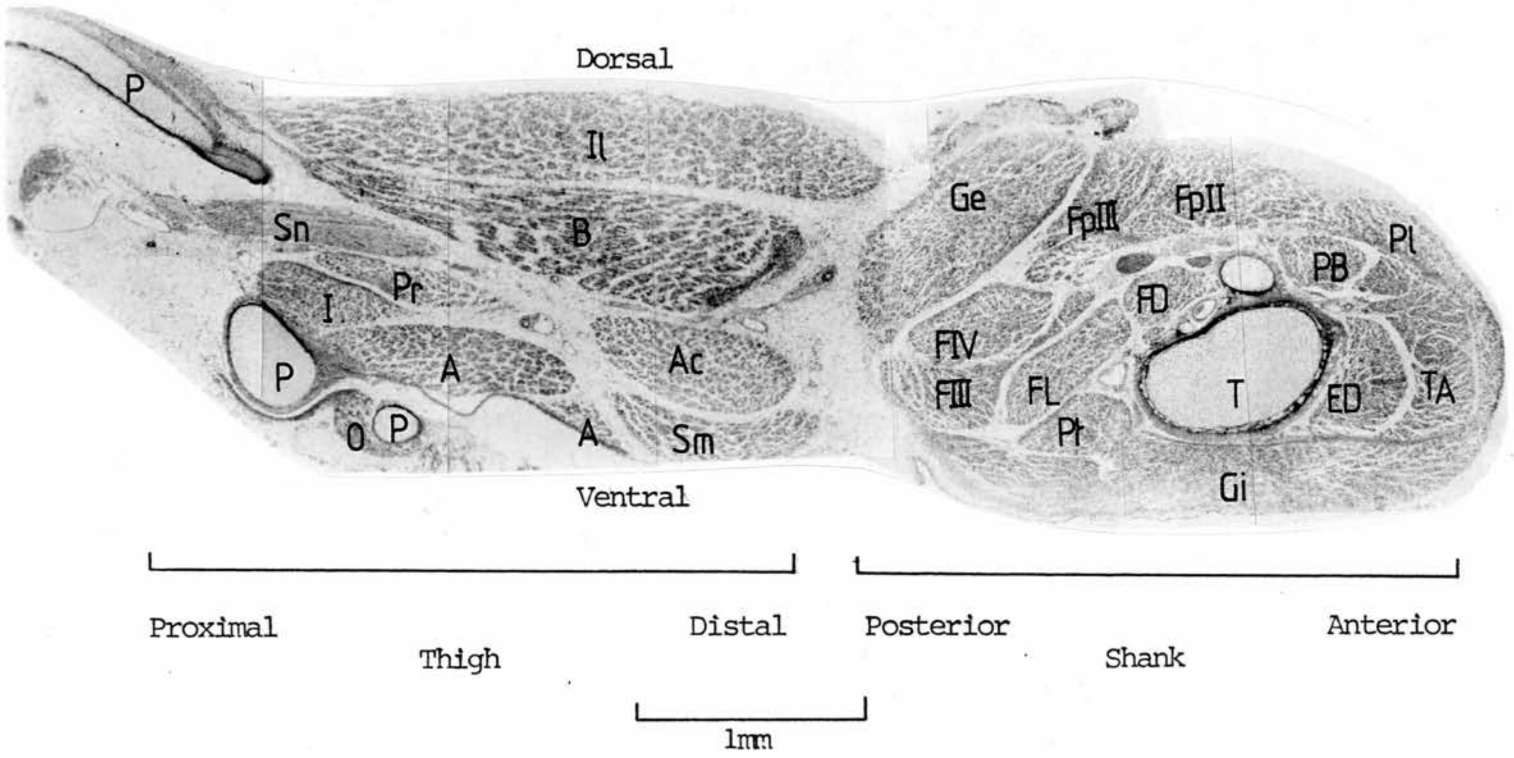


Figure 3.7
Photograph of the control limb section from which the camera lucida drawing in figure 3.6c was made.

Key: A- adductor longus et brevis. Ac- acessory. B- biceps femoris. ED- extensor digitorum longus. FD- flexor digitorum longus. FL- flexor hallcis longus. FIII- flexor perforatus digiti III. FIV- flexor perforatus digiti IV. Fp II- flexor perforans et perforatus digiti II. Fp III- flexor perforans et perforatus digiti III. Ge- gastronemius pars externa. Gi- gastronemius pars interna. Il- iliotibialis. P- pelvis. PB- peronaeus brevis. Pl- peronaeus longus. Pr- piriformis. Pt- plantaris. Sm- semimembranosus. Sn- sciatic nerve. St- semitendinosus. T- tibia. TA- tibialis anterior.

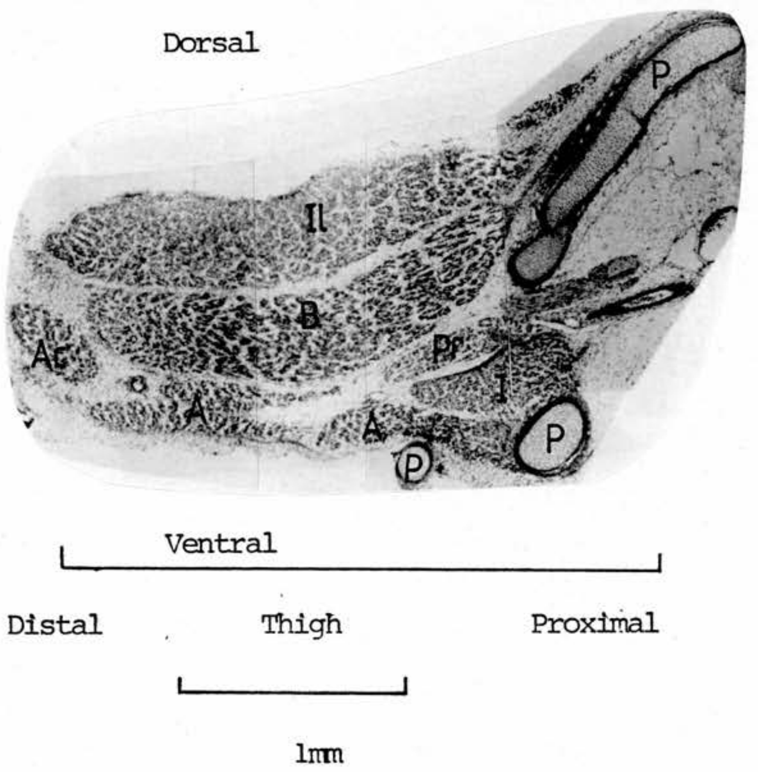


Figure 3.8. Photograph of the amputated limb section from which the camera lucida drawing in figure 3.6c was made. Only thigh muscle remains. Shank muscle is absent.

Key: see fig. 3.7.

Embryo	Control Muscle Volume mm^3	Thigh Muscle Volume mm^3		Shank Muscle Volume mm^3	
		Control	Amputated	Control	Amputated
P181	21.0	12.0	13.44	8.4	3.19
P230	20.6	13.9	13.9	6.7	3.8
P231	25.7	16.4	14.7	9.2	6.5
P232	18.2	12.1	10.6	6.1	5.1
P234	17.3	12.3	11.4	5.0	7.1

Table (3.2)

Volumes of thigh and shank muscle in control and amputated limbs. Embryos at day 10 of incubation. Transverse amputation of the right hind limb on day 4.

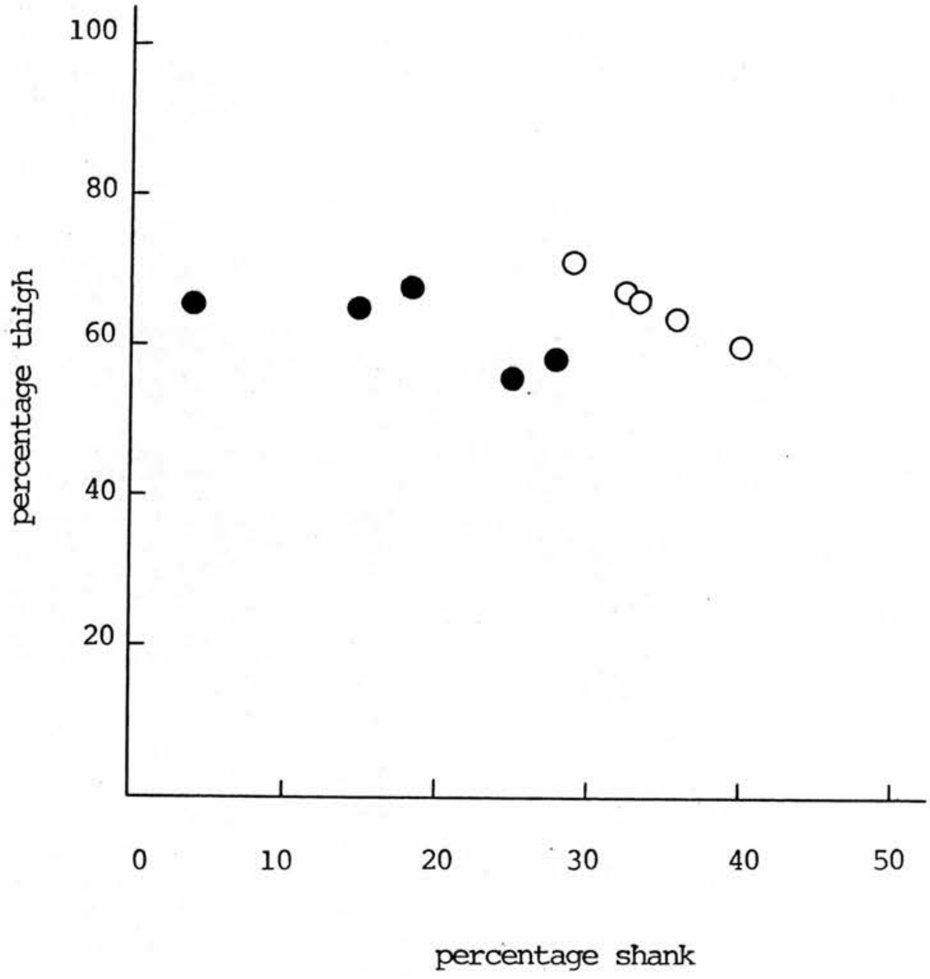


Figure 3.9

The effect of amputation of the shank on the volume of thigh muscle. Shank and thigh muscle volumes expressed as a percentage of the control limb total muscle volume. Open circles the percentage of thigh and shank muscle in control limbs; filled circles the percentage of shank and thigh muscle in amputated limbs. Transverse amputation on day 4 of incubation. Embryos fixed on day 10 of incubation. Each point represents one embryo. Muscle volume in mm3.

elements following partial amputation of the shank does not affect the percentage volume of thigh muscle on day 10 of incubation.

3.2.2. Ventral Amputations

Morphology

Shown in fig (3.10) is a photograph of an embryo on day 10 of incubation with almost total absence of ventral muscle. The skeleton of the entire limb is intact, although the limb is shorter than the contra-lateral control limb.

A series of camera lucida drawings of the control and amputated limbs of such an embryo are shown in fig (3.11). Photographs of the sections from which the drawings in fig (3.11c) were made are shown in figs (3.12) and(3.13). The morphology of the amputated and control limbs are similar in sections through, or anterior to, the femur, fig (3.11 a, b). Sections of limb posterior to the femur show that very little ventral thigh muscle remains. A little ventral shank muscle is present, fig (3.11 c, d)

Muscle Volumes

Removal of the ventral muscle mass on day 4 of incubation does not significantly affect the volume of dorsal muscle remaining on day 10 of incubation. The volumes of dorsal and ventral muscle in control and amputated limbs, are shown in table (3.3) and expressed as a percentage of control muscle volume in fig (3.14). The arrow in fig (3.14) marks the mean relationship between the percentage of dorsal and ventral muscle in control limbs (see fig (3.4).

The data shown in fig (3.14) suggest that removal of a large proportion of ventral muscle is accompanied by a reduction in

1.5cm.

Figure 3.10.

Photograph of an embryo on day 10 of incubation following ventral amputation of the right hind limb bud on day 4 of incubation. The amputated limb is somewhat shorter than the contralateral control limb. The skeletal system is intact. The embryo has been partly eviscerated.

Figure 3.11.

A series of camera lucida drawings through the control and amputated limbs of an embryo on day 10 of incubation following ventral amputation of the right limb bud on day 4 of incubation.

a/ Longitudinal section through the anterior thigh. Dorsal thigh muscle is present in both limbs.

b/ Longitudinal section of the thigh through the femur. Dorsal thigh muscle is present.

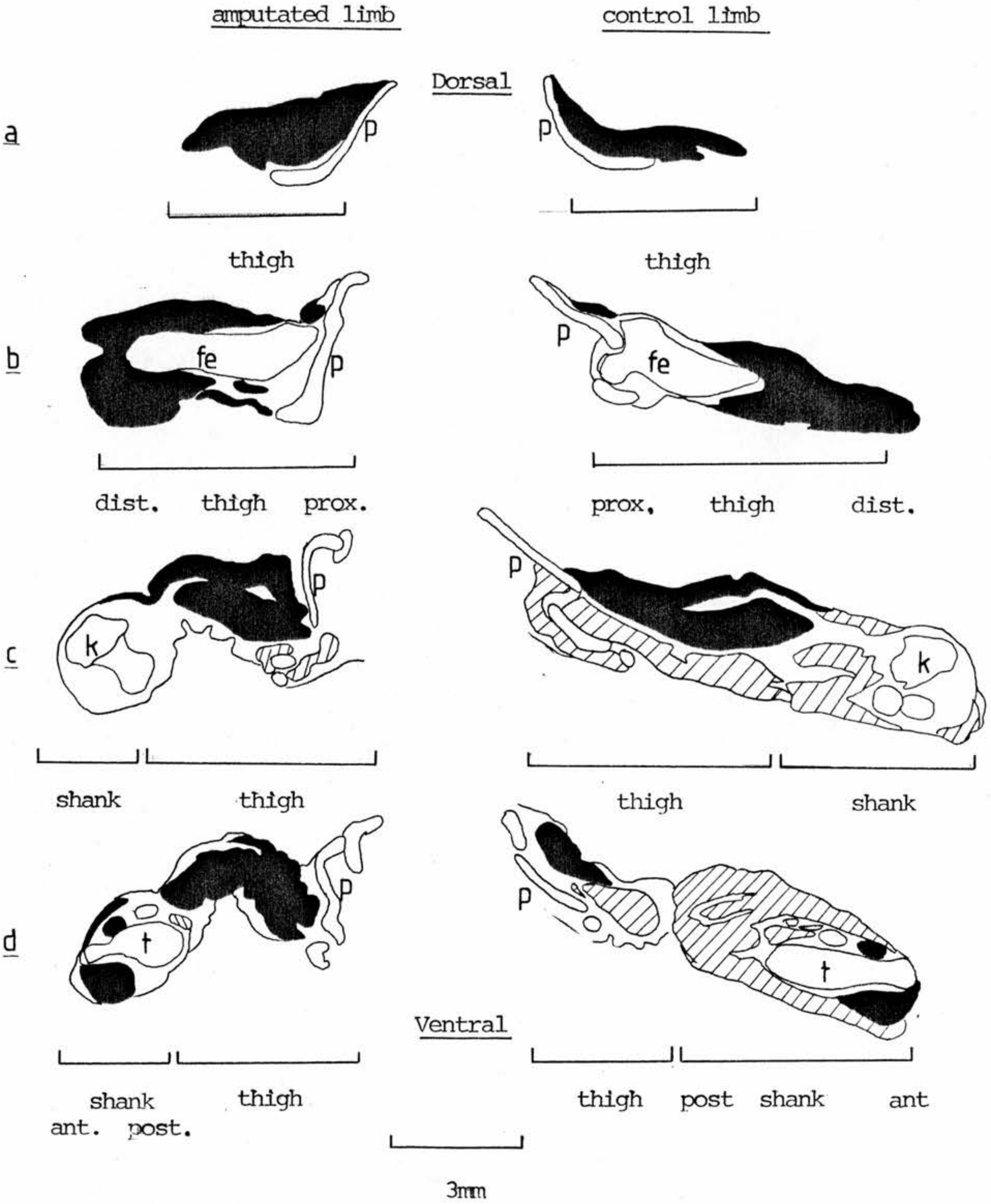
c/ Longitudinal section of mid-thigh and transverse section of the extreme proximal shank. Little ventral thigh muscle remains in the amputated limb compared to the control limb. No ventral shank muscle is present in the amputated limb.

d/ Longitudinal section through posterior thigh and transverse section of the mid-shank. A very little ventral shank muscle is present in the amputated limb. There is no ventral thigh muscle.

Key

■ Dorsal muscle
▨ Ventral muscle
◌ Nerve

p- pelvic girdle
fe- femur
k- knee
t- tibia.
ant.- anterior.
post.- posterior.
prox.- proximal.
dist.- distal.


amputated limb
control limb
Dorsal
a
p
p
thigh
thigh
b
fe
p
p
fe
dist. thigh prox.
prox, thigh dist.
c
k
p
p
k
shank thigh
thigh shank
d
t
p
p
t
Ventral
shank thigh
ant. post.
thigh post shank ant
3mm

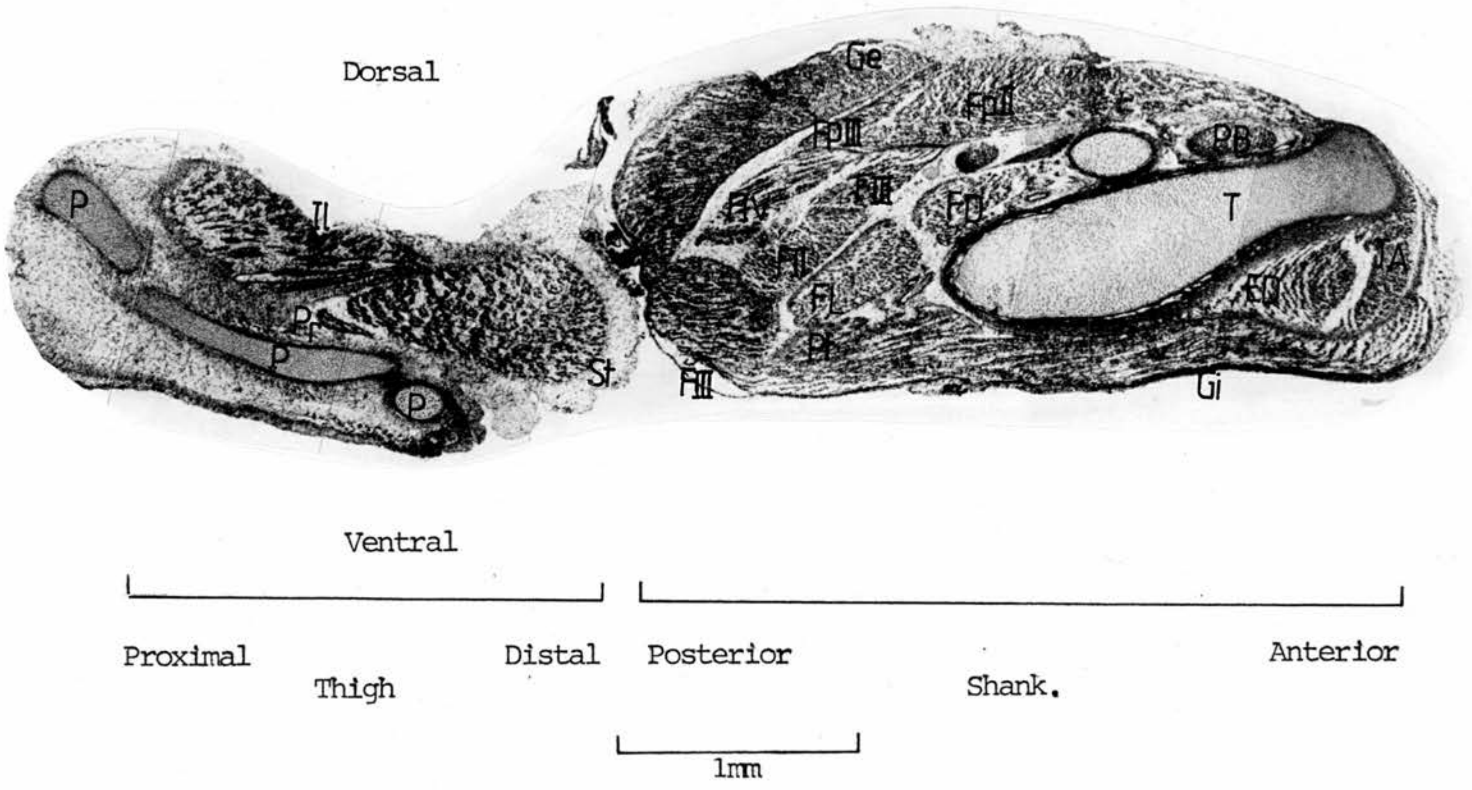


Figure 3.12.
Photograph of the control limb section from which the camera lucida drawing in figure 3.11d was made.

Key: B- biceps femoris. ED- extensor digitorum longus. FD- flexor digitorum longus. FL- flexor hallucis longus. FII- flexor perforatus digiti II. FIII- flexor perforatus digiti III. FIV- flexor perforatus digiti IV. FpII- flexor perforans et perforatus digiti II. FpIII- flexor perforans et perforatus III. Ge- gastronemius pars externa. Gi- gastronemius pars interna. Il- iliotibialis. P- pelvis. PB- peroaeus brevis. Pl- peronaeus longus. Pr- piriformis. Pt- plantaris. St- semitendinosus. T- tibia. TA- tibialis anterior.

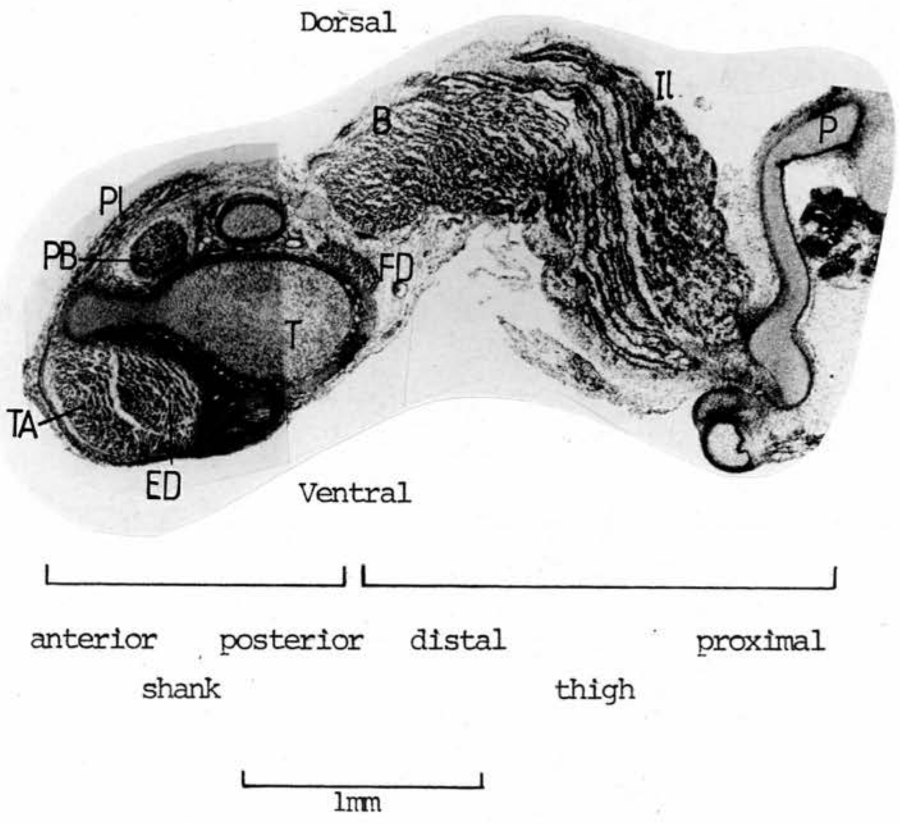


Figure 3.13.
Photograph of the amputated limb section from which the camera lucida drawing in figure3.11d was made. Almost all ventral muscles situated on the posterior border of the shank are missing. A small remnant of flexor digitorum longus remains. There is no ventral thigh muscle.
Key: see fig. 3.12.

Embryo	Control Muscle Volume in mm3	Dorsal Muscle Volume in mm3		Ventral Muscle Volume in mm3	
		Control	Amputated	Control	Amputated
P33	19.6	11.1	10.4	8.5	6.3
P34	15.4	8.7	8.0	6.7	4.0
P38	17.5	10.2	10.2	7.3	4.8
P88	19.8	10.9	9.0	8.9	3.1
P104	14.4	8.7	7.7	5.7	0.7
P165	20.6	12.0	8.9	8.6	1.4
P176	12.1	7.4	5.7	4.7	1.6
P296	23.3	12.6	11.8	10.7	2.9
P318	12.4	7.3	6.2	5.1	0.3
T177	14.0	8.3	5.0	5.7	0.1
T178	16.6	9.5	8.6	7.1	0.4

Table 3.3

Dorsal and ventral muscle volume in control and amputated limbs of embryos at day 10 of incubation. Amputation of the ventral muscle mass of the right hind limb on day 4 of incubation.

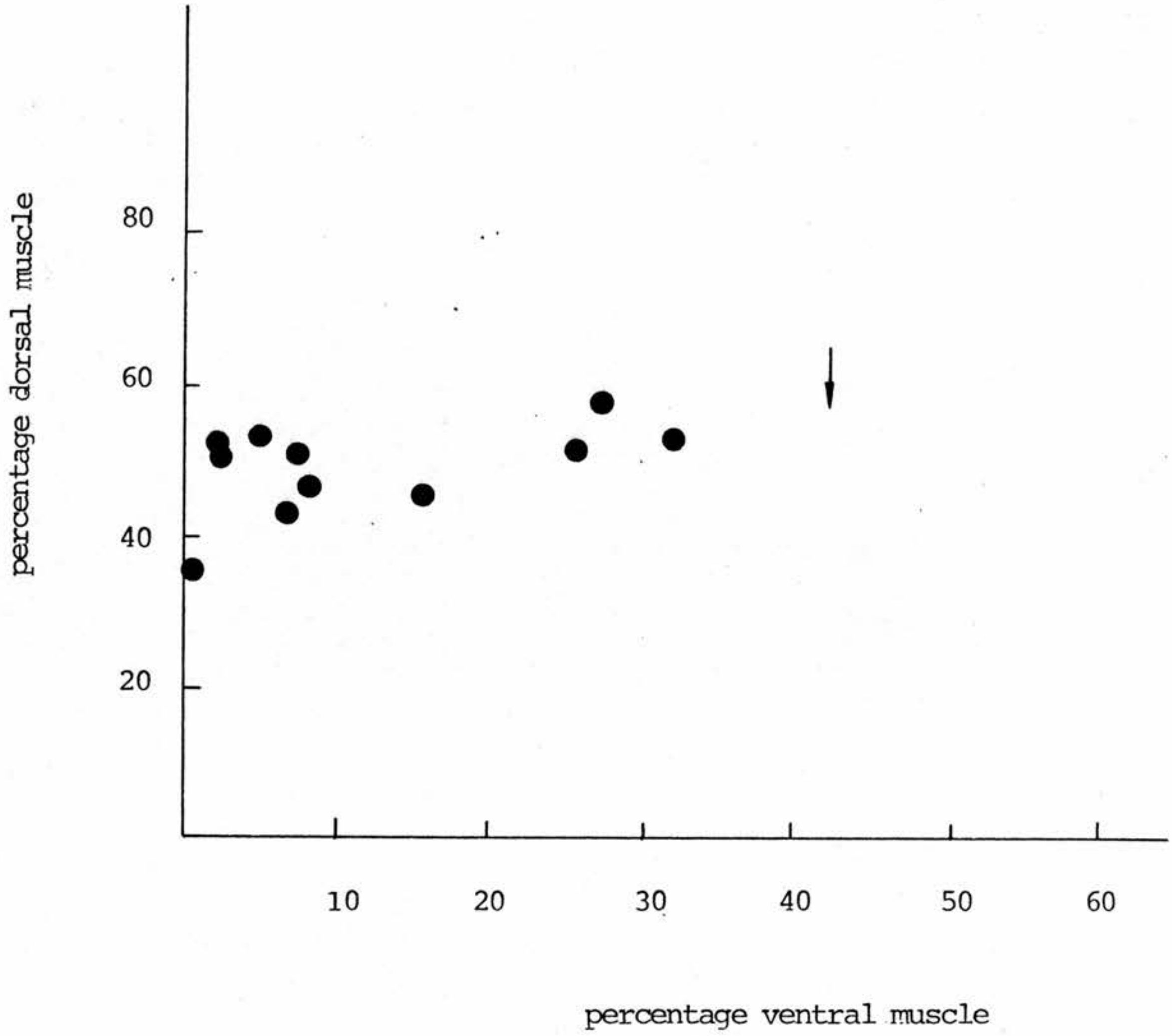


Figure 3.14

The effect of amputation of the ventral muscle mass on day 4 of incubation on the volume of dorsal muscle on day 10 of incubation. Dorsal and ventral muscle volume expressed as a percentage of the control limb total muscle volume. The arrow marks the mean relationship between dorsal and ventral muscle volumes in control limbs. Each point is one embryo. Muscle volume in mm3.

dorsal muscle volume. The apparent reduction in dorsal muscle volume is however not significant. (Regression coefficient = 0.29 ± 0.15 (SE); $p < 0.10 > 0.05$, Student's t-test)

3.2.3. Dorsal Amputations

Morphology

Fig (3.15) shows the photograph of an embryo on day 10 of incubation after removal of the dorsal muscle mass on day 4 of incubation.

As seen after ventral amputation, the skeletal system of the entire limb is intact. The amputated limb is again slightly shorter than the contra-lateral control limb.

A series of camera lucida drawings through the control and amputated limbs of an embryo with partial dorsal muscle removal are shown in fig (3.16). Photographs of the sections from which the drawings shown in fig (3.16 c) were made are shown in fig (3.17) and (3.18). In this embryo dorsal thigh muscle is much reduced in volume compared to the control side, although not entirely absent. Some remains, especially anterior to the femur, fig (3.16 a). The entire dorsal shank musculature is missing (fig 3.16 d)

Muscle Volumes

Amputation of the dorsal muscle mass on day 4 of incubation reduces the volume of ventral as well as dorsal muscle remaining in the amputated limb compared to the control limb on day 10 of incubation.

The volumes of dorsal and ventral muscle in control and amputated limbs are given in table (3.4), and expressed as a percentage of control muscle volume in fig (3.19). The arrow in fig (3.19) marks the mean percentage of dorsal and ventral muscle in control embryos fig (3.4). A reduction in the percentage of

Figure 3.15

Photograph of an embryo on day 10 of incubation following dorsal amputation of the right hind limb bud on day 4 of incubation. The amputated limb is slightly shorter than the contralateral control limb. The skeletal system is intact.

Figure 3.16

A series of camera lucida drawings through the control and amputated limbs of an embryo on day 10 of incubation following dorsal amputation of the right limb bud on day 4 of incubation.

a/ Longitudinal section of the anterior thigh. The volume of dorsal thigh muscle is much reduced on the amputated side compared to the control side.

b/ Longitudinal section of the thigh through the femur. Again dorsal thigh muscle is much reduced in the amputated limb.

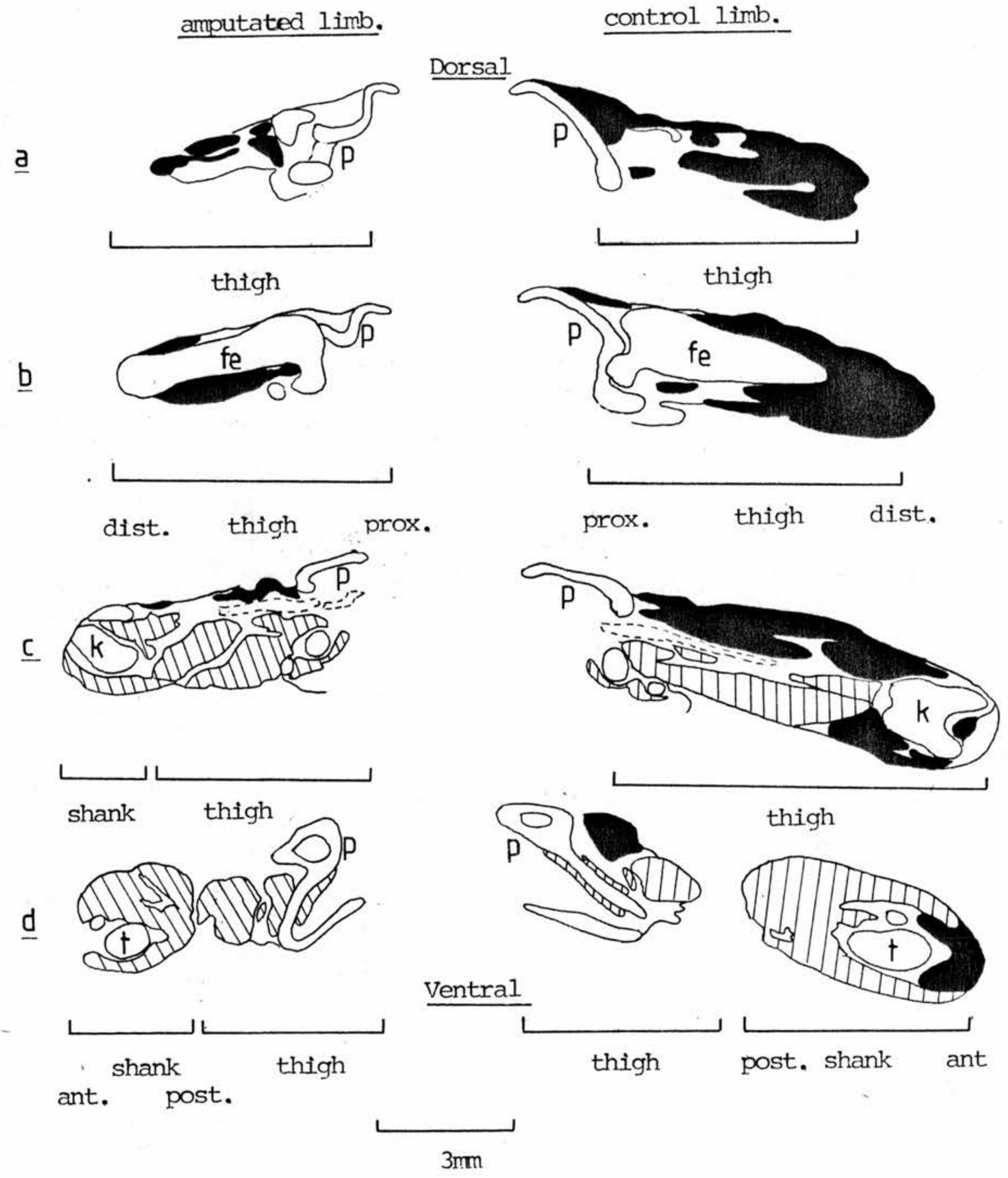
c/Longitudinal section of the thigh through the knee. Some dorsal thigh remnants are present in the amputated limb.

d/ Longitudinal section through the posterior thigh and transverse section of the mid-shank. No dorsal muscle is present in the amputat limb. Only ventral muscle remains.

Key.

■ dorsal muscle
▨ ventral muscle
◌ Nerve

p- pelvic girdle
fe- femur
k- knee
t- tibia
ant.-anterior
post -posterior
prox.-proximal
dist -distal


amputated limb.
control limb.
Dorsal
a
p
p
thigh
thigh
b
fe
p
p
fe
dist.
thigh
prox.
prox.
thigh
dist.
c
p
k
p
k
shank
thigh
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Ventral
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post. shank
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ant.
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3mm

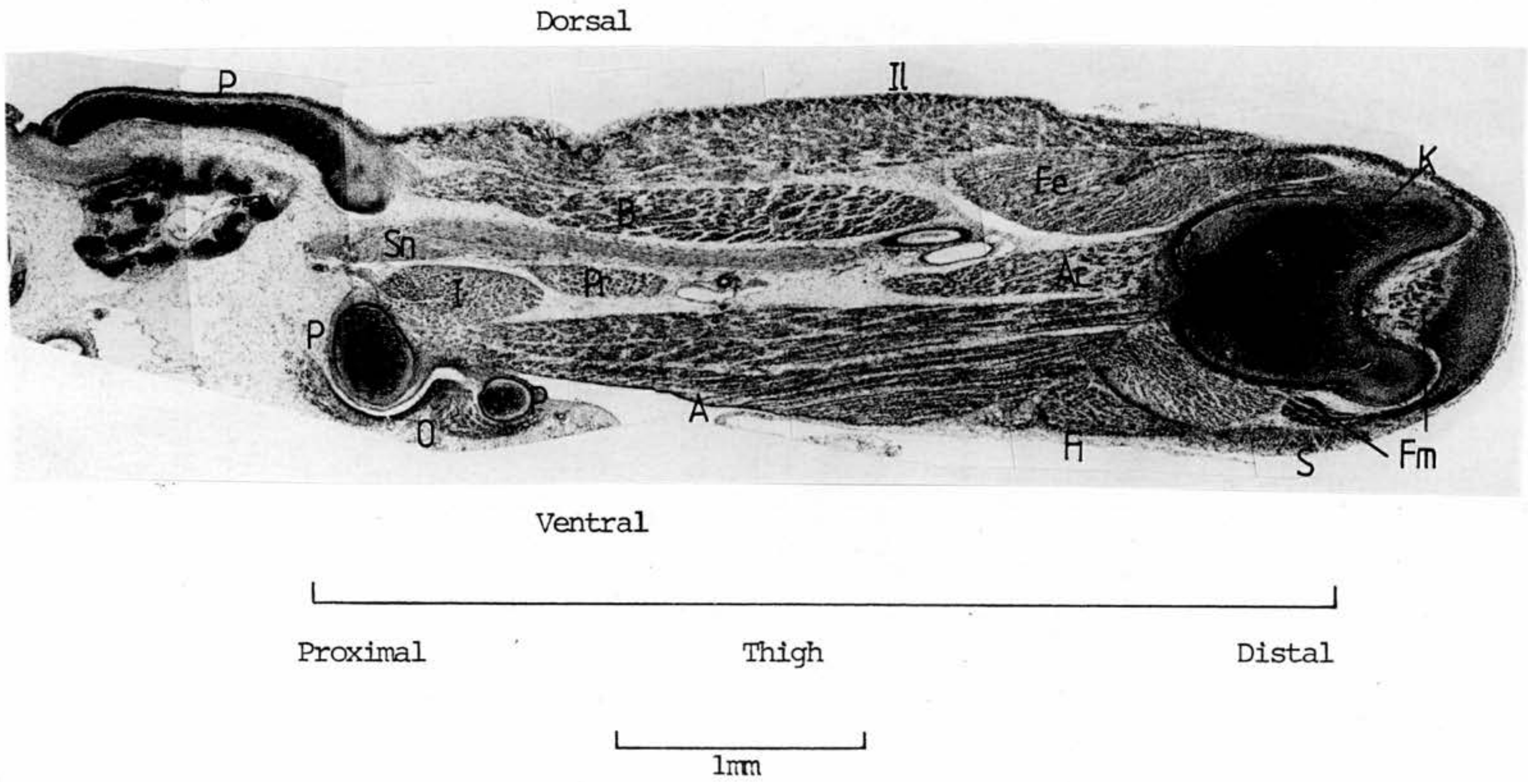


Figure 3.17. Photograph of the control limb section from which the camera lucida drawing in figure 3.16c was made.

Key: A- adductor longus et brevis. Ac- accessory. B- biceps femoris. FD- flexor digitorum longus. Fe- femorotibialis externus. Fi- femoritibialis internus. Fm- femoritibialis medius. FIV- flexor perforatus digiti IV. Ge- gastronemius pars externa. Gm- gastronemius pars media. I- ischiofemoralis. Il- iliotibialis. O- obturator internus. P- pelvic girdle. Pr- piriformis. S- sartorius. Sn- sciatic nerve.

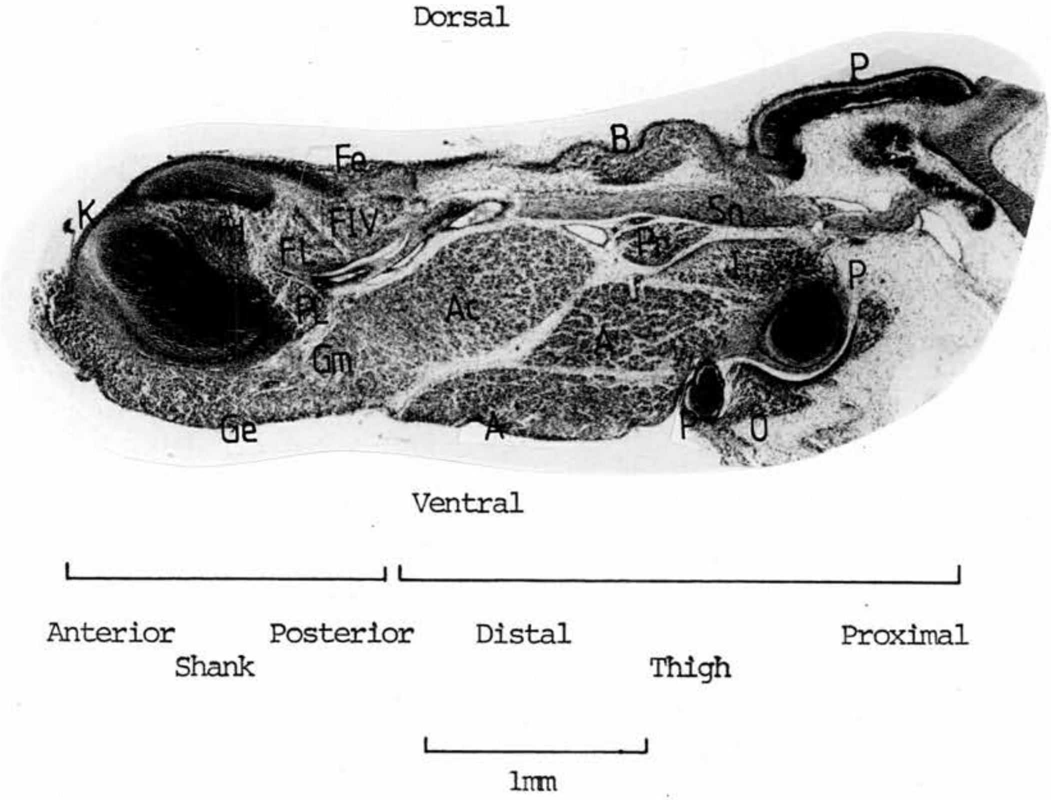


Figure 3.18.
Photograph of the amputated limb section from which the camera lucida drawing in figure 3.16c was made. A little dorsal thigh muscle remains - biceps femoris and femoritibialis externus - although much reduced compared to the control limb. Iliotibialis is absent.
Key: see fig. 3.17.

Embryo	Control Muscle Volume in mm^3	Dorsal Muscle Volume in mm^3		Ventral Muscle Volume in mm^3	
		Control	Amputated	Control	Amputated
A278	16.3	9.4	0.9	6.9	5.0
P23	16.8	9.7	5.8	7.0	6.1
P29	17.0	10.1	4.6	6.9	5.5
P37	18.5	11.1	6.0	7.4	7.5
P48	12.1	7.0	0.7	5.1	3.7
P49	17.5	9.8	1.7	7.7	6.0
P61	20.8	11.4	2.0	9.4	5.7
P67	17.1	10.0	5.8	7.1	6.4
P71	12.0	6.7	0.6	5.3	3.3
P75	17.9	10.2	5.1	7.7	6.2
T73	15.8	9.5	4.8	6.3	4.0
T147	17.9	9.9	1.6	8.0	4.4
T165	24.9	13.9	6.2	11.0	7.8
T172	16.3	9.4	4.6	6.9	6.2

Table (3.4)

Volume of dorsal and ventral muscle in control and amputated limbs. Embryo at day 10 of incubation. Amputation of the dorsal muscle mass of right hind limb on day 4 of incubation.

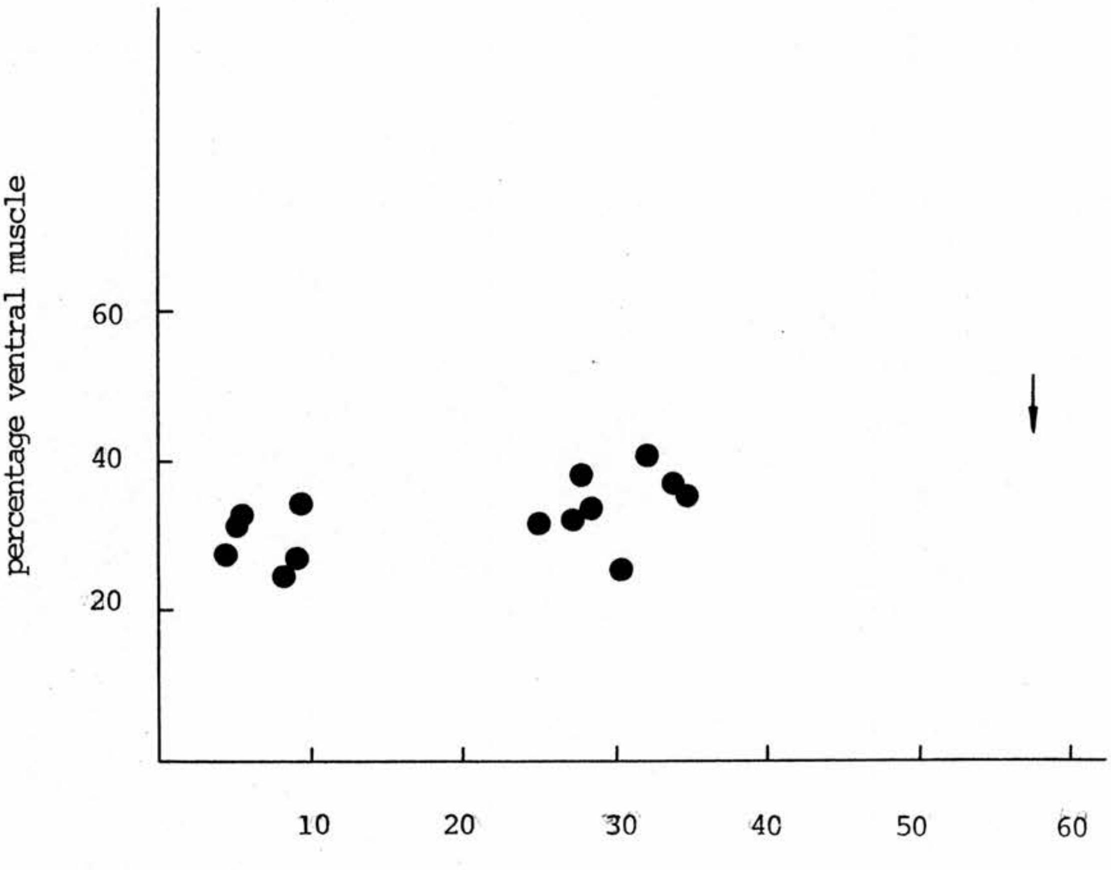


Figure 3.19

The effect of amputation of the dorsal muscle mass on day 4 of incubation on the volume of ventral muscle on day 10 of incubation. Dorsal and ventral muscle volume expressed as a percentage of the control limb total muscle volume. The arrow marks the mean relationship between dorsal and ventral muscle volumes in control limbs. Each point is one embryo. Muscle volume in mm3.

dorsal muscle in amputated limbs is accompanied by a significant reduction in the percentage of ventral muscle. (Regression coefficient = 0.25 ± 0.10 (SE); $p < 0.05 > 0.02$, Student's t-test)

3.3 Interpretation of the Results

One possible reason for the significant decrease in ventral muscle volume from control levels that is associated with dorsal amputations fig (3.19), is that both muscle masses were accidently amputated on day 4 of incubation. Two reasons make this possibility unlikely.

The first is that the two muscle masses are separated from one another by the prospective skeleton on day 4 of incubation (Hamburger 1975). Amputation of both dorsal and ventral muscle masses should therefore result in removal of the skeleton as well as the muscle masses. Since all embryos used in this study had their skeletal systems intact it is unlikely that this occurred. The second is that the reduction in ventral muscle volume also occurs following partial amputations of the dorsal muscle mass. It is difficult to see how partial amputation of the dorsal muscle mass could accidently result in amputation of the ventral muscle mass.

An alternative explanation for the decrease in ventral muscle volume is that it is due to atrophy of the remaining muscle. This atrophy could be due to reduced limb movements. Although muscles derived from the dorsal and ventral muscle masses cannot be divided into flexor or extensor in function on the basis of their embryological origin (Landmesser 1978a), removal of the dorsal muscle mass could conceivably result in an imbalance of muscles acting on any given joint. This imbalance may lead to reduced movement of the remaining muscles resulting in a failure to develop to the size they would normally do so. That this is a plausible explanation is suggested by the results of transverse amputation. Amputation of the shank does not reduce the volume of thigh muscle remaining in the amputated limb from control levels, fig (3.9). Amputation of the shank leaves intact antagonistic pairs of thigh muscles whose actions will therefore be balanced. However amputation of the ventral muscle mass does not result in a significant decrease in dorsal muscle volume - fig (3.14). This may be related to the different contributions of the dorsal and ventral muscle masses to muscle volumes in limbs on day 10 of incubation. Dorsal muscle forms about 58% of total limb volume whereas ventral muscle only 42%, fig (3.4). Removal of the smaller volume of ventral muscle may still leave intact sufficient antagonistic pairs of muscles to prevent any significant atrophy.

CHAPTER 4

RESULTS

THE EFFECT OF AMPUTATION ON THE LATERAL MOTOR COLUMNS

4.1 Morphology

The effect of all three types of amputation is to reduce the size of the ipsilateral lateral motor column compared to the contra-lateral motor column on day 10 of incubation fig (4.1).

4.2 Motoneurone Number

Motoneurone number is reduced in the ipsilateral lateral motor column on day 10 of incubation with all three types of amputation. The extent of the loss is directly related to the extent of muscle loss. Motoneurone number is unaffected in the contra-lateral lateral motor column, fig (4.2). In order to compensate for the large variation in motoneurone numbers in control sides, fig (4.3), motoneurone number on the amputated side was expressed as a percentage of that on the contra-lateral control side. Similarly muscle volume in the amputated limb was expressed as a percentage of that in the contra-lateral control limb. The data are given in tables (4.1, 4.2, 4.3) and depicted graphically in figs. (4.4, 4.5 & 4.6).

Fig (4.4) shows that on day 10 of incubation there is a linear relationship between the percentage of motoneurones in the ipsilateral lateral motor column and the percentage of muscle remaining in the amputated limb following transverse amputation. (Regression coefficient = 0.95 ± 0.06 (SE) $p < 0.001$ Student's t-test).

A linear relationship also exists between the percentage of motoneurones and the percentage of ventral muscle remaining on day 10 of incubation following ventral amputation on day 4 fig (4.5),

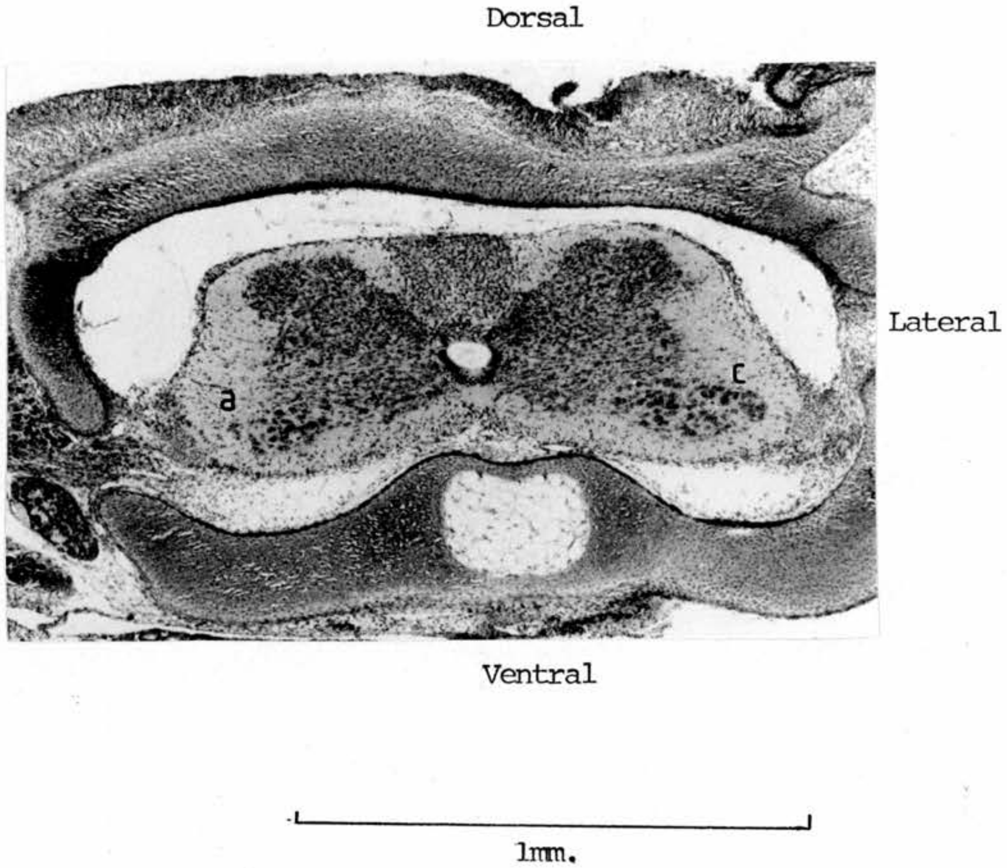


Figure 4.1.

The effect of amputation of the right hind limb bud on day 4 of incubation on the morphology of the lateral motor column on day 10 of incubation. The lateral motor column is reduced on the amputated side (a) compared to the control side (c).

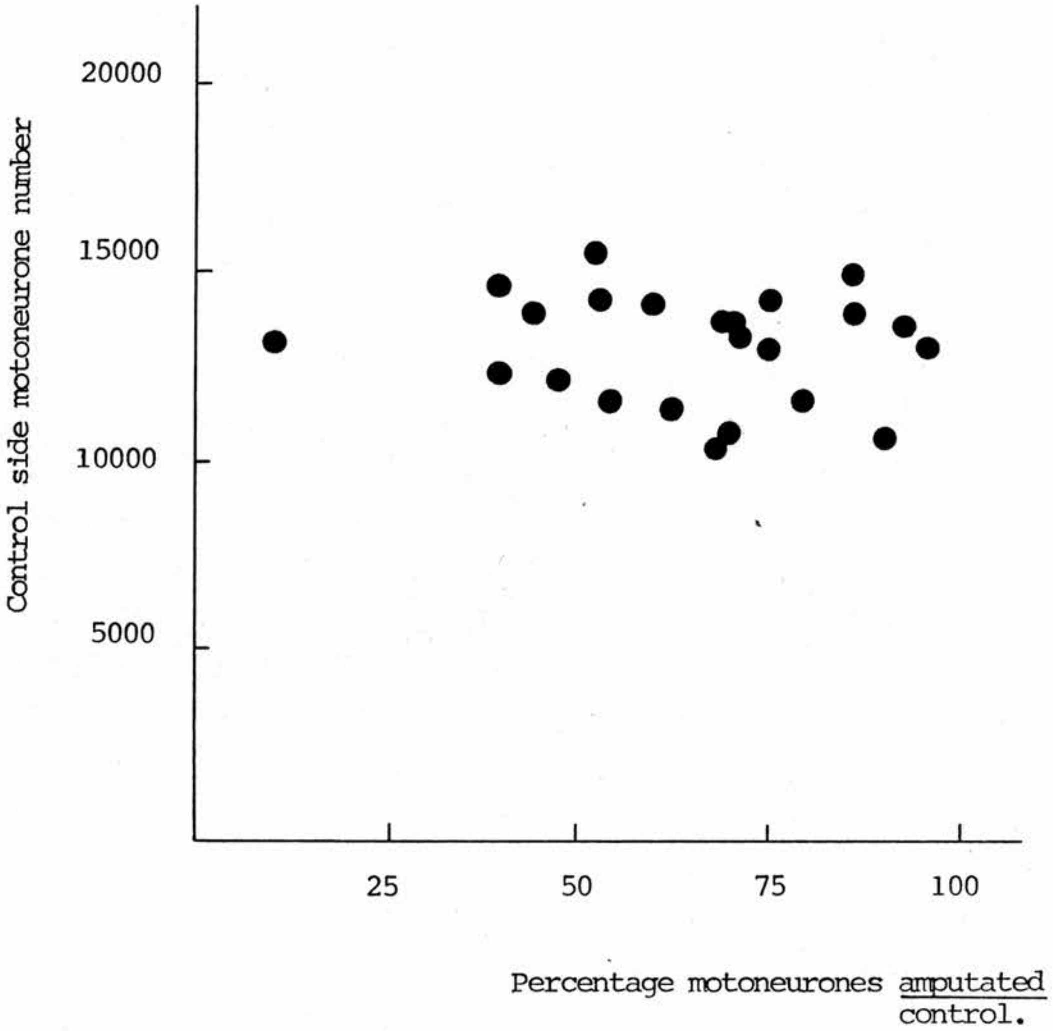


Figure 4.2 Number of motoneurones innervating limb contralateral to amputation compared with the degree of amputation. Ampuation on day 4 of incubation. Embryos fixed on day 10 of incubation. Each point is one embryo.

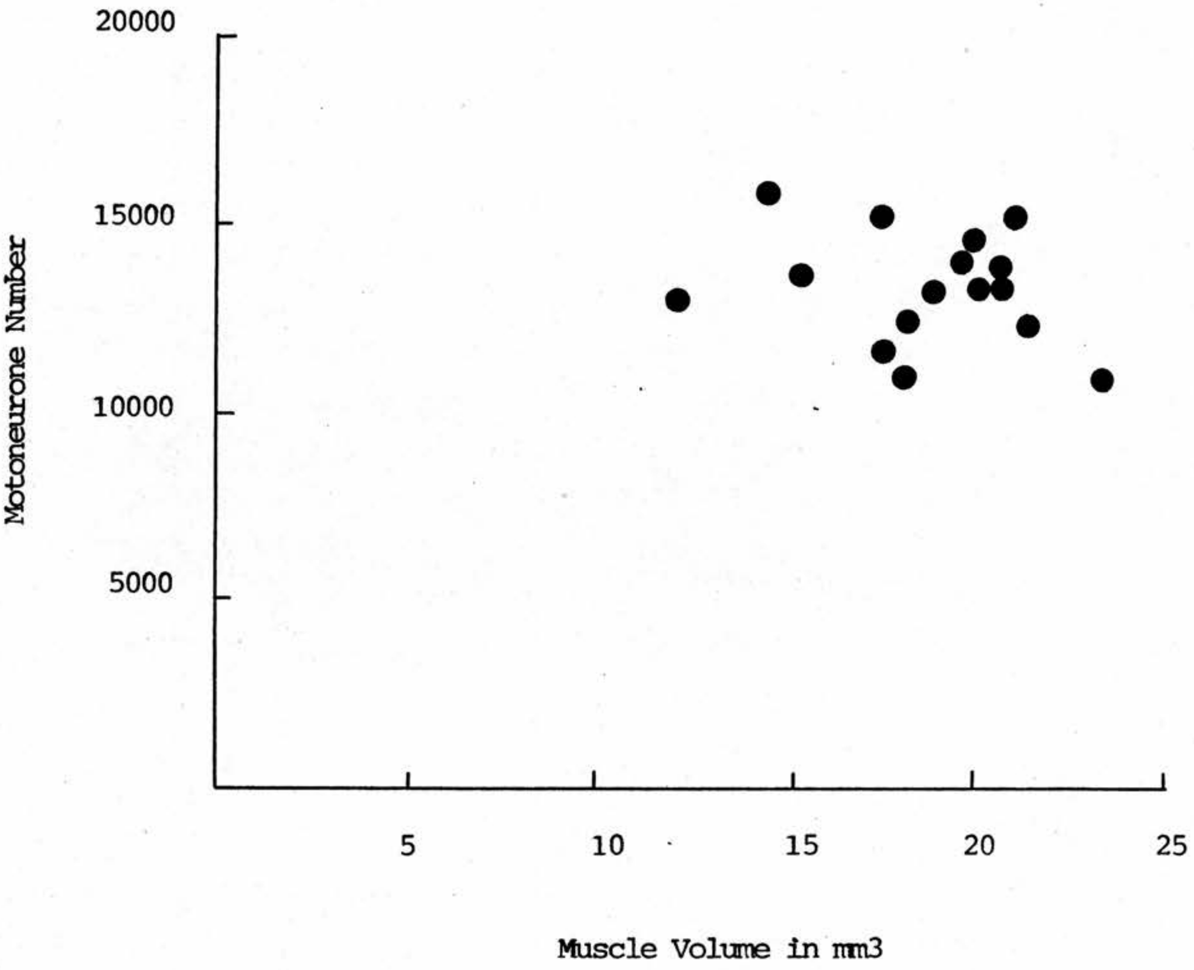


Figure 4.3

Motoneurone number and muscle volume in mm3 in the control sides of embryos on day 10 of incubation. Each point is one embryo.

	Motoneurone Number		Muscle Volume in mm^3	
No.	Control	Amputated	Control	Amputated
P181	15.280	12,080	21.0	16.7
P182	13.160	1,530	18.8	0.5
P186	14.480	5.800	19.9	6.5
P187	12,200	5,890	21.3	10.2
P229	10,810	7,720	18.0	12.4
P230	13,360	11,290	20.6	17.7
P231	10,730	9.760	25.7	21.1
P232	12,240	11,480	18.2	15.7
P234	11,580	8,140	17.3	12.1

Table (4.1)

Motoneurone number and muscle volume in control and amputated sides of embryos on day 10 of incubation. Transverse amputation on day 4 of incubation.

Embryo	Motoneurone Number		Muscle Volume in mm3			
	Control	Ammputated	Control		Amputated	
			Total	Ventral	Total	Ventral
P33	13,950	12,200	19.6	8.5	16.9	6.5
P34	13,600	12,750	15.5	6.7	12.0	4.0
P38	15,120	13,100	17.5	7.3	15.0	4.8
P88	13,390	9,520	19.8	8.9	12.1	3.1
P104	15,630	8,450	14.4	5.7	8.4	0.7
P165	13,700	9,220	20.6	8.6	10.3	1.4
P176	13,000	9,730	12.1	4.7	7.3	1.6
P296	10,890	7,860	23.3	10.7	14.7	2.9
P318	11,650	6,380	12.4	5.1	6.5	0.3
T177	13,820	6,180	14.0	5.7	5.1	0.1
T178	14,320	7,770	16.6	7.1	9.0	0.4

Table 4.2

Motoneurone number, total and ventral muscle volume in the control and amputated sides of embryos at day 10 of incubation. Ventral amputation on day 4 of incubation.

No.	Motoneurone No.		Muscle Volume in mm^3			
	Control	Amputated	Control		Amputated	
			Total	Dorsal	Total	Dorsal
A278	15,610	8.460	16.3	9.4	5.9	0.9
P23	17,000	13,440	16.8	9.8	11.9	5.8
P29	14,630	11,360	17.0	10.1	10.1	4.6
P37	14,440	10,730	18.5	11.1	13.5	6.0
P48	15,510	7.820	12.1	7.0	4.4	0.7
P49	15,080	8,580	17.5	9.8	7.7	1.7
P61	14,230	8,480	20.8	11.4	7.7	2.0
P67	14,570	12,260	17.1	10.0	12.2	5.8
P71	14,660	7,640	12.0	6.7	3.9	0.6
P75	14,900	12,020	17.9	10.2	11.3	5.1
T73	14.360	10,890	15.8	9.5	8.8	4.8
T147	11,920	7,380	17.4	9.9	6.0	1.6
T165	13,810	9,660	24.9	13.9	14.0	6.2
T173	11,620	9,520	16.3	9.4	10.8	4.6

Table (4.3)

Motoneurone number, total and dorsal muscle volumes in control and amputated sides of embryos on day 10 of incubation.

Dorsal amputation on day 4 of incubation.

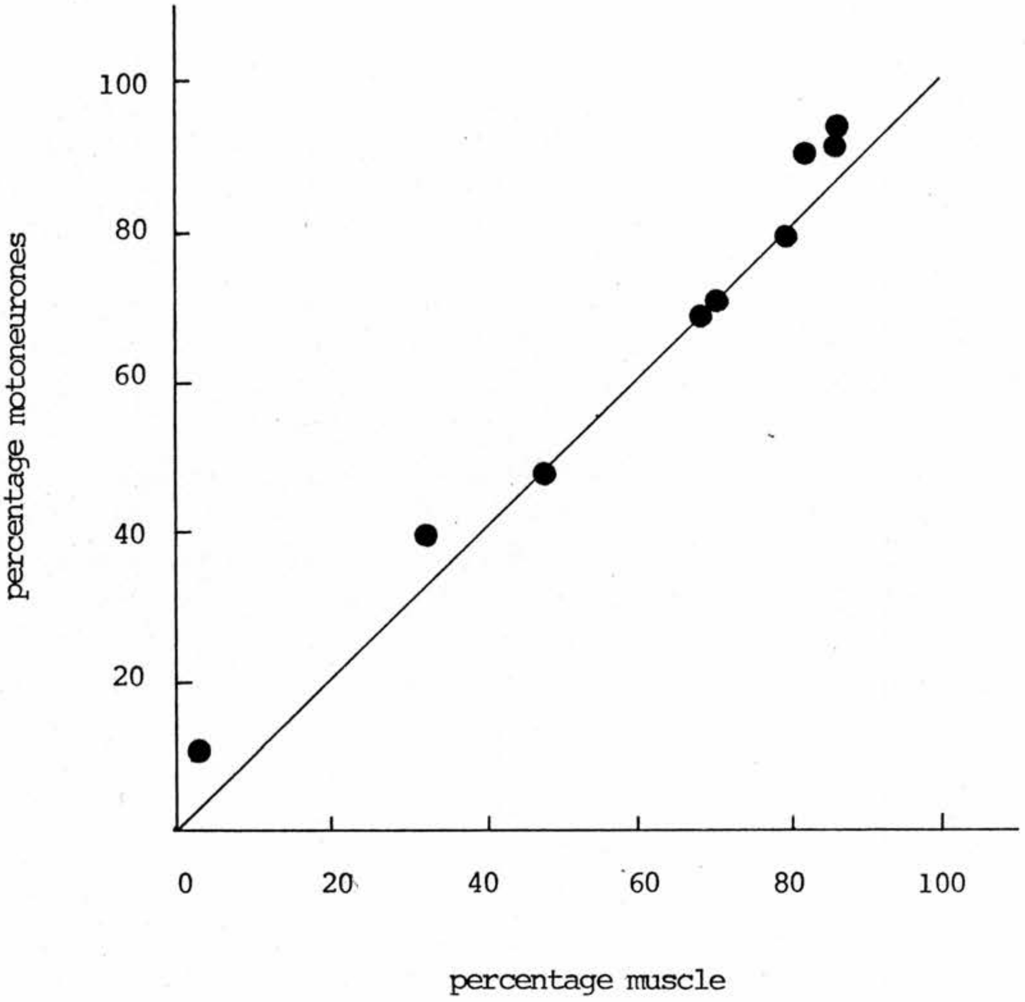


Figure 4.4.

The number of motoneurones remaining in the amputated side lateral motor column on day 10 of incubation compared to the volume of muscle remaining in the amputated limb. Transverse amputation on day 4 of incubation. Motoneurone number expressed as a percentage of the control side motoneurone number. Muscle volume expressed as a percentage of the control limb total muscle volume. Each point is one embryo. Solid line is a theoretical regression line - see text. Muscle volume in mm3.

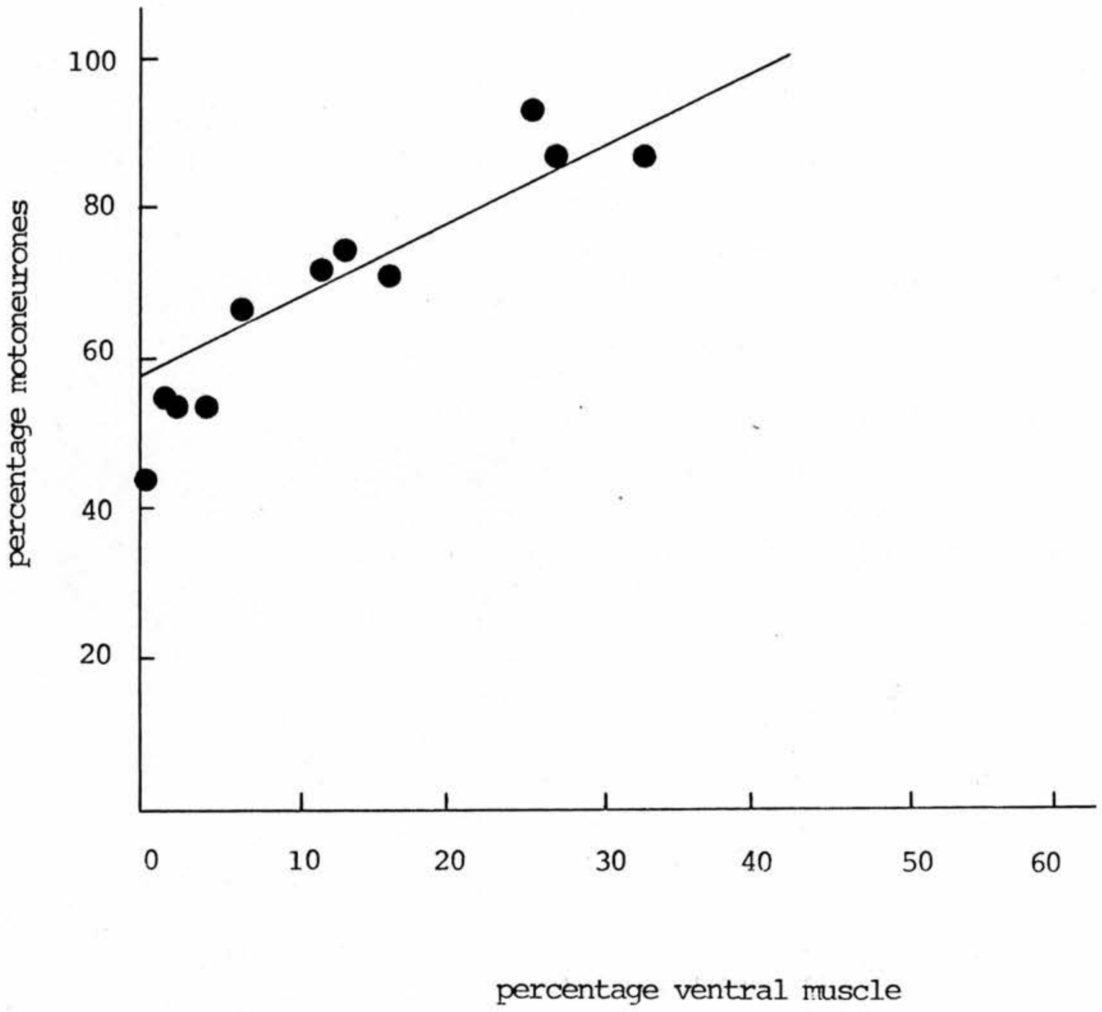


Figure 4.5.

The number of motoneurones remaining in the amputated side lateral motor column on day 10 of incubation compared to the volume of ventral muscle remaining in the amputated limb. Ventral amputation on day 4 of incubation. Motoneurone number expressed as a percentage of the control side motoneurone number. Ventral muscle volume expressed as a percentage of the control limb total muscle volume. Each point is one embryo. Solid line is a theoretical regression line - see text. Muscle volume in mm3.

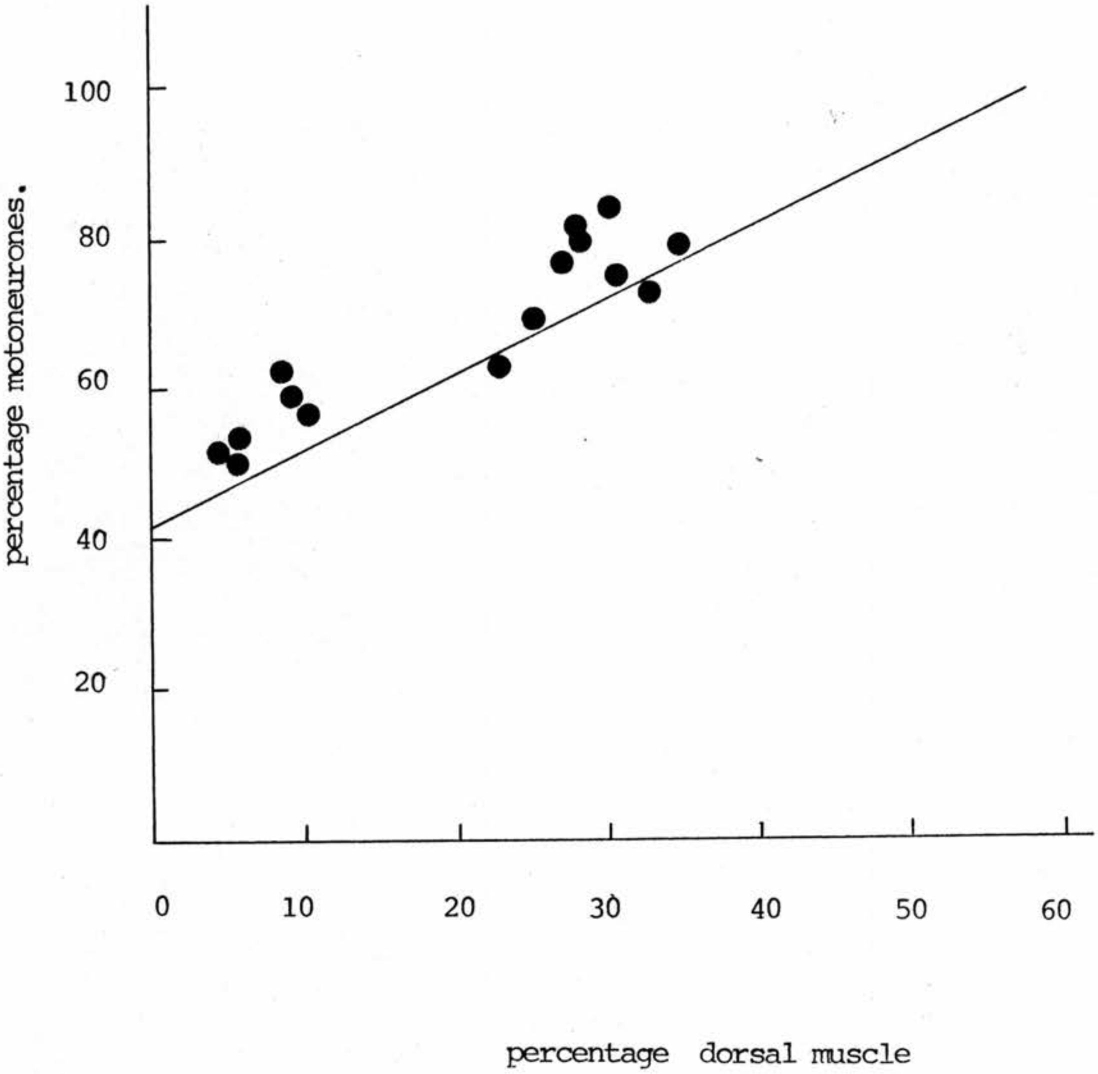


Figure 4.6.

The number of motoneurones remaining in the amputated side lateral motor column on day 10 of incubation compared to the volume of dorsal muscle remaining in the amputated limb. Dorsal amputation on day 4 of incubation. Motoneurone number expressed as a percentage of the control side motoneurone number. Dorsal muscle volume expressed as a percentage of the control limb total muscle volume. Each point is one embryo. Solid line is a theoretical regression line - see text . Muscle volume in mm3.

(Regression coefficient = 1.33 ± 0.16 (SE) $p < 0.001$ Student's t-test); and between the percentage of motoneurones and the percentage of dorsal muscle remaining on day 10 following dorsal amputation on day 4 fig (4.6), (Regression coefficient = 0.95 ± 0.10 (SE) $p < 0.001$ Student's t-test).

Theoretical regression lines, represented by the solid lines in figs (4.4 - 4.6) have been drawn based on the following assumptions:

(1) A complete limb supports the control number of motoneurones

(2) Motoneurones must contact limb muscle in order to survive

(3) There is a constant innervation density (defined as motoneurone number per unit muscle volume)

(4) Motoneurones do not innervate other muscles in the absence of their normal muscle targets

Assumption (1) defines the upper limits of the lines. In terms of the abscissae in fig (4.4) a complete limb composed of a 100% muscle will support the control number of motoneurones - a 100%. The line will pass through the 100% : 100% point. In terms of the abscissae in figs (4.5) and (4.6) the line will pass through the 100% : 42.4% and the 100% : 57.6% point respectively. This is because a complete limb supporting the control number of motoneurones - a 100%, will be composed of about 42.4% ventral muscle and about 58.6% dorsal muscle - see fig (3.4).

Assumptions (2), (3) and (4) define the behaviour of the lines from the upper limits. Since motoneurones do not innervate other muscles in the absence of their normal targets and must contact muscle in order to survive, and since there is a constant innervation density throughout the limb, then loss of muscle will be associated with an equal proportional loss of motoneurones. The predicted lines will therefore be straight with a slope of 1.0. For fig (4.4) relating the percentages of motoneurones to muscle volume remaining after transverse amputation, the predicted line will pass through zero. For fig (4.5) relating the percentage of ventral muscle to the percentage of motoneurones remaining after ventral amputation the predicted line will cross the y-axis at 57.6%. This is because a limb containing no ventral muscle will be composed entirely of dorsal muscle - about 57.6% (the mean control value of dorsal muscle, see fig (3.4)), and should therefore support an equal proportional percentage of motoneurones. Similarly for fig (4.6) relating the percentage of dorsal muscle remaining to the percentage of motoneurones remaining following dorsal amputation the predicted line will cross the y-axis at 42.4%. This is because a limb containing no dorsal muscle will be composed entirely of ventral muscle (42.4% - the mean control value of ventral muscle - fig (3.4)) and should therefore support an equal percentage of motoneurones.

A comparison of the observed and predicted regression lines for the three types of amputation is given in table (4.4) along with a Student's t-test for significant difference between them. For transverse amputations, neither the slope nor the intercept of the observed line differs significantly from those of the predicted line ($p > 0.1$ and $p > 0.5$ respectively, Student's t-test). Similarly for ventral amputations there are no significant differences between the slopes and the intercepts of the observed and predicted

Type of Amputation	Io	I_E	ti	So	S_E	ts
Transverse	6.86 ± 4,04	0	1.69 $(p>0.5)$	0.95 ± 0.06	1.0	0.833 $(p>0.1)$
Ventral	51.85 ± 2.73	57.6 ± 1.5	1.85 $(p> 0.05)$	1.33 ± 0.16	1.0	2.06 $(p>0.05$
Dorsal	48.61 ± 2.28	42.4 ± 1.5	2.27 $p>0.02<0.05$	0.95 ± 0.10	1.0	0.5 $(p>0.5)$

Table (4.4)

Comparison of the observed and expected regression lines between percentage of motoneurones and the percentage of muscle remaining on day 10 of incubation.

Transverse, dorsal and ventral amputation on day 4 of incubation.

Io - observed intercept with its standard error

I_E - expected intercept with its standard error

ti - Student's t-test of significant difference between Io & I_E

So - observed slope with its standard error

S_E - expected slope with its standard error

ts - Student's t-test of significant difference between So & S_E

lines ($p > 0.05$ and $p > 0.05$ respectively, Student's t-test). However while the slopes of the observed and predicted lines do not differ significantly for dorsal amputations ($p > 0.5$, Student's t-test), there is a significant difference between the intercepts of the two lines ($p < 0.05$, Student's t-test). The y-axis intercept of the observed regression line is 48.6% and not 42.4% as predicted. This means that 6% more motoneurones survive than predicted after any given dorsal amputation.

The behaviour of the observed regression lines for transverse and ventral amputations are thus consistent with the predicted lines that fulfil the afore mentioned criteria. The behaviour of the observed regression line for dorsal amputation is not consistent in that 6% more motoneurones survive than predicted following any given dorsal amputation.

4.3 Interpretation of Results

Motoneurone loss from the lateral motor column on day 10 of incubation is in direct proportion to the loss of muscle for transverse amputations, fig (4.4). This result confirms the findings of Laing (1979) who also noted a linear relationship between the two variables on day 10 of incubation following transverse amputation of the chick hind limb on day 4. Similarly a linear relationship between number of innervating motoneurones, as estimated by HRP uptake labelling, and wet weight of muscle for the chick hind limb on day 10 of incubation has been noted by Landmesser (1978a). There is therefore good evidence for the relationship.

The regression lines relating the percentage of remaining motoneurones to the percentage of total muscle or ventral muscle remaining for transverse and ventral amputations respectively, do not differ significantly from predicted lines based on certain assumptions, outlined in the results. (p 107) . However the regression line relating the percentage of remaining motoneurones to the percentage of dorsal muscle remaining after dorsal amputation does differ from the predicted line in that it crosses the y-axis at 48.6% and not 42.4% as predicted. This difference is significant at the 5% level. The slopes of the calculated and predicted lines do not, however, differ. This means that for any given dorsal amputation some 6% more motoneurones survive than predicted.

There are two possible reasons for this other than that the assumptions upon which the predicted line is based are not valid.

One is that the increase is artifact due to errors in motoneurone number and muscle volume estimations. These errors become important in estimations of low motoneurone numbers, (which are overestimated) and small muscle volumes (which are underestimated) - see methods (2.6.1) and (2.7.1). Errors in small muscle volumes may be equally important for transverse, dorsal and ventral amputations. Error in estimation of low motoneurone numbers is undoubtedly important for radical transverse amputations and may be more important in influencing motoneurone counts remaining after dorsal than ventral amputations. This is because of the different rostro-caudal distribution of motoneurones innervating dorsal and ventral muscle, see section (5.1.1), and the greater number of motoneurones innervating dorsal than ventral muscle. Dorsal amputation results in fewer motoneurones remaining per section, especially in the rostral parts of the lateral motor column than ventral amputation. However it is difficult to see how this overestimation could play a role after small partial amputations of the dorsal muscle mass since large numbers of motoneurones survive per section after this type of amputation.

Another possible reason for the apparent 6% increase in motoneurone survival is that there is a delay in naturally occurring motoneurone death. Paralysis of chick embryos with α-bungarotoxin (Laing & Prestige 1978) and cobratoxin (Pittman & Oppenheim 1978) before and during the period of motoneurone death almost entirely prevents motoneurone degeneration so long as the paralysis is maintained. However Pittman & Oppenheim (1979) found 'no clear graded increase in (motoneurone) survival with decreasing activity' for the chick hind limb and suggested that motoneurone survival is only affected once the decrease in activity falls below a threshold level. If this is the case it may be that a drop in muscle activity below threshold level occurs following dorsal amputation, related perhaps to the previously discussed atrophy of ventral muscle that is associated with dorsal amputations. This would then lead to increased motoneurone survival.

Discussion of Assumptions

The assumptions upon which the predicted regression lines relating percentage of motoneurones remaining to the percentage of muscle remaining are based, will now be discussed for all three types of amputation.

A Complete Limb Supports the Control Number of Motoneurones

This is not really an assumption. A complete limb supports, by definition, the control number of motoneurones.

Motoneurones must Contact Muscle in Order to Survive

The effect of limb amputation on motoneurone survival is well documented (Hamburger 1934, 1958, Oppenheim et al 1978; Prestige 1967b) and has led to the belief that motoneurones are dependant on contact with limb muscle for their survival. The results of radical transverse amputation reported here confirm this. Almost all motoneurones have disappeared from the lateral motor column on day 10 of incubation. However a few motoneurones do remain - the regression line relating motoneurone number to muscle volume crosses the y axis at a positive value of 6%, fig (4.4), but this does not differ significantly from a line passing through zero. The most likely explanation for the observed regression line crossing the y-axis at a positive value is that it is due to error in motoneurone number estimations (see methods 2.6). However another possibility is that a few motoneurones can survive independently of the limb. A similar explanation could account for the 6% of motoneurones that survive above the expected number following dorsal amputation.

Other workers have also found that some motoneurones persist in the chick lateral motor column after early radical amputation of the limb (Bueker 1947; Hamburger 1958; Oppenheim et al 1978; Laing 1979). Oppenheim investigated the possibility that they represent ectopic medial motor column motoneurones (that normally innervate trunk musculature) by injecting HRP into the trunk musculature. In no instance did these motoneurones become labelled with HRP although medial motor column motoneurones proper did so. Another possibility is that they represent motoneurones that have innervated muscles in the contra-lateral limb. In

Xenopus both sides of the spinal cord can innervate a single hind limb (Lamb 1981a)and amputation of both hind limbs in Xenopus results in total absence of ventral horn motoneurones (Lamb 1981b). Whether this is true for chick motoneurones is not known. The possibility that some chick motoneurones are limb-independent requires further investigation.

Innervation Density

The linear relationship between muscle volume and motoneurone number remaining on day 10 of incubation after transverse amputation shows that there is a constant innervation density throughout the proximo-distal axis of the limb. Although this may be so it is conceivable that there is a differential innervation density between muscles derived from the dorsal and ventral muscle masses such that they average at 1.0. If the innervation density was less in dorsal muscle than in ventral muscle this could account for the apparent 6% increase in motoneurone survival. However this cannot be the explanation because graded amputation of the ventral muscle mass results in an equal proportional loss of motoneurones, not a greater than equal proportional loss which would be expected if there was a differential innervation density between the two muscle masses.

Although these results show that the innervation density is constant throughout the proximo-distal as well as the dorso-ventral axis of the limb it is impossible to say anything about motor unit size from the findings. The size of individual muscle fibres, and the ratio of fibres to connective tissue may differ between muscles and vary the relationship between muscle volume, as measured by camera lucida drawings, and the number of motor units contained in the muscle. Indeed there is evidence in the adult (Sissons 1974) that motor unit size is smaller in distal muscles concerned with fine movements than in more proximal muscles. Whether this is also true for the chick on day 10 of incubation will require electrophysiological investigation.

Motoneurones do not Innervate Other Muscles in the Absence of Their Normal Muscle Targets

The linear relationship between motoneurone number and muscle volume remaining after transverse, ventral and dorsal amputation suggests that in the absence of their normal muscle targets the vast majority of motoneurones do not innervate other muscles and thus survive the period of motoneurone death. However another possible explanation that is also compatable with the results is that motoneurones do innervate other muscles in the absence of their normal muscle targets but in doing so displace the original innervation. In this case there would still be a linear relationship between motoneurone number remaining and muscle volume remaining but the pattern of innervation of the remaining muscle would be abnormal. The analysis of the position of the remaining motoneurones in the lateral motor columns however shows that this is not so. (Chapter 5).

One possible explanation for the apparent 6% increase in motoneurone survival observed after dorsal amputation is that these are motoneurones that do innervate other muscles in the absence of their normal muscle targets but in doing so do not displace the original innervation. If this is so why these 6% of motoneurones should differ from the remaining 94% is not clear, nor is why certain muscles should accept additional innervation after dorsal amputations when they do not do so after transverse or ventral amputations.

CHAPTER 5

RESULTS

THE POSITION OF REMAINING MOTONEURONES IN THE LATERAL MOTOR COLUMN

The position of motoneurones remaining in the medio-lateral and rostro-caudal axes of the lateral motor column on day 10 of incubation following transverse, dorsal and ventral amputation on day 4 of incubation will now be considered.

5.1 Rostro-Caudal Position

Dorsal and Ventral Amputations

The rostro-caudal distribution of motoneurones innervating both the control and amputated sides of : a) embryos with almost total amputation of the dorsal muscle mass are given in table (5.1) and shown in figs (5.1a-5.6a); and embryos with almost total amputation of the ventral muscle mass are given in table (5.2) and shown in figs (5.1b-5.6b). In figs (5.1a,b-5.6a,b) the solid lines represent the control sides of embryos, the dashed lines the amputated sides and the shaded areas the position and extent of motoneurone loss between the two sides.

Almost complete amputation of the dorsal muscle mass on day 4 of incubation (all embryos had some dorsal muscle remnants remaining varying from 5.0 to 5.8% of control limb total muscle volume - table (5.1)) results in loss of motoneurones from the entire rostro-caudal extent of the lateral motor column, but with greatest loss of motoneurones occurring in the rostral tenths. In each case the peak of motoneurone survival occurs in caudal tenths 5-7.

This contrasts to the rostro-caudal distribution of motoneurones remaining after almost total amputation of the ventral muscle mass on day 4 of incubation (ventral muscle

Embryo	A278		P48		P71	
	Control	Amputated	Control	Amputated	Control	Amputated
Motoneurone Number	15,600	8,460	11,640	6,370	14,760	7,630
% motoneurones per rostro-caudal tenth						
1	2.5	1.5	4.6	2.5	4.1	2.1
2	10.3	5.7	11.6	5.2	8.6	5.2
3	14.6	4.9	14.1	3.5	12.3	2.3
4	14.7	3.9	14.1	4.9	12.2	4.2
5	15.2	6.8	14.3	6.6	15.8	9.6
6	13.9	9.2	12.6	8.4	16.2	9.5
7	14.2	9.0	11.9	7.9	13.8	8.3
8	7.8	6.7	8.6	5.3	8.5	5.3
9	5.2	3.8	4.8	3.7	5.4	3.9
10	1.6	2.8	3.5	2.5	3.7	1.6
% dorsal muscle	57.6	5.5	57.9	5.8	55.8	5.0

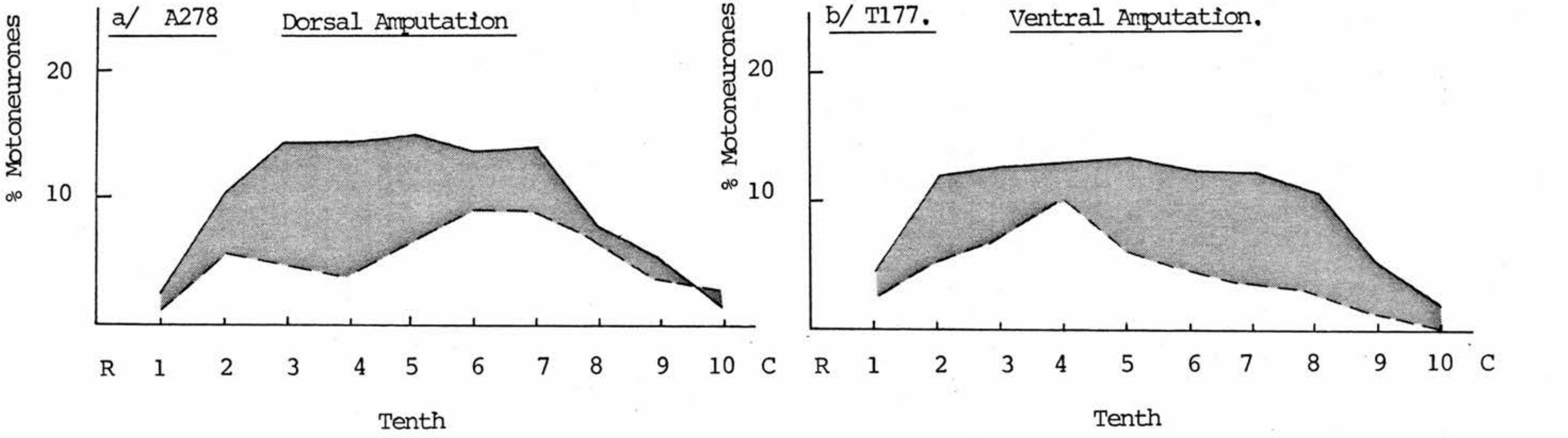
Table (5.1)

Motoneurone number, percentage of dorsal muscle and percentage of motoneurones per rostro-caudal tenth in control and amputated sides of embryos on day 10 of incubation. Radical dorsal amputation on day 4 of incubation. Dorsal muscle volume expressed as a percentage of control limb total muscle volume. Motoneurone number per rostro-caudal tenth expressed as a percentage of the control side total motoneurone number.

Embryo	T177		P318		T178	
	Control	Amputated	Control	Amputated	Control	Amputated
Motoneurone number	13,910	6,190	11,640	6,380	14,320	7,770
% Motoneurones per rostro-caudal tenth						
1	4.5	2.2	4.7	2.6	3.8	3.9
2	12.0	5.4	11.2	5.7	10.3	6.8
3	12.9	7.2	11.5	7.0	13.0	10.9
4	13.2	10.4	13.8	9.0	14.7	9.8
5	13.6	6.4	11.0	7.0	14.9	7.9
6	12.6	4.6	15.5	6.3	14.9	6.1
7	12.6	3.8	11.2	4.9	13.1	4.9
8	10.9	3.1	13.8	7.1	8.4	1.3
9	5.5	1.3	4.6	3.4	4.7	0.6
10	2.2	0.1	2.6	1.7	2.2	0.6
% ventral muscle	40.7	0.7	41.1	2.4	40.7	2.4

Table 5.2

Motoneurone number, percentage ventral muscle and percentage of motoneurones per rostro-caudal tenth in control and amputated sides of embryos at day 10 of incubation. Radical ventral amputation on day 4 of incubation. Ventral muscle volume expressed as a percentage of control limb total muscle volume. Motoneurone number per rostro-caudal tenth expressed as a percentage of the control side total motoneurone number.



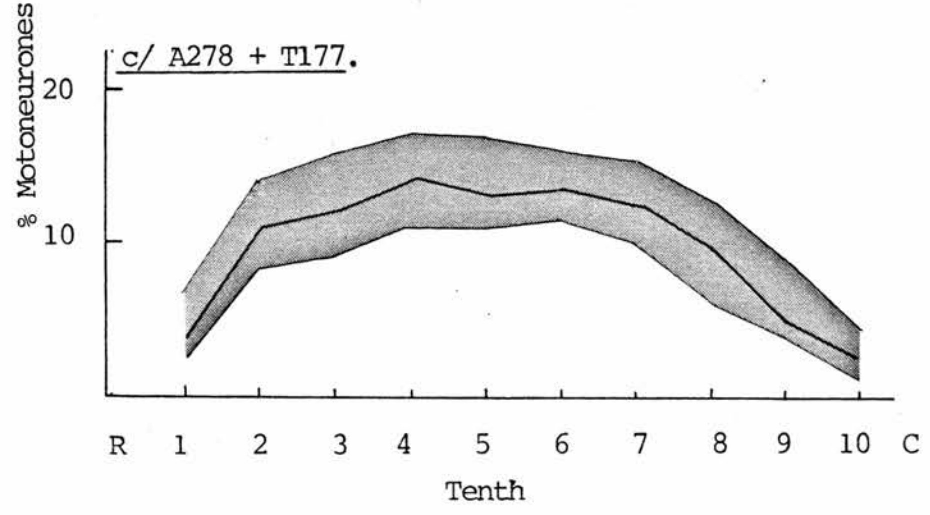


Figure 5.1.

a/ & b/: Motoneurone number per rostro-caudal tenth in control (solid line) and amputated side (dashed line) lateral motor columns of embryos on day 10 of incubation following a/ dorsal amputation on day 4 of incubation and b/ventral amputation on day 4 of incubation. The shaded area represents the position and extent of motoneurone loss between the control and amputated sides. Motoneurone number expressed as a percentage of the control side total motoneurone number.

c/ The result of adding together the percentage of motoneurones per rostro-caudal tenth in the amputated side lateral motor columns of embryos A278 and T177 (solid line). The shaded area represents the variation in the percentage of motoneurones per rostro-caudal tenth for control embryos. R-rostral. C-caudal.

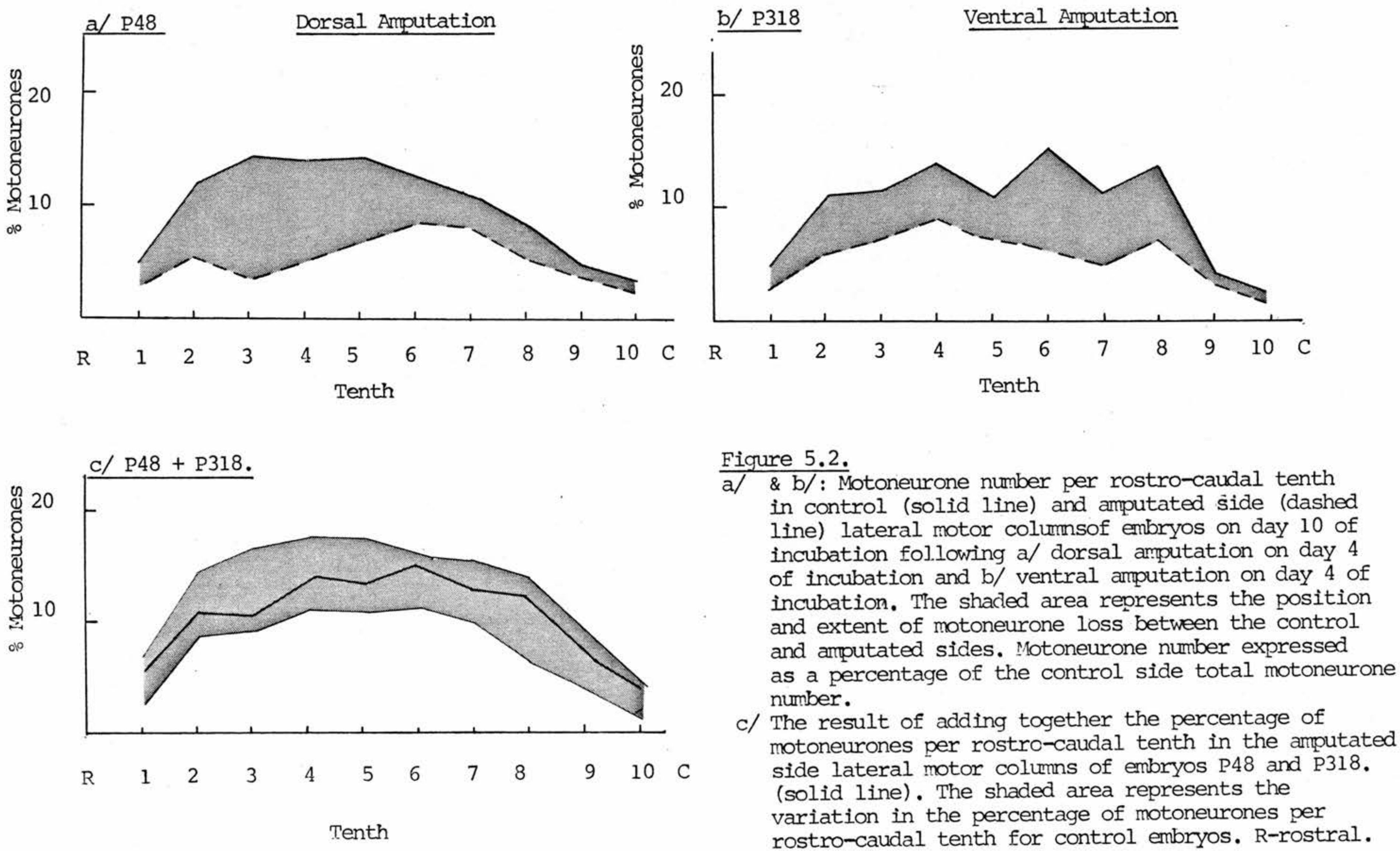
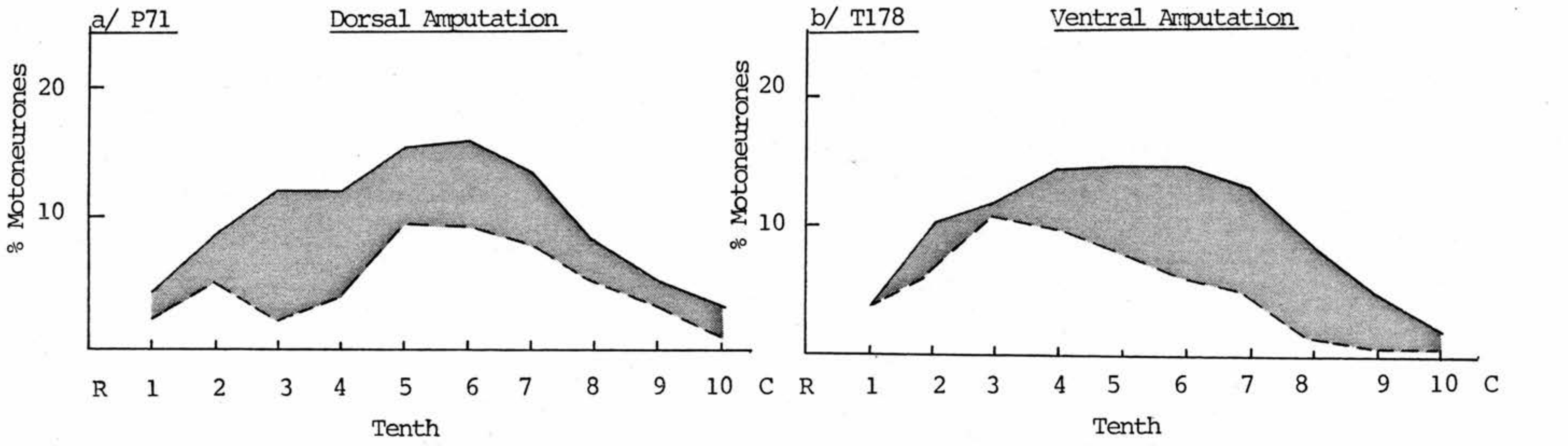


Figure 5.2.

a/ & b/: Motoneurone number per rostro-caudal tenth in control (solid line) and amputated side (dashed line) lateral motor columnsof embryos on day 10 of incubation following a/ dorsal amputation on day 4 of incubation and b/ ventral amputation on day 4 of incubation. The shaded area represents the position and extent of motoneurone loss between the control and amputated sides. Motoneurone number expressed as a percentage of the control side total motoneurone number.

c/ The result of adding together the percentage of motoneurones per rostro-caudal tenth in the amputated side lateral motor columns of embryos P48 and P318. (solid line). The shaded area represents the variation in the percentage of motoneurones per rostro-caudal tenth for control embryos. R-rostral. C- caudal.



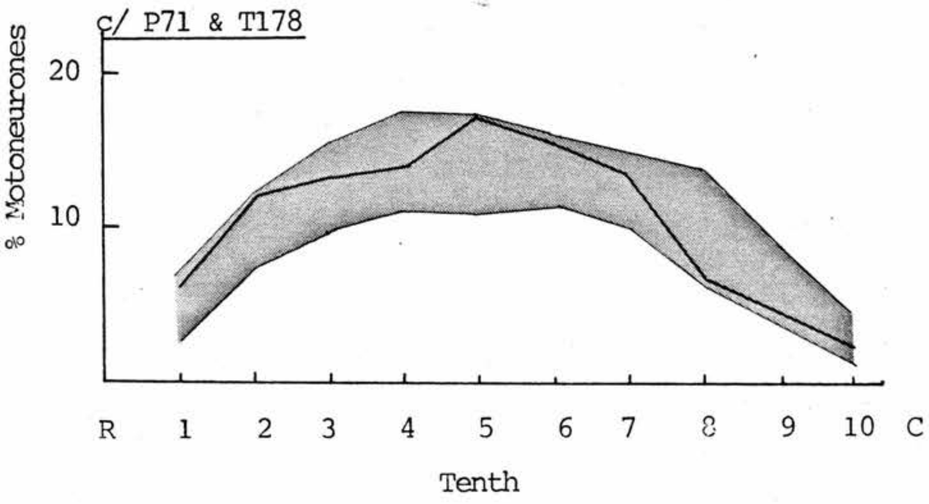
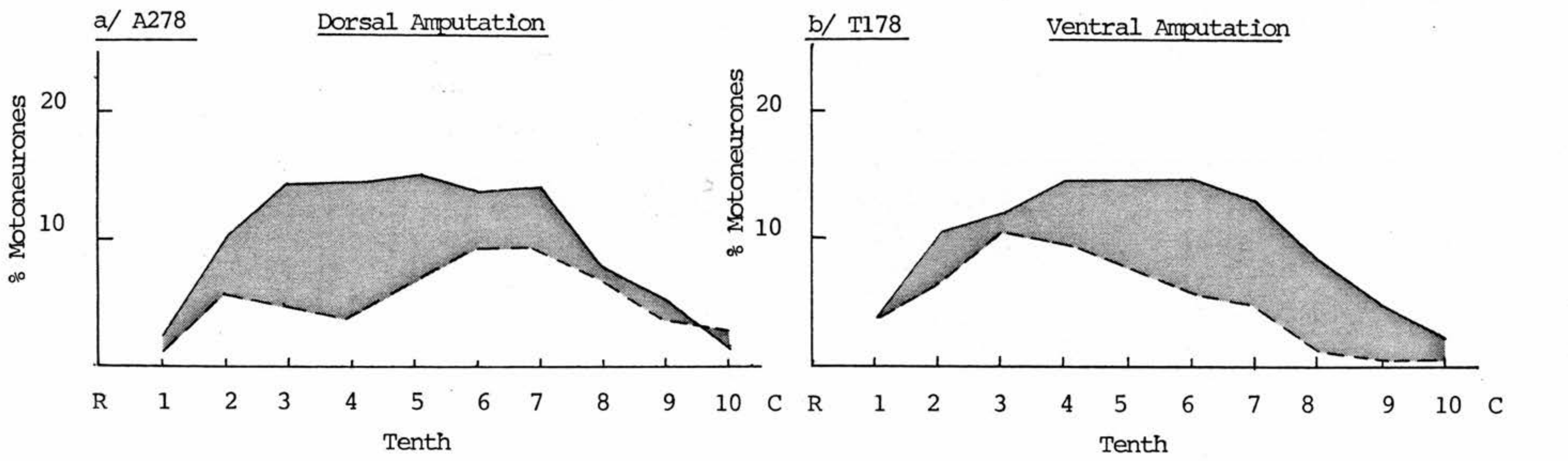


Figure 5.3

a/ & b/: Motoneurone number per rostro-caudal tenth in control (solid line) and amputated side (dashed line) lateral motor columns of embryos on day 10 of incubation follwing a/ dorsal amputation and b/ ventral amputation on day 4 of incubation. The shaded area represents the position and extent of motoneurone loss between the control and amputated sides. Motoneurone number expressed as a percentage of the control side total motoneurone number.

c/ The result of adding together the percentage of motoneurones per rostro-caudal tenth in the amputated side lateral motor columns of embryos P71 and T178 (solid line). The shaded area represents the variation in the percentage of motoneurones per rostro-caudal tenth for control embryos. R-rostral. C- caudal.



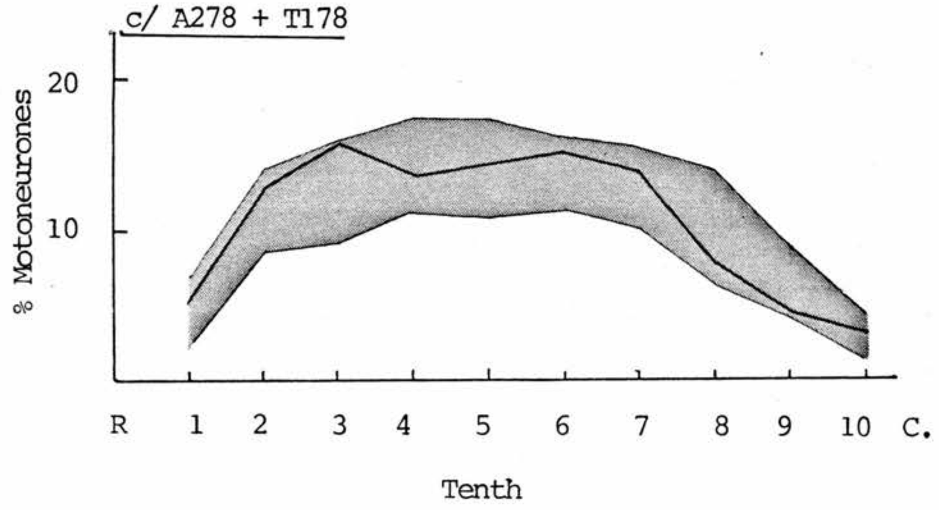
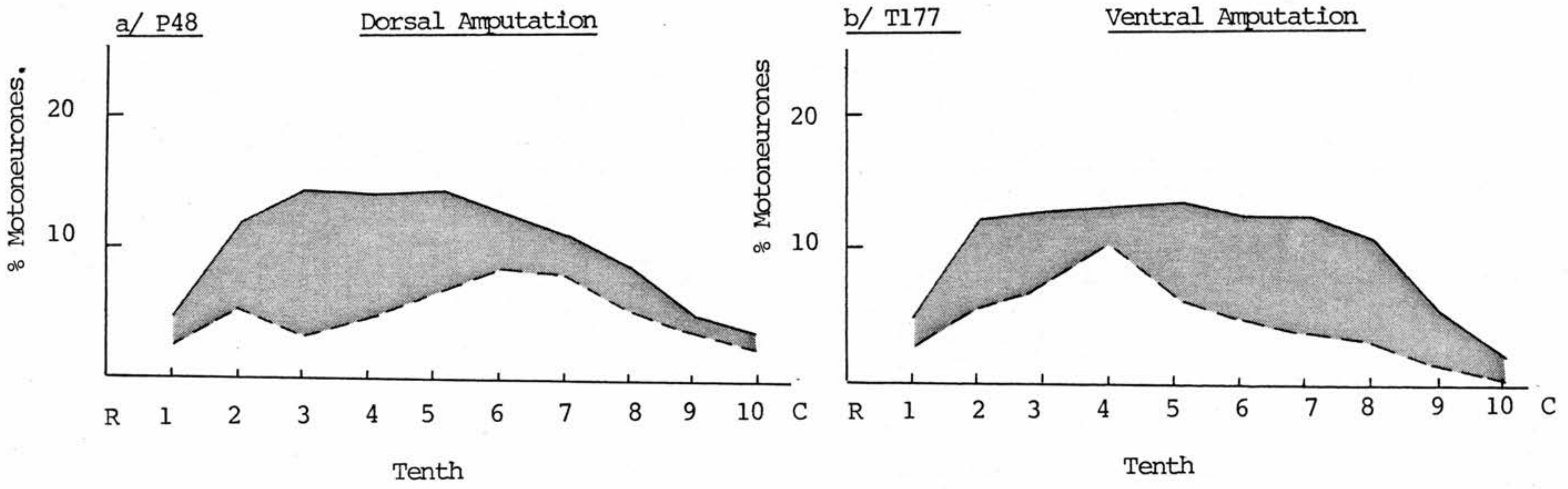


Figure 5.4.

a/ & b/: Motoneurone number per rostro-caudal tenth in control (solid line) and amputated side (dashed line) lateral motor columns of embryos on day 10 of incubation following a/ dorsal amputation on day 4 of incubation and b/ ventral amputation on day 4 of incubation. The shaded area represents the position and extent of motoneurone loss between the control and amputated sides. Motoneurone number expressed as a percentage of the control side total motoneurone number.

c/ The result of adding together the percentage of motoneurones per rostro-caudal tenth in the amputated side lateral motor columns of embryos A278 and T178 (solid line). The shaded area represents the variation in the percentage of motoneurones per rostro-caudal tenth for control embryos. R- rostral. C- caudal.



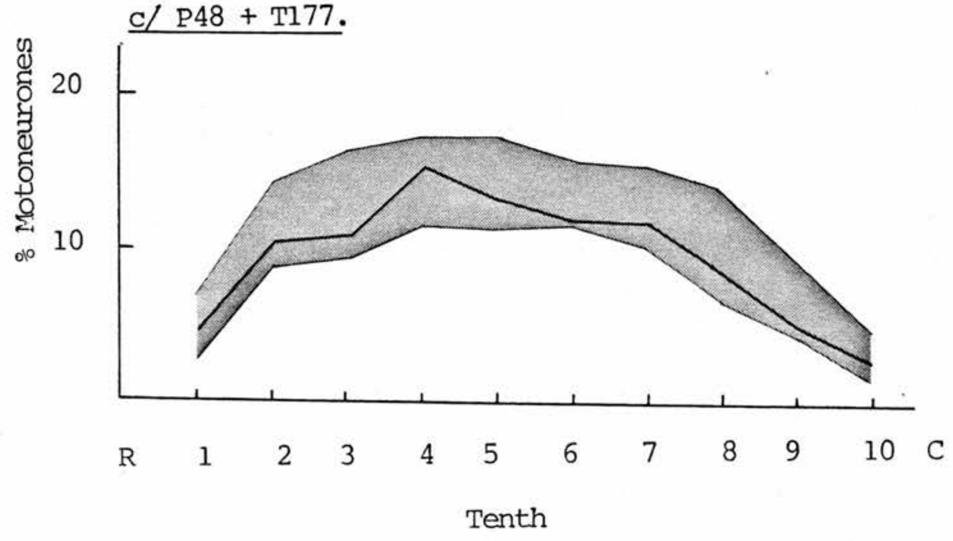
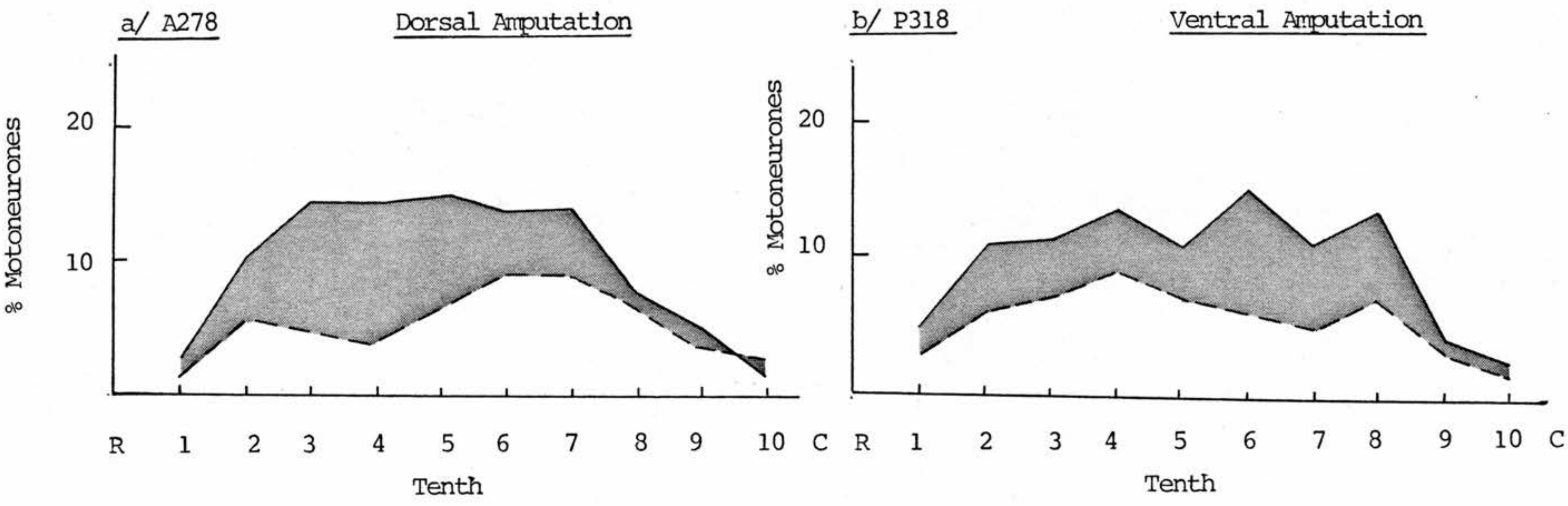


Figure 5.5.
a/ & b/: Motoneurone number per rostro-caudal tenth in control (solid line) and amputated side (dashed line) lateral motor columns of embryos on day 10 of incubation following a/ dorsal amputation on day 4 of incubation and b/ ventral amputation on day 4 of incubation. The shaded area represents the position and extent of motoneurone loss between the control and amputated sides. Motoneurone number expressed as a percentage of the control side total motoneurone number.

c/ The result of adding together the percentage of motoneurones per rostro-caudal tenth in the amputated side lateral motor columns of embryos P48 and T177 (solid line). The shaded area represents the variation in the percentage of motoneurones per rostro-caudal tenth for control embryos. R- rostral. C- caudal.



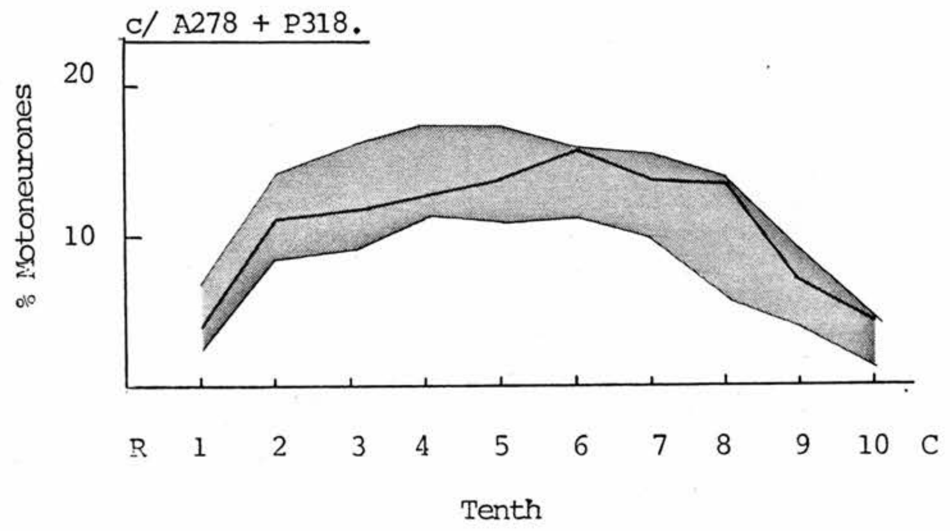


Figure 5.6

a/ & b/: Motoneurone number per rostro-caudal tenth in control (solid line) and amputated side (dashed line) lateral motor columns of embryos on day 10 of incubation following a/ dorsal amputation and b/ ventral amputation on day 4 of incubation. The shaded area represents the position and extent of motoneurone loss between the control and amputated sides. Motoneurone number expressed as a percentage of the control side motoneurone number.

c/ The result of adding together the percentage of motoneurones per rostro-caudal tenth in the amputated side lateral motor columns of embryos A278 and P318 (solid line). The shaded area represents the variation in the percentage of motoneurones per rostro-caudal tenth for control embryos. R- rostral. C- caudal.

remnants varying from 0.7 to 2.4% of the control limb total muscle volume - table (5.2)). Although motoneurone loss again occurs from the entire rostro-caudal extent of the lateral motor column, greatest motoneurone loss occurs in the caudal tenths. The peak of motoneurone survival occurs in rostral tenths 3 or 4.

Comparing figs (5.1a-5.6a) to figs (5.1b-5.6b) the motoneurones remaining after almost total amputation of the dorsal muscle mass have a different rostro-caudal distribution from the remaining motoneurones of embryos with almost total amputation of the ventral muscle mass.

The effect of adding together the percentage of motoneurones remaining in the amputated sides of embryos with almost total amputation of the dorsal muscle mass to those of embryos with almost total amputation of the ventral muscle mass, for 9 embryo pairs, is given in table (5.3). Six such additions are represented graphically in figs (5.1c-5.6c) - the shaded areas represent the variation in the percentage of motoneurones per rostro-caudal tenth for control embryos, table (5.4).

In all cases the rostro-caudal distribution of motoneurones, calculated by adding together the amputated sides of embryo pairs, falls within the control range of rostro-caudal distributions.

Transverse Amputations

The rostral-caudal distribution of motoneurones innervating the control and amputated sides of embryos with varying degrees of transverse amputation are given in table (5.5) and shown in figs (5.76-5.12b). The volumes of thigh and shank muscle in control and amputated limbs of each embryo are shown in figs (5.7a-5.12a). Motoneurone loss per rostro-caudal tenth is shown in figs (5.7c-5.12c).

Rostro-caudal Tenth	T177 + A278	P318 + P48	T178 + P71	T177 + P48	P318 + A278	T178 + A278	T177 + P71	P318 + P71	T178 + P48	Control Range
1	3.7	5.1	6.0	4.7	4.1	5.4	4.3	4.7	6.4	2.5- 6.8
2	11.1	10.9	12.0	10.6	11.4	12.5	10.6	10.9	12.0	8.6- 14.2
3	12.1	10.5	13.2	10.7	11.9	15.8	9.5	9.3	14.4	9.3- 16.0
4	14.3	13.9	14.0	15.3	12.9	13.7	14.6	13.2	14.7	11.4- 17.3
5	13.2	13.6	17.5	13.0	13.8	14.7	16.0	16.6	14.5	11.0- 17.5
6	13.8	14.7	15.6	13.0	15.5	15.3	14.1	15.8	14.5	11.5- 16.2
7	12.8	13.2	11.7	13.9	13.9	13.9	12.1	13.2	12.8	10.0- 15.4
8	9.8	12.4	6.6	8.4	13.8	8.0	8.4	12.4	6.6	6.3- 13.8
9	5.1	7.1	4.5	5.0	7.2	4.4	5.2	7.3	4.3	4.2- 9.1
10	2.9	4.2	2.2	2.6	4.5	3.4	1.7	3.3	3.1	1.4- 4.5

Table 5.3. The percentage of motoneurones per rostro-caudal tenth of embryo pairs and the control range of percentage motoneurones per rostro-caudal tenth of embryos on day 10 of incubation. Motoneurone number per rostro-caudal tenth expressed as a percentage of the control side total motoneurone number. The percentage of motoneurones per rostro-caudal tenth of embryos with radical ventral amputation has been added to the percentage of motoneurones per rostro-caudal tenth of embryos with radical dorsal amputation.

	Percentage motoneurones per rostro-caudal tenth										Total
Embryo	1	2	3	4	5	6	7	8	9	10	M.N. No.
A278	2.5	10.3	14.6	14.7	15.2	13.9	14.2	7.8	5.2	1.6	15,600
A280	4.6	12.6	15.6	13.8	15.5	12.4	11.1	6.3	5.1	3.0	15,950
P23	4.8	12.2	15.4	14.3	13.7	12.5	10.2	8.8	5.3	3.0	17,000
P29	4.0	10.3	12.3	13.8	14.6	13.1	12.1	10.1	5.3	4.4	14,620
P33	5.4	9.8	13.7	12.1	14.7	14.3	12.6	9.5	5.7	2.2	14,020
P34	3.2	8.9	13.3	13.3	13.3	15.6	14.2	9.7	5.6	3.0	13,000
P37	3.4	10.3	12.1	15.3	12.7	14.4	12.5	9.5	6.3	3.6	14,440
P38	4.8	10.0	13.1	14.8	14.4	14.6	13.0	7.9	5.3	2.6	15,120
P48	4.6	11.5	14.1	14.1	14.3	12.6	11.9	8.6	4.8	3.5	15,510
P49	4.2	9.4	14.5	17.3	15.2	12.5	13.6	6.8	4.7	2.0	15,100
P61	4.2	10.7	15.1	14.6	13.4	14.3	12.3	7.6	4.5	3.4	14,230
P67	3.1	8.7	13.4	14.3	12.7	12.9	12.8	11.5	5.9	4.5	14,570
P71	4.1	8.6	12.3	12.2	15.8	16.2	13.8	8.5	5.4	3.7	14,760
P75	4.5	11.1	13.4	14.2	14.4	13.9	12.1	8.6	5.1	2.7	15,040
P88	5.5	11.5	14.3	14.5	12.8	11.5	12.0	8.3	6.7	3.0	13,400
P104	3.7	8.8	12.3	11.9	12.8	13.4	14.3	13.5	6.3	2.9	15,730
P165	5.0	12.3	14.5	14.1	14.1	13.5	12.1	7.6	5.5	1.4	13,700
P176	3.7	9.1	12.7	14.0	13.9	14.0	13.1	11.1	5.7	2.6	13,000
P181	3.3	9.7	12.7	14.3	13.0	14.7	14.4	9.0	5.6	3.4	15,280
P182	5.4	11.7	12.9	13.3	13.5	14.6	11.9	8.2	4.2	4.4	13,160
P186	3.9	9.2	12.8	13.8	14.2	14.1	13.4	10.9	5.5	2.3	14,480
P189	4.9	10.8	12.8	11.9	15.3	15.5	11.3	9.8	4.8	2.9	12,200
P229	5.2	14.2	14.4	15.6	13.1	12.3	11.2	6.8	4.2	3.1	10,800
P230	6.2	10.9	14.7	14.1	15.5	13.2	12.7	6.5	4.3	2.0	13,260
P231	6.7	12.0	15.2	12.5	13.9	12.7	12.2	8.7	3.8	2.5	10,770
P232	6.8	10.5	16.2	15.3	14.2	14.8	10.0	6.6	3.5	2.2	12,240
P234	2.8	7.4	12.6	14.7	17.5	14.5	12.4	10.4	4.7	3.0	11,560
P296	3.8	7.8	13.1	13.9	13.0	14.7	15.4	9.4	5.5	3.5	11,220
P318	4.7	11.2	11.5	13.8	11.0	15.5	11.2	13.8	4.6	2.7	11,640
T73	4.3	7.8	9.3	12.8	14.6	14.8	13.6	12.6	7.6	2.4	14,360
T95	6.3	11.9	9.9	11.4	11.4	12.7	13.8	10.5	9.1	3.0	15,640
T147	3.6	10.2	14.5	15.9	14.3	14.1	11.2	9.0	4.3	3.1	11,480
T165	5.9	10.4	11.6	13.8	14.1	13.7	14.0	9.8	4.6	2.1	13,910
T173	4.9	8.1	12.6	13.1	13.7	12.3	12.5	11.9	7.5	3.4	11,610
T177	4.5	12.0	12.9	13.2	13.6	12.6	12.6	10.9	5.5	2.2	13,910
Range	2.5-	8.6-	9.3	11.4-	11.0-	11.5-	10.0-	6.3-	4.2-	1.4-	
	6.8	14.2	16.0	17.3	17.5	16.2	15.4	13.8	9.1	4.5	

Table 5.4.

Total motoneurone number and the percentage of motoneurones per rostro-caudal tenth in control sides of embryos on day 10 of incubation. Motoneurone number expressed as a percentage of the control side total motoneurone number.

Table 5.5

Total motoneurone number and motoneurone number per rostro-caudal tenth on control and amputated sides of embryos on day 10 of incubation Transverse amputation on day 4 of incubation. R- rostral. C- caudal.

Embryo	Total Motoneurone Number.	Motoneurone number per rostro-caudal tenth									
		R. 1	2	3	4	5	6	7	8	9	10 C.
P181 control	15,280	498	1,478	1,944	2,180	1,990	2,242	2,204	1,372	854	518
amp	12,060	480	1,406	1,875	1,875	1,592	1,430	1,445	965	731	279
P182 control	13,160	704	1,540	1,694	1,752	1,770	1,924	1,572	1,072	556	576
amp	1,530	20	118	125	123	104	260	206	134	298	142
P186 control	14,480	556	1,328	1,850	1,998	2,058	2,036	1,940	1,576	792	336
amp	5,800	376	772	1,026	964	722	434	462	380	384	280
P189 control	12,200	592	1,322	1,567	1,449	1,870	1,888	1,372	1,200	590	350
amp	5,890	404	812	1,260	986	528	436	352	322	510	280
P230 control	13,200	816	1,450	1,952	1,866	2056	1,746	1,680	868	566	266
amp	11,290	586	1,658	1,550	1,612	1,534	1,316	1,498	758	536	242
P231 control	10,770	724	1,288	1,633	1,343	1,497	1,367	1,310	936	408	246
amp	9,760	540	1,318	1,460	1,184	1,408	1,204	1,296	830	336	184
P232 control	12,240	830	1,290	1,980	1,870	1,735	1,815	1,220	810	425	265
amp	11,380	628	1,400	2,060	1,520	1,652	1,502	1,256	620	434	308
P234 control	11,560	328	854	1,461	1,733	1,989	1,677	1,439	1,197	539	343
amp	8,160	336	938	1,536	1,564	1,216	744	492	564	452	318

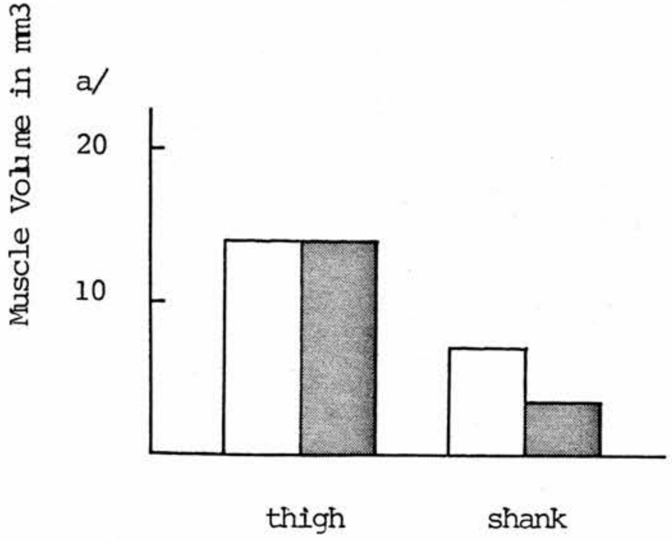
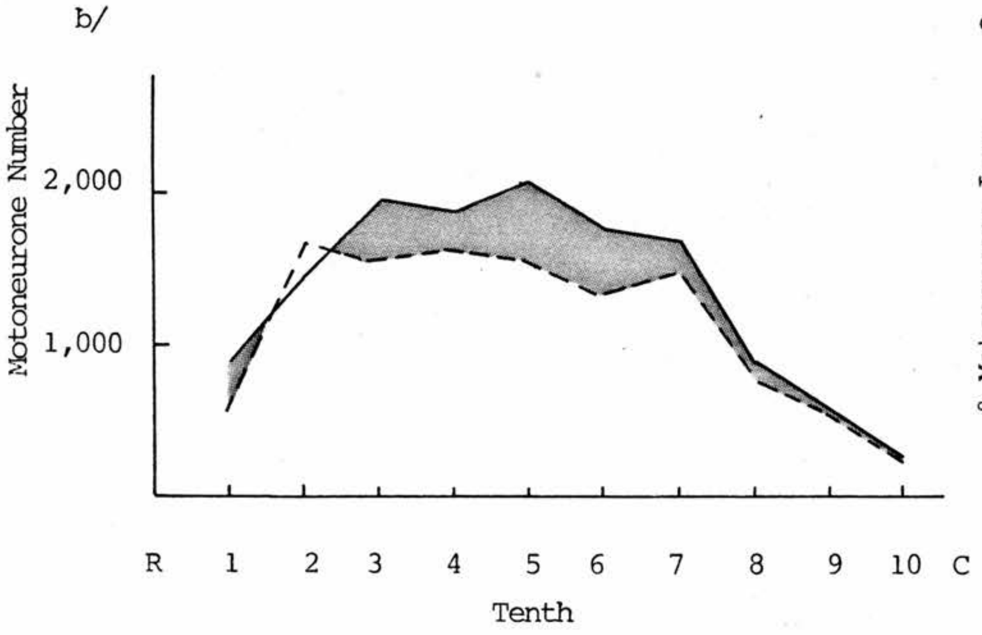
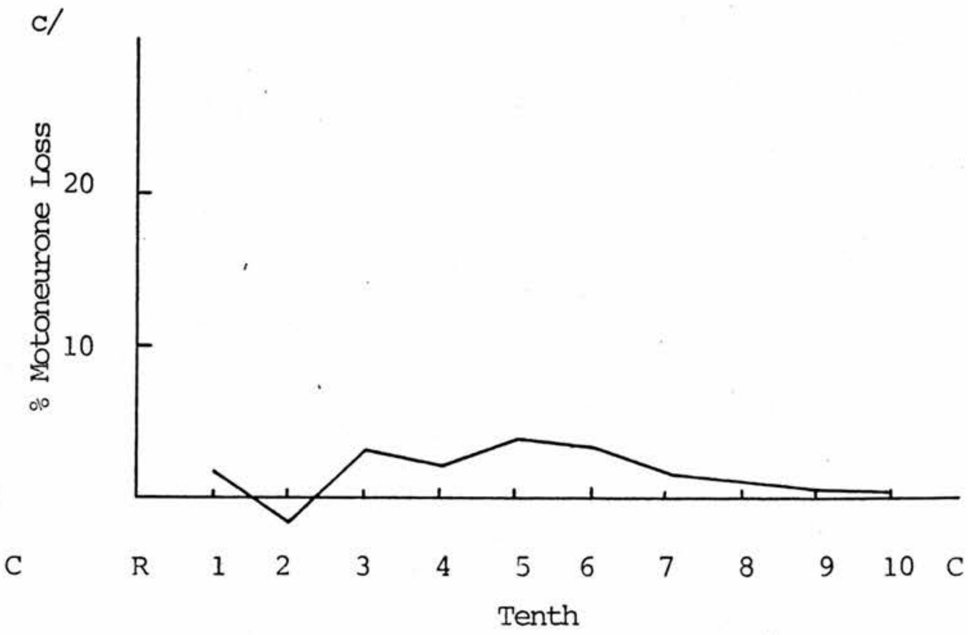


Figure 5.7 Embryo P230. Day 10 of incubation.
a/ Thigh and shank muscle volumes in control (clear area) and amputated (shaded area) limbs.
b/ Motoneurone number per rostro-caudal tenth in control (solid line) and amputated (dashed line) side lateral motor columns. Shaded area represents position and extent of motoneurone loss between the two sides.
c/ Motoneurone loss per rostro-caudal tenth in the amputated side lateral motor column compared to the control side. Motoneurone loss expressed as a percentage of the control side total motoneurone number.
R- rostral. C- caudal.

Transverse amputation on day 4 of incubation.





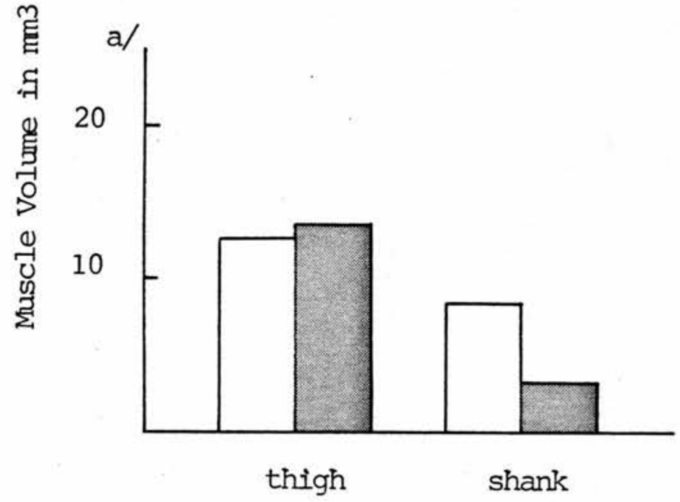
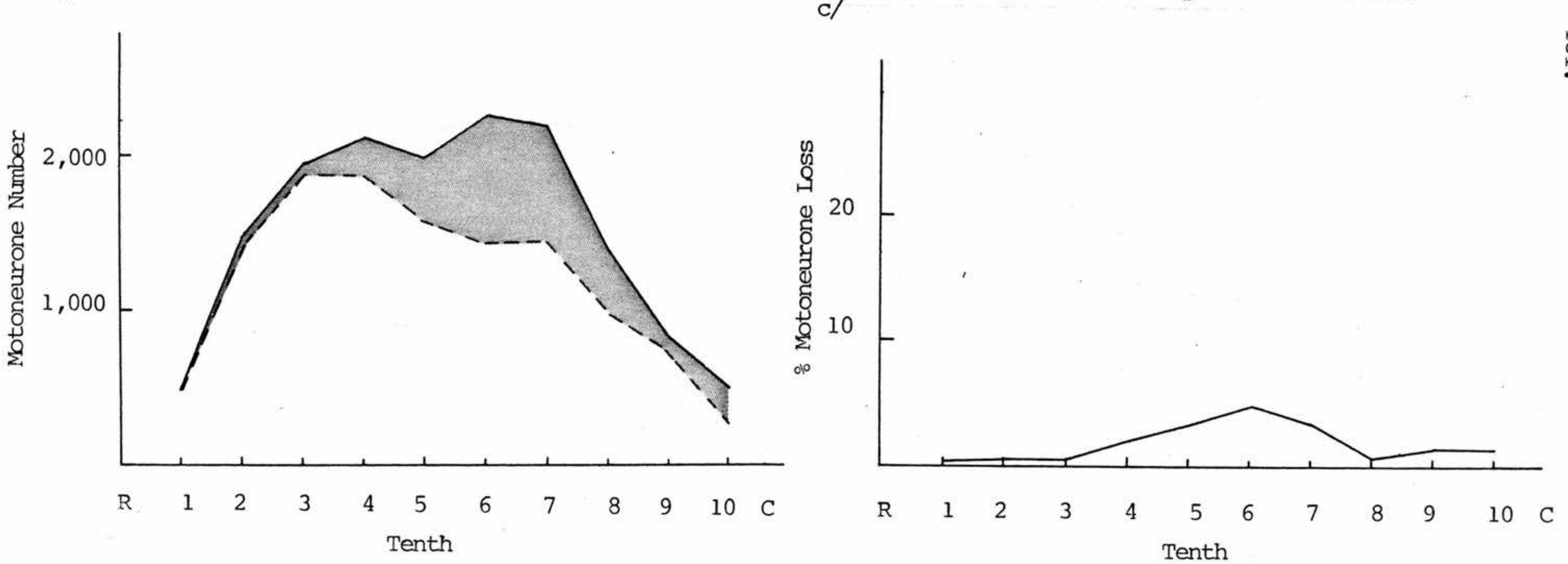
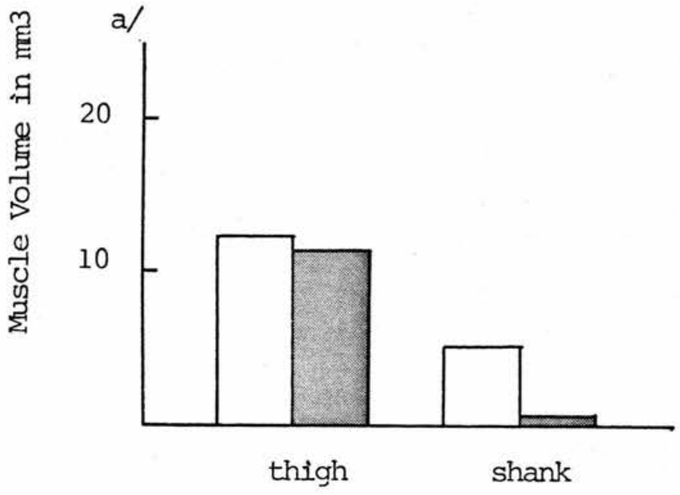
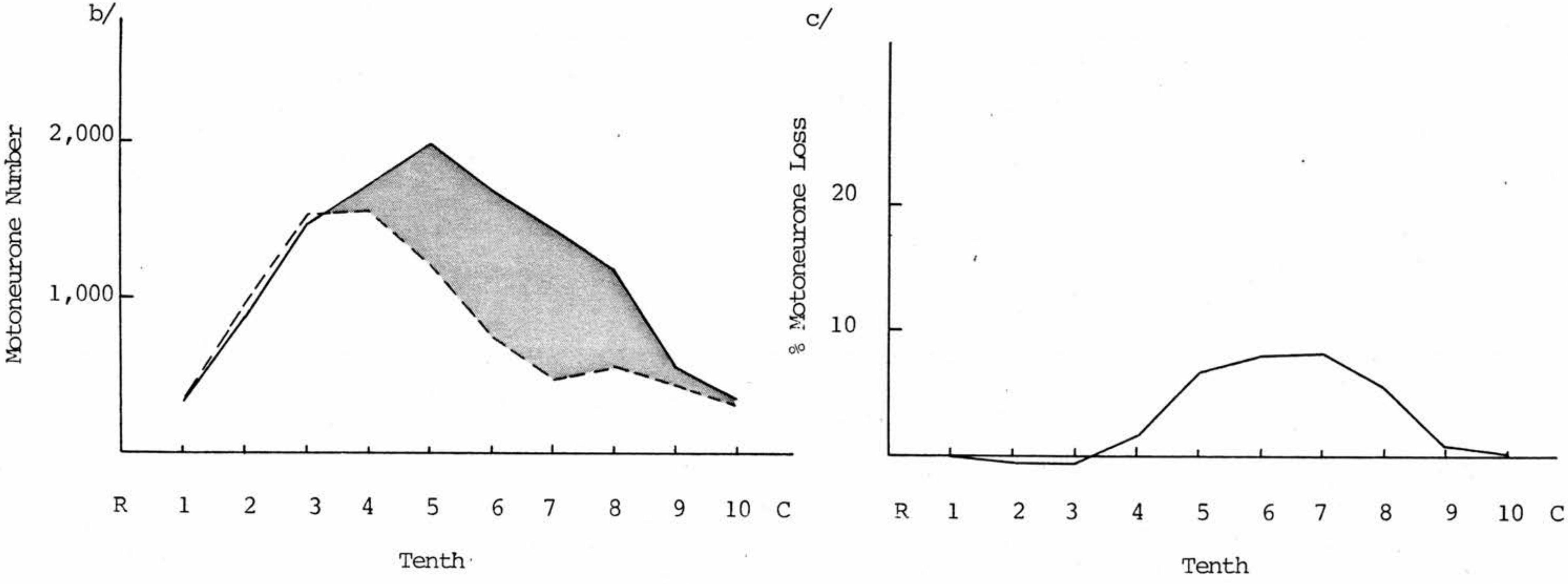


Figure 5.8 . Embryo P181. Day 10 of incubation.
a/ Thigh and shank muscle volumes in control (clear area) and amputated (shaded area) limbs.
b/ Motoneurone number per rostro-caudal tenth in control (solid line) and amputated side (dashed line) lateral motor columns. Shaded area represents position and extent of motoneurone loss between the two sides.
c/ Motoneurone loss per rostro-caudal tenth in the amputated side lateral motor column compared to the control side. Motoneurone loss expressed as a percentage of the control side total motoneurone number. R-rostral. C-caudal.

Transverse amputation on day 4 of incubation.







<u>Figure 5.9 . Embryo P234. Day 10 of incubation.</u>

a/ Thigh and shank muscle volume in control (clear area) and amputated (shaded area) limbs.

b/ Motoneurone number per rostro-caudal tenth in control (solid line) and amputated side (dashed line) lateral motor columns. Shaded area represents position and extent of motoneurone loss between the two sides.

c/ Motoneurone loss per rostro-caudal tenth in the amputated side lateral motor column compared to the control side. Motoneurone loss expressed as a percentage of the control side total motoneurone number.

R-rostral. C-caudal.

Transverse amputation on day 4 of incubation.

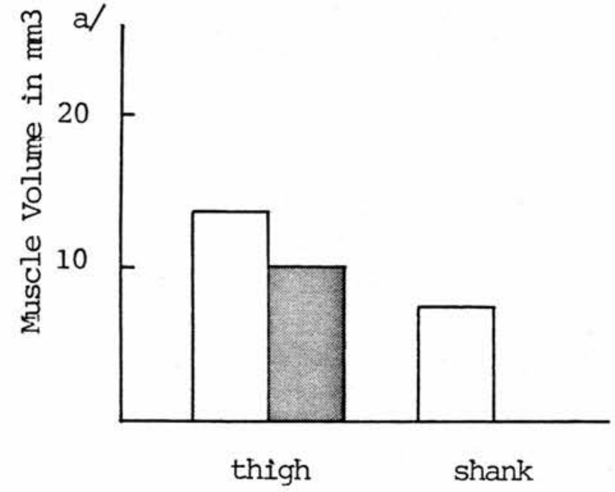


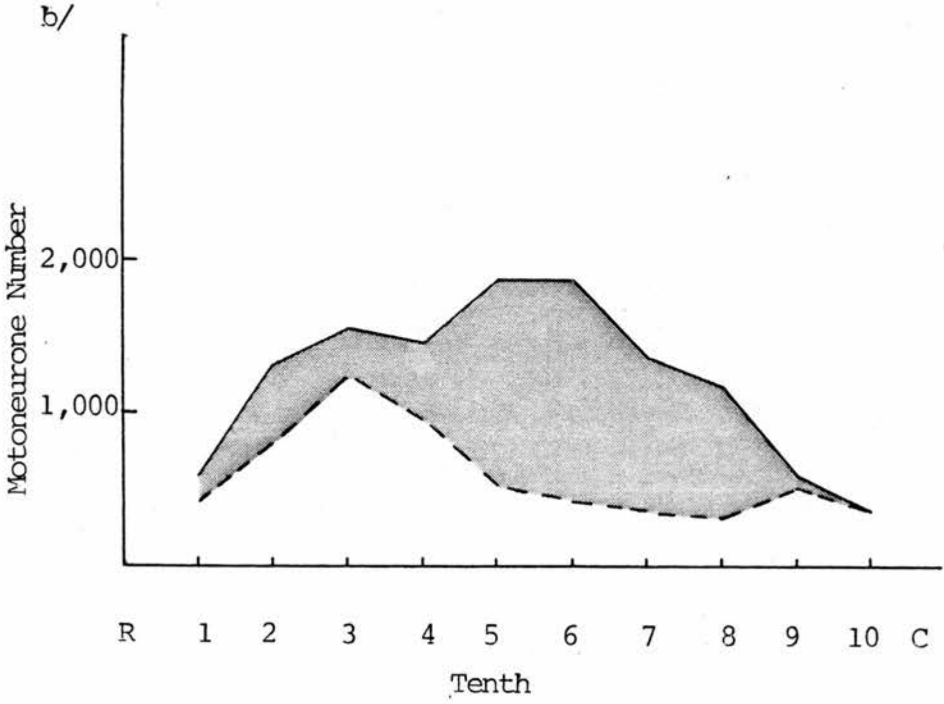
Figure 5.10. Embryo P189. Day 10 of incubation.

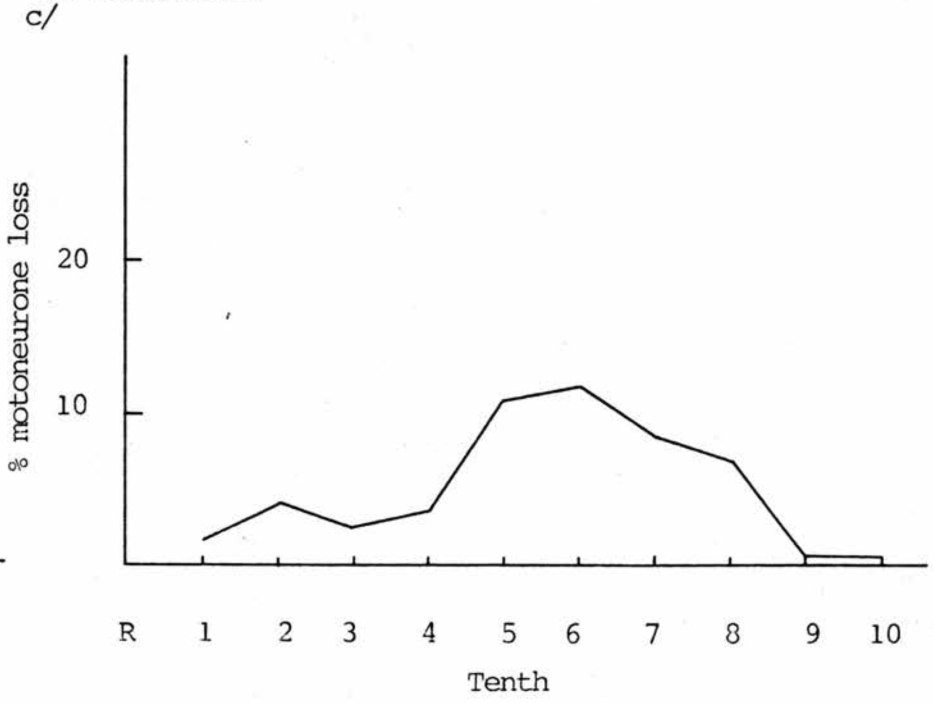
a/ Thigh and shank muscle volume in control (clear area) and amputated (shaded area) limbs.

b/ Motoneurone number per rostro-caudal tenth in control (solid line) and amputated side (dashed line) lateral motor columns. Shaded area represents position and extent of motoneurone loss between the two sides.

c/ Motoneurone loss per rostro-caudal tenth in the amputated side lateral motor column compared to the control side. Motoneurone loss expressed as a percentage of the control side total motoneurone number.

R-rostral. C-caudal. Transverse amputation on day 4 of incubation.





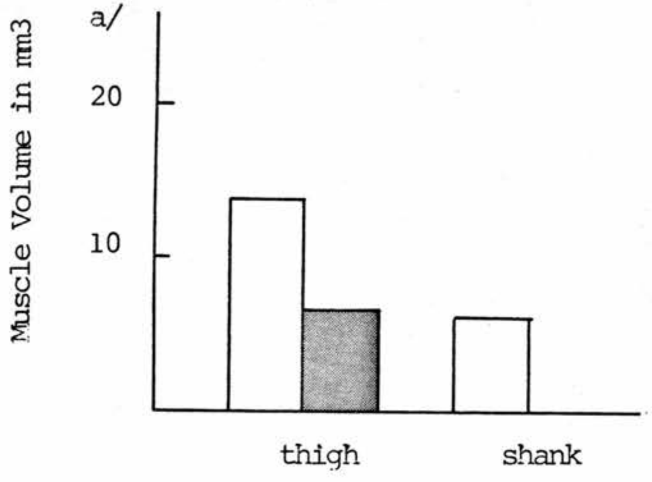
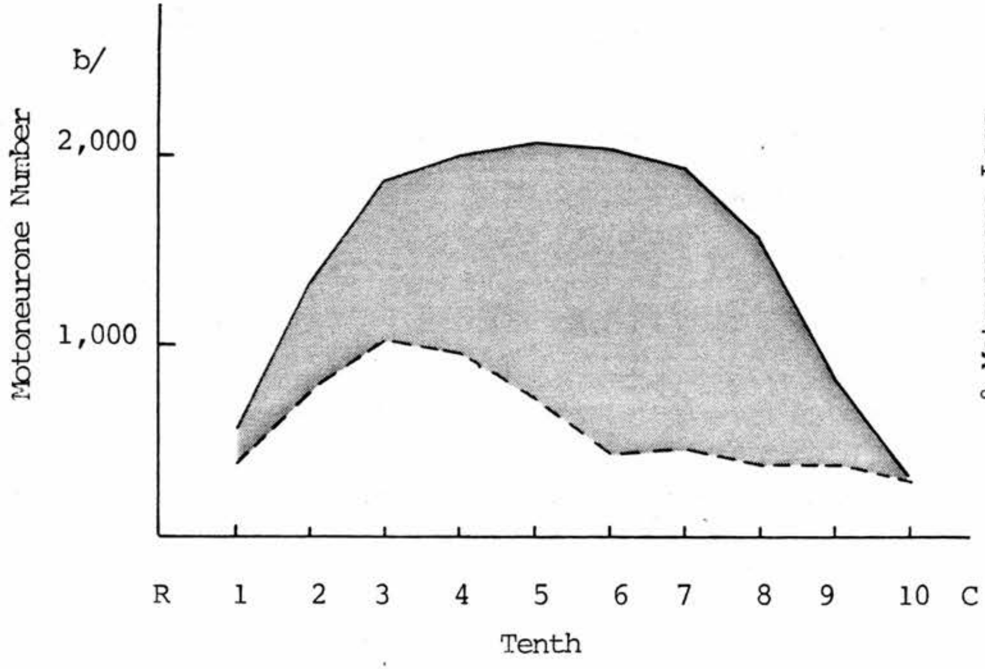
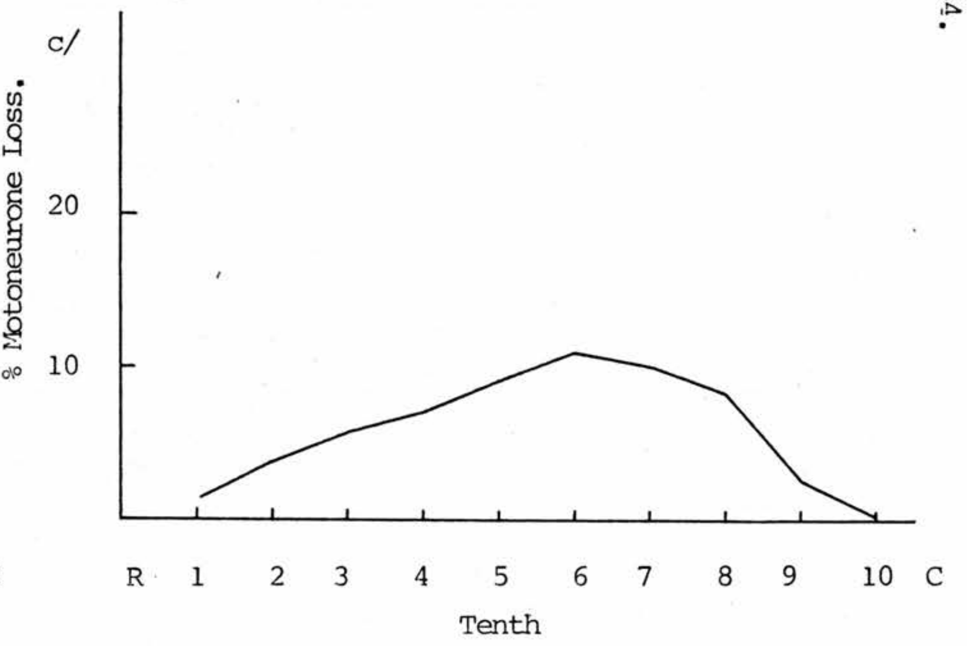
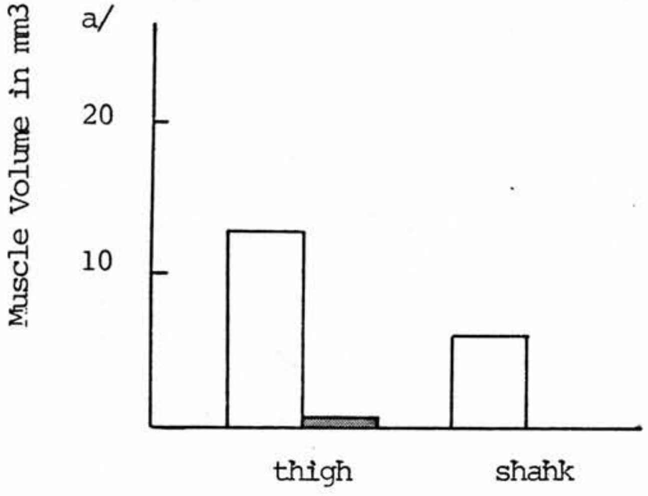


Figure 5.11 Embryo P186. Day 10 of incubation.
a/ Thigh and shank muscle volume in control (clear area) and amputated (shaded area) limbs.
b/ Motoneurone number per rostro-caudal tenth in control (solid line) and amputated side (dashed line) lateral motor columns. Shaded area represents position and extent of motoneurone loss between the two sides.
c/ Motoneurone loss per rostro-caudal tenth in the amputated side lateral motor column compared to the control side. Motoneurone loss expressed as a percentage of the control side total motoneurone number.
R-rostral. C-caudal. Transverse amputation on day 4 of incubation.







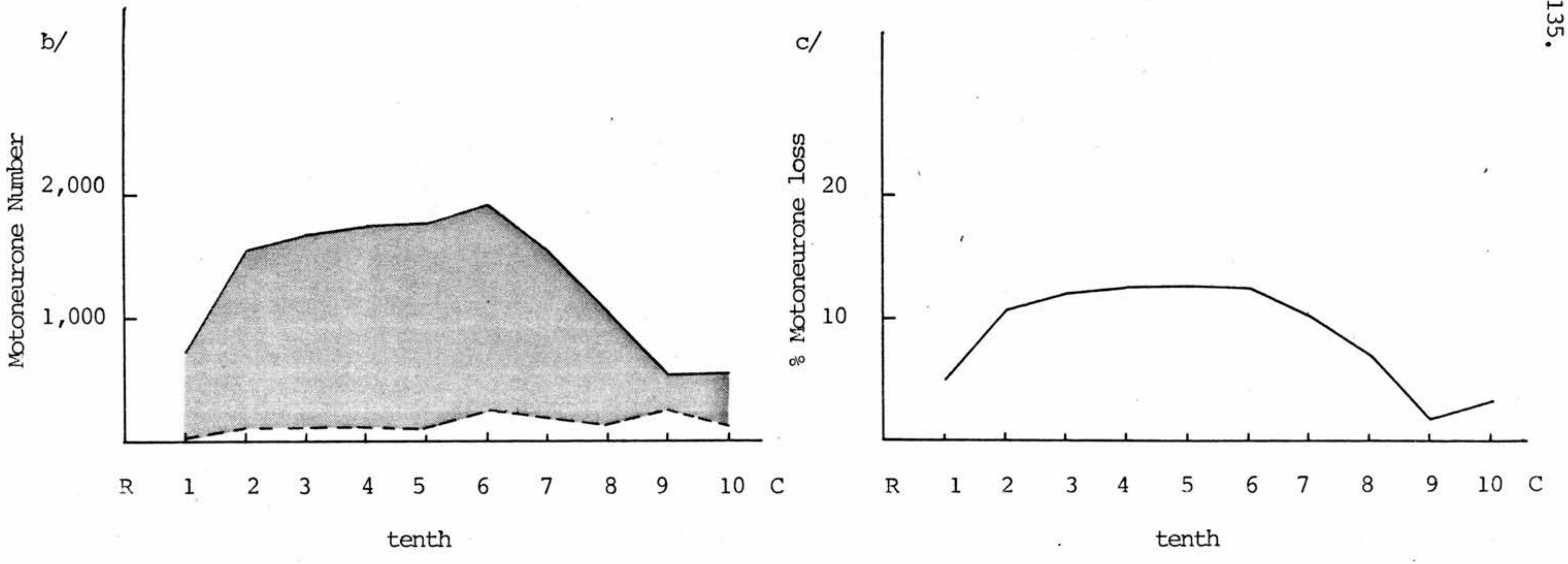


Figure 5.12. Embryo P182. Day 10 of incubation.

a/ Thigh and shank muscle volume in control (clear area) and amputated (shaded area) limbs.
b/ Motoneurone number per rostro-caudal tenth in control (solid line) and amputated side (dashed line) lateral motor columns. Shaded area represents position and extent of motoneurone loss between the two sides.
c/ Motoneurone loss per rostro-caudal tenth in the amputated side lateral motor column compared to the control side. Motoneurone loss expressed as a percentage of the control side total motoneurone number.
R-rostral. C-caudal. Transverse amutation on day 4 of incubation.

Figs (5.7-5.9) show the results of three embryos with varying degrees of amputation of the shank ranging from under 50% less to almost entire loss. Thigh volumes remain unaffected. In all three embryos motoneurone loss occurs from mid to caudal tenths 4-10, with little or no motoneurone loss from rostral tenths 1-2. However there is some motoneurone loss from rostro-caudal tenth 3 for embryo P230 - fig (5.7c).

Figs (5.10-5.12) show the results of three embryos with varying degrees of amputation of the thigh as well as the entire shank. Loss of some 25% of the thigh, fig (5.10), is associated with loss of motoneurones from rostral tenths 1,3 as well as extensive loss from mid to caudal tenths 4-10. Loss of 50% of the thigh is associated with greater motoneurone loss from tenths 1-4, fig (5.11), than for the previous embryo. Almost entire amputation of the limb, fig (5.12), results in extensive motoneurone loss from the entire rostro-caudal extent of the lateral motor column.

Motoneurone loss due to amputation of the shank only therefore occurs predominantly from mid to caudal tenths of the lateral motor column. Amputation of the thigh and the shank results in motoneurone loss from rostral as well as mid to caudal tenths of the lateral motor column. The position of loss of motoneurones due to amputation of the thigh may therefore be deduced to occur predominantly from rostral tenths, although some loss may occur from more mid to caudal tenths.

5.2 Medio-Lateral Position

Motoneurone position in the medio-lateral axis of the lateral motor column was determined by motoneurone 'birthdate'.

Motoneurones that normally innervate ventral muscles can be distinguished from motoneurones that normally innervate dorsal muscles by an injection of [3H] - thymidine at an appropriate developmental stage. Accordingly embryos were injected with 10μCi [3H] - thymidine at stage 19 of development. Autoradiographs of control sides of two such embryos are shown in fig (5.13a,b). The lateral motor column motoneurones are identified by their well-defined nucleii and darkly staining cytoplasm. The motoneurones that underwent their final division before the injection of [3H] - thymidine and which therefore appear 'unlabelled' on the autoradiograph lie in a cluster on the medial border of the lateral motor column. The motoneurones that underwent their final division after the injection of [3H] - thymidine and which are therefore 'labelled' with silver grains on the autoradiograph lie more laterally within the lateral motor column. An injection of [3H] - thymidine at stage 19 of development therefore distinguishes, by leaving 'unlabelled' on an autoradiograph, the first-born motoneurones which lie on the medial border of the lateral motor column, from the later-born, more laterally positioned motoneurones within the lateral motor column.

However injection of [3H] - thymidine at stage 19 of development does not distinguish the entire motoneurone population that normally innervates ventral muscle from the motoneurone population that normally innervates dorsal muscle. The number of motoneurones remaining 'unlabelled' by the injection schedule are shown in table (5.6). Between 11% and 25% of motoneurones are 'unlabelled'. However ventral muscle normally supports 42.4% of

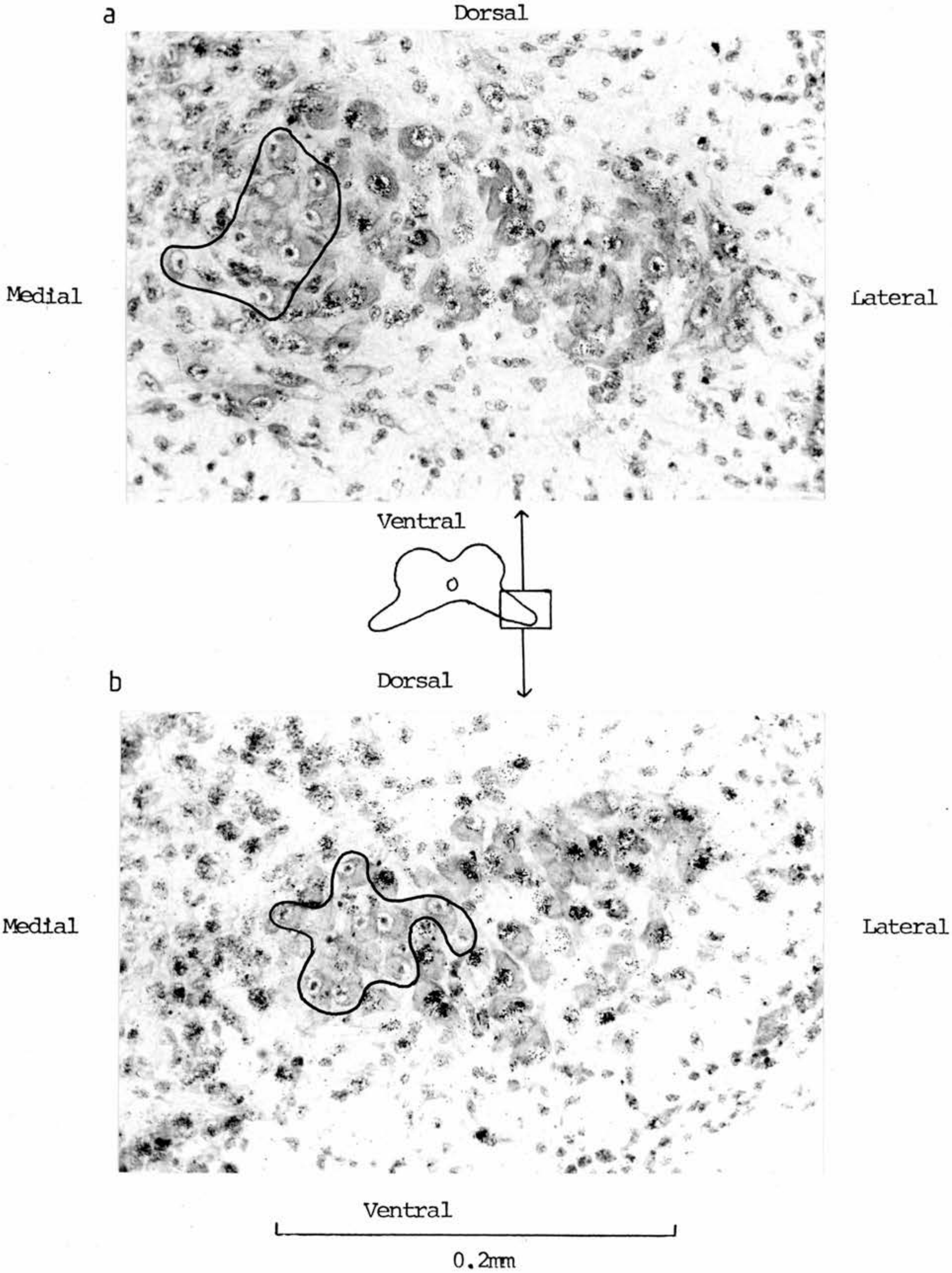


Figure 5.13 a,b.

Autoradiographs of the control sides of embryos on day 10 of incubation. 10μCi 3H thymidine at stage 19 of development. Motoneurones that underwent their final division before the injection of thymidine appear 'unlabelled' with silver grains and lie in a cluster on the medial border of the lateral motor column. They are outlined in black.

Embryo	Total Motoneurone Number	'Unlabelled' Motoneurone Number	% 'Unlabelled' Motoneurones
T73	14,360	3,540	24.7
T95	15,640	2,480	15.9
T147	11,480	1,360	11.8
T177	13,900	3,060	22.0

Table 5.6

Motoneurone number, 'unlabelled' motoneurone number and the percentage of 'unlabelled' motoneurones in control sides of embryos on day 10 of incubation. 10μCi [3H] - thymidine at stage 19 of development. 'Unlabelled' motoneurone number expressed as a percentage of total motoneurone number.

motoneurones on day 10 of incubation, fig (4.5). The injection schedule used therefore only distinguishes a proportion of the motoneurone population that normally innervates ventral muscle - that is the first-born motoneurones that come to occupy an extreme medial position within the lateral motor column.

An injection schedule that distinguishes the entire motoneurone population that normally innervates ventral muscle is not possible because the lateral motor column is laid down in rostro-caudal as well as medio-lateral temperal sequence. Motoneurones that normally innervate dorsal muscle are formed in the rostral segments at the same time as motoneurones that normally innervate ventral muscle are formed in the caudal segments (Hollyday & Hamburger 1977; Hollyday 1978).

5.2.1. The Effect of Amputation on 'Unlabelled' and 'Labelled' Motoneurone Populations

Ventral Amputation

Amputation of the ventral muscle mass on day 4 of incubation results in almost total disappearance of the 'unlabelled' motoneurone population on day 10 of incubation as well as a reduction in the 'labelled' motoneurone population.

Fig (5.14a) shows that almost total ventral amputation (99.3% loss) reduces the 'unlabelled' motoneurone population from 22% on the control side to 2.9% on the amputated side.*
The 'labelled' motoneurone population is reduced from 78% on the control side to 41.7% on the amputated side.

* Numbers of motoneurones are expressed as a percentage of the total number of motoneurones on the control side.

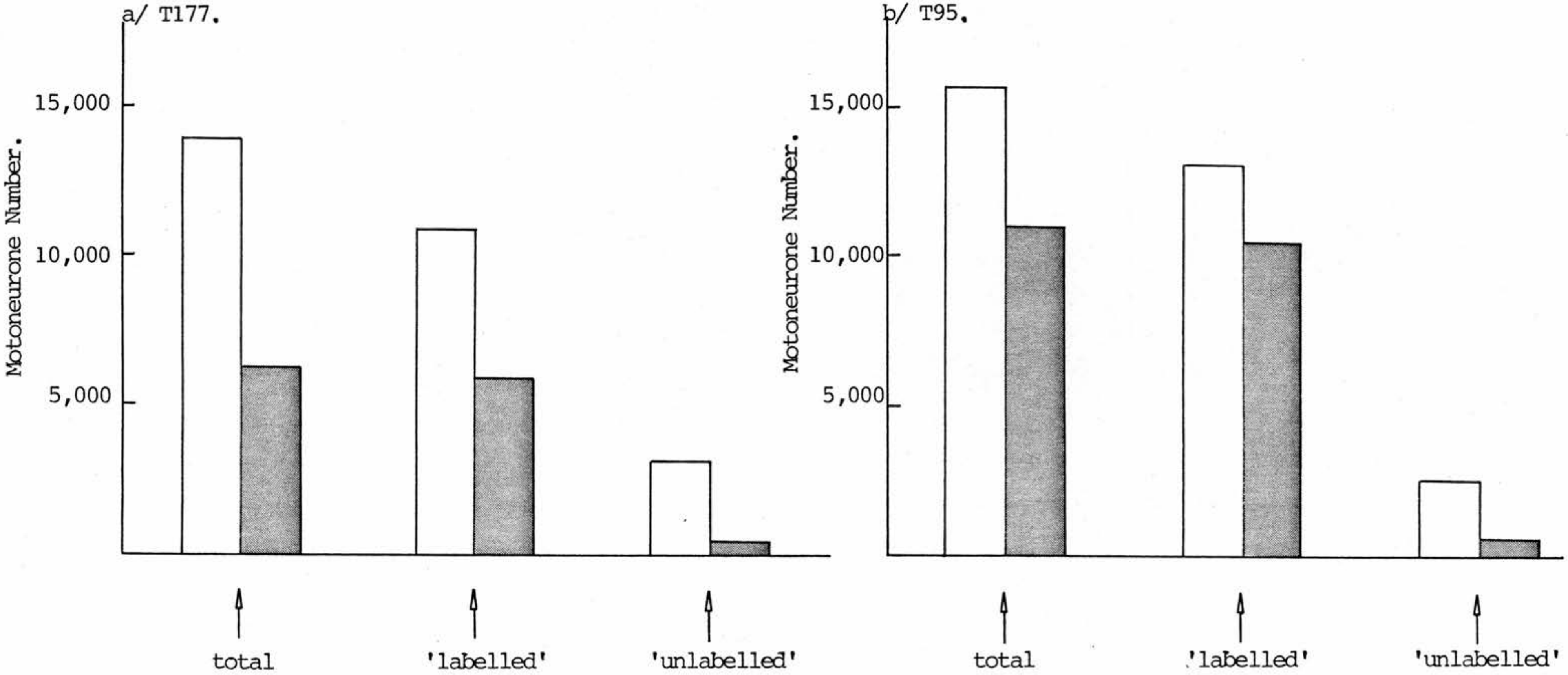


Figure 5.14.

Total, 'labelled', and 'unlabelled' motoneurone number in embryos at day 10 of incubation. 10µCi 3H-thymidine at stage 19 of development. Ventral amputation on day 4 of incubation. Clear area represents the control side, the shaded area the amputated side of embryos.

Similarly fig (5.14) shows that amputation of some 30% of ventral muscle reduces the 'unlabelled' motoneurone population from 15.9% on the control side to 3.5% on the amputated side. The 'labelled' motoneurone population is reduced from 84.1% on the control side to 66.8% on the amputated side.

The almost total reduction of the 'unlabelled' motoneurone population and the partial reduction of the 'labelled' motoneurone population following ventral amputation is illustrated by the autoradiographs shown in fig (5.15a,b). Note:

(1) The presence of medially positioned 'unlabelled' motoneurones in the control side lateral motor columns.

(2) The absence of the 'unlabelled' motoneurone population in the amputated side lateral motor column

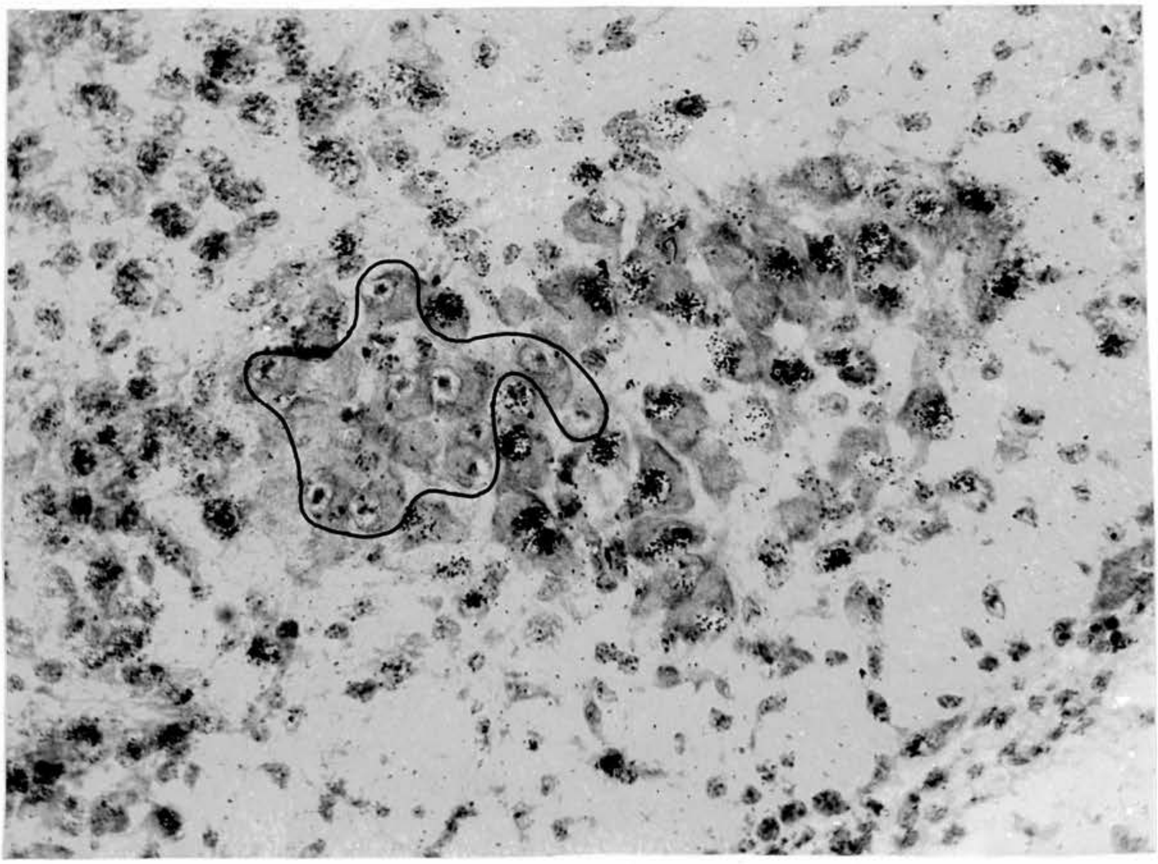
(3) The partial reduction in the 'labelled' motoneurone population in the amputated side lateral motor column

The distribution of 'unlabelled' and total motoneurones along the rostro-caudal axis for the control and amputated sides of embryos, given as motoneurone number per rostro-caudal tenth, is shown in figs (5.16) and (5.17).

The control side lateral motor columns of embryos show that 'unlabelled' motoneurones occur along the entire rostro-caudal extent of the lateral motor column but with greater numbers rostrally than caudally. Ventral amputation reduces the numbers of 'unlabelled' motoneurones along the entire rostro-caudal extent of the lateral motor column. In fig (5.16) the few remaining 'unlabelled' motoneurones lie in rostral tenths 1-6. In fig (5.17) the remaining 'unlabelled' motoneurones lie predominantly in rostro-caudal tenth 9, although they do occur along the entire rostro-caudal extent of the lateral motor column.

Dorsal

M.



L.

Ventral

0.2mm

Figure 5.15a.

Autoradiograph of the control lateral motor column of an embryo on day 10 of incubation following ventral amputation of the right hind limb bud on day 4 of incubation. 10μCi 3H-thymidine at stage 19 of development. The 'unlabelled' motoneurones lie in a cluster on the medial border of the lateral motor column, and are outlined in black. M- medial. L- lateral.

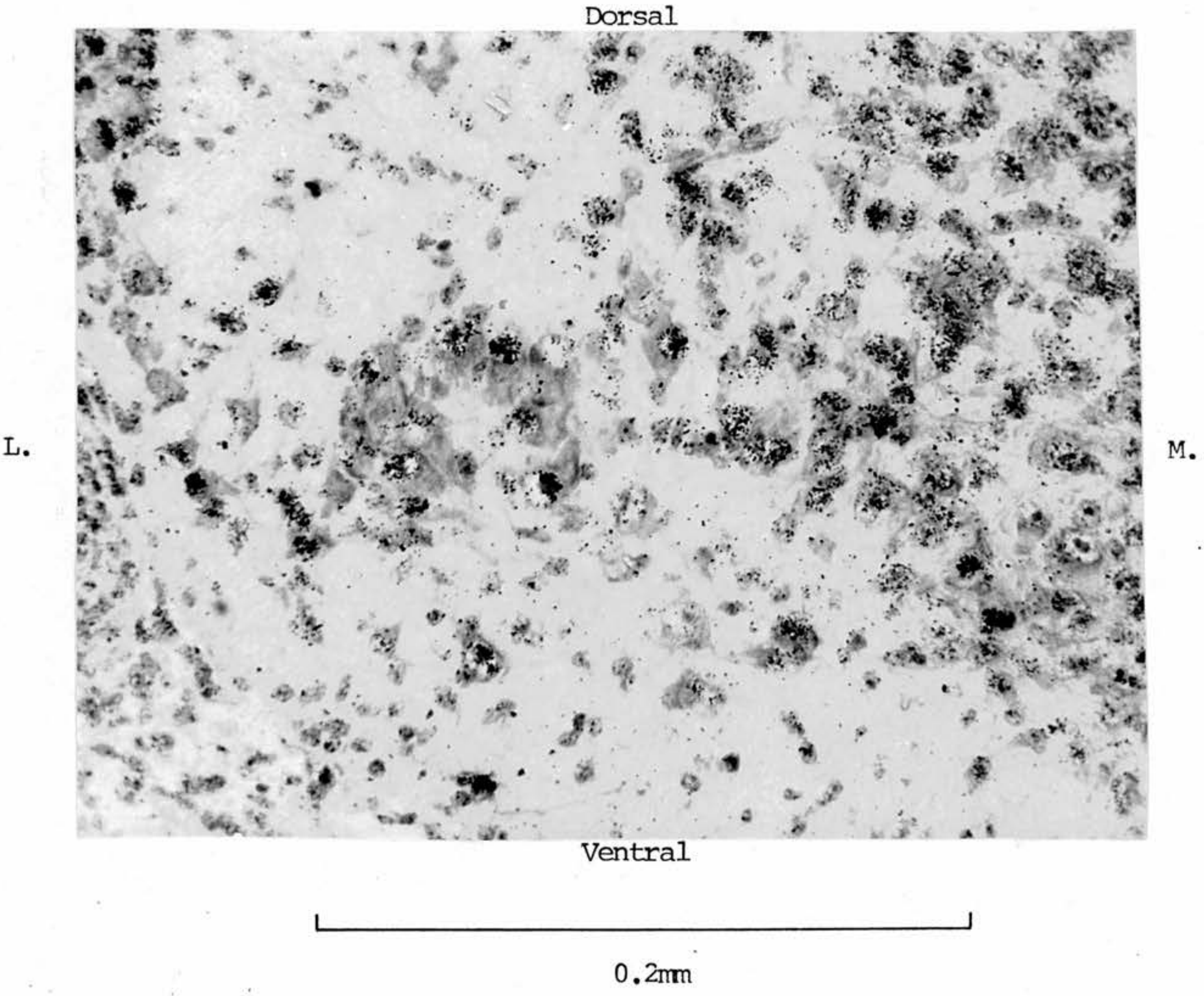


Figure 5.15b.

Autoradiograph of the amputated side lateral motor column of an embryo on day 10 of incubation following ventral amputation of the right hind limb bud on day 4 of incubation. 10µCi 3H- thymidine at stage 19 of development. The 'unlabelled' motoneurone population has disappeared from the lateral motor column. The remaining motoneurones are 'labelled' although their number is reduced compared to the control side. M- medial. L- lateral.

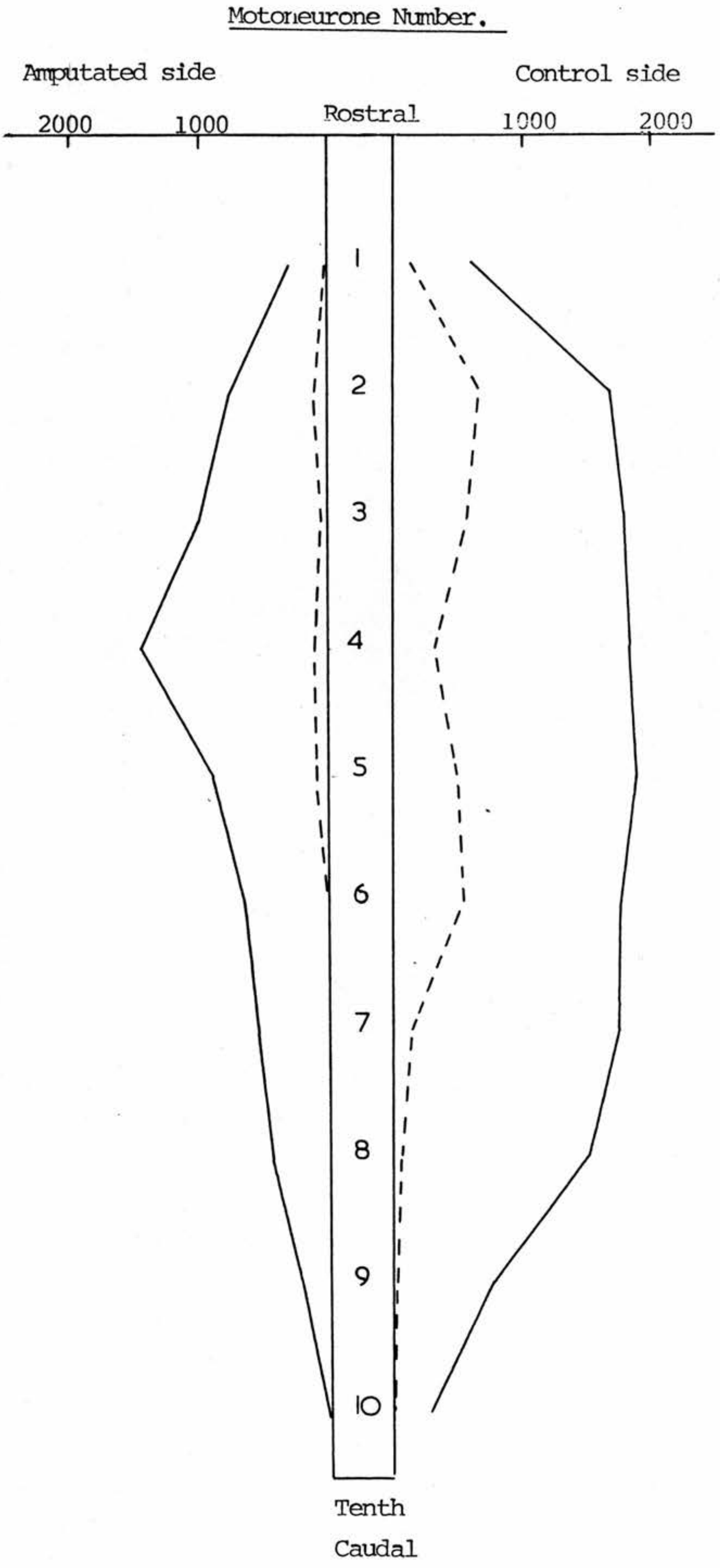


Figure 5.16. Motoneurone number (solid line) and 'unlabelled' motoneurone number (dashed line) per rostro-caudal tenth in embryo T177 on day 10 of incubation. 10μCi 3H-thymidine at stage 19 of development. Ventral amputation on day 4 of incubation.

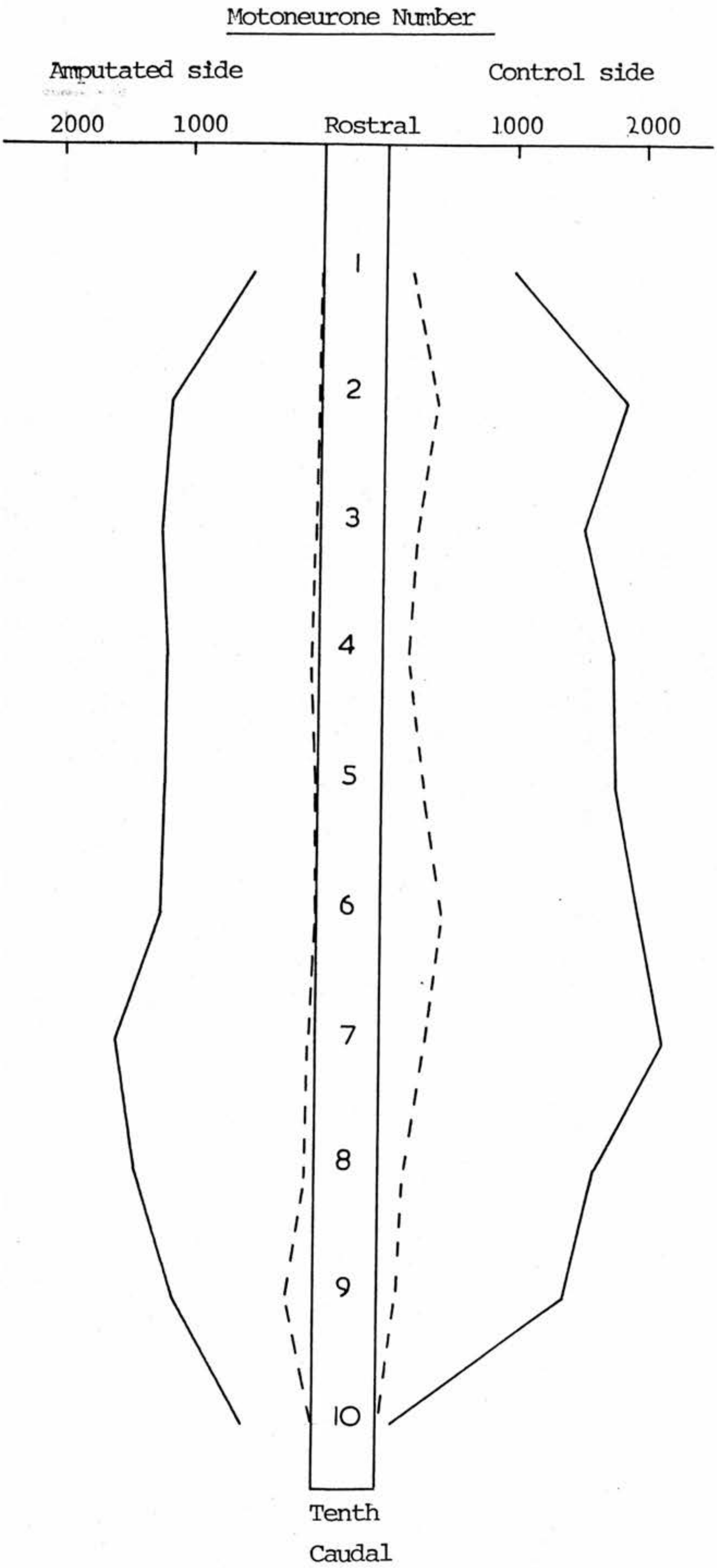


Figure 5.17.
Motoneurone number (solid line) and 'unlabelled' motoneurone number (dashed line) per rostro-caudal tenth in embryo T95 on day 10 of incubation. 10μCi 3H-thymidine at stage 19 of development. Ventral amputation on day 4 of incubation.

Amputation of the ventral muscle mass is therefore associated with loss of the first-born 'unlabelled' motoneurones from the entire rostro-caudal extent of the lateral motor column. The remaining muscle is innervated by later-born 'labelled' motoneurones.

Dorsal Amputation

Amputation of the dorsal muscle mass on day 4 of incubation has no effect on the 'unlabelled' motoneurone population on day 10, but results in a reduction of the 'labelled' motoneurone population.

Fig (5.18a) shows that a marked reduction in dorsal muscle volume (86%) reduces the number of 'labelled' motoneurones from 88.2% on the control side to 53.7% on the amputated side but does not affect the 'unlabelled' motoneurone population - 11.8% and 10% on the control and amputated sides respectively.*

Similarly fig (5.18b) shows that a 50% reduction in dorsal muscle volume reduces the 'labelled' motoneurone number from 75.3% on the control side to 51.3% on the amputated side. There is no effect on 'unlabelled' motoneurone number - 24.7% on the control side and 24.5% on the amputated side.

That amputation of the dorsal muscle mass has no effect on the 'unlabelled' motoneurone population but reduces 'labelled' motoneurone number is illustrated by the autoradiographs shown in fig (5.19a,b). Note:

(1) The presence of 'unlabelled' motoneurones in both control and amputated side lateral motor columns.

(2) The reduction in the number of 'labelled' motoneurones in the amputated side lateral motor column compared to the control side.

* See foot note at the bottom of page 140.

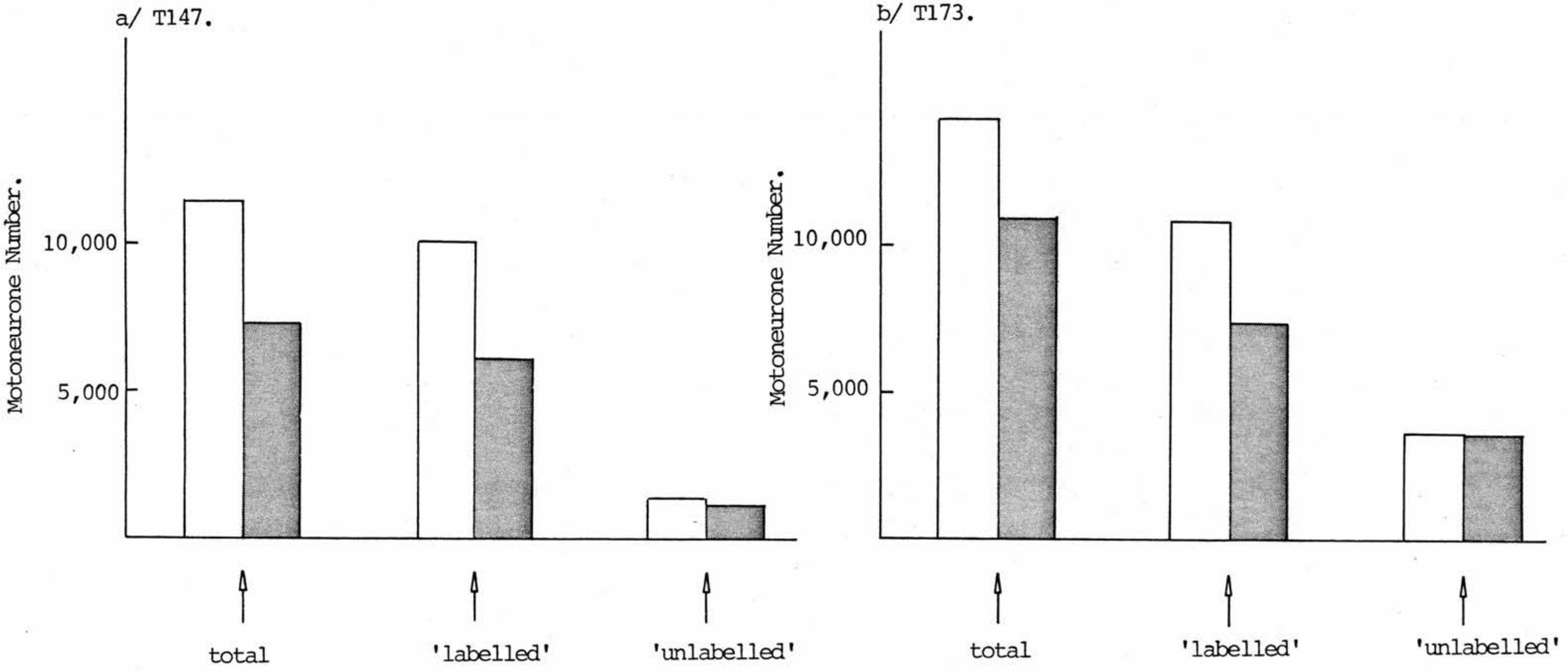


Figure 5.18.

Total, 'labelled', and 'unlabelled' motoneurone number in embryos at day 10 of incubation. 10μCi 3H-thymidine at stage 19 of development. Dorsal amputation on day 4 of incubation. Clear area represents the control side, the shaded area the amputated side of embryos.

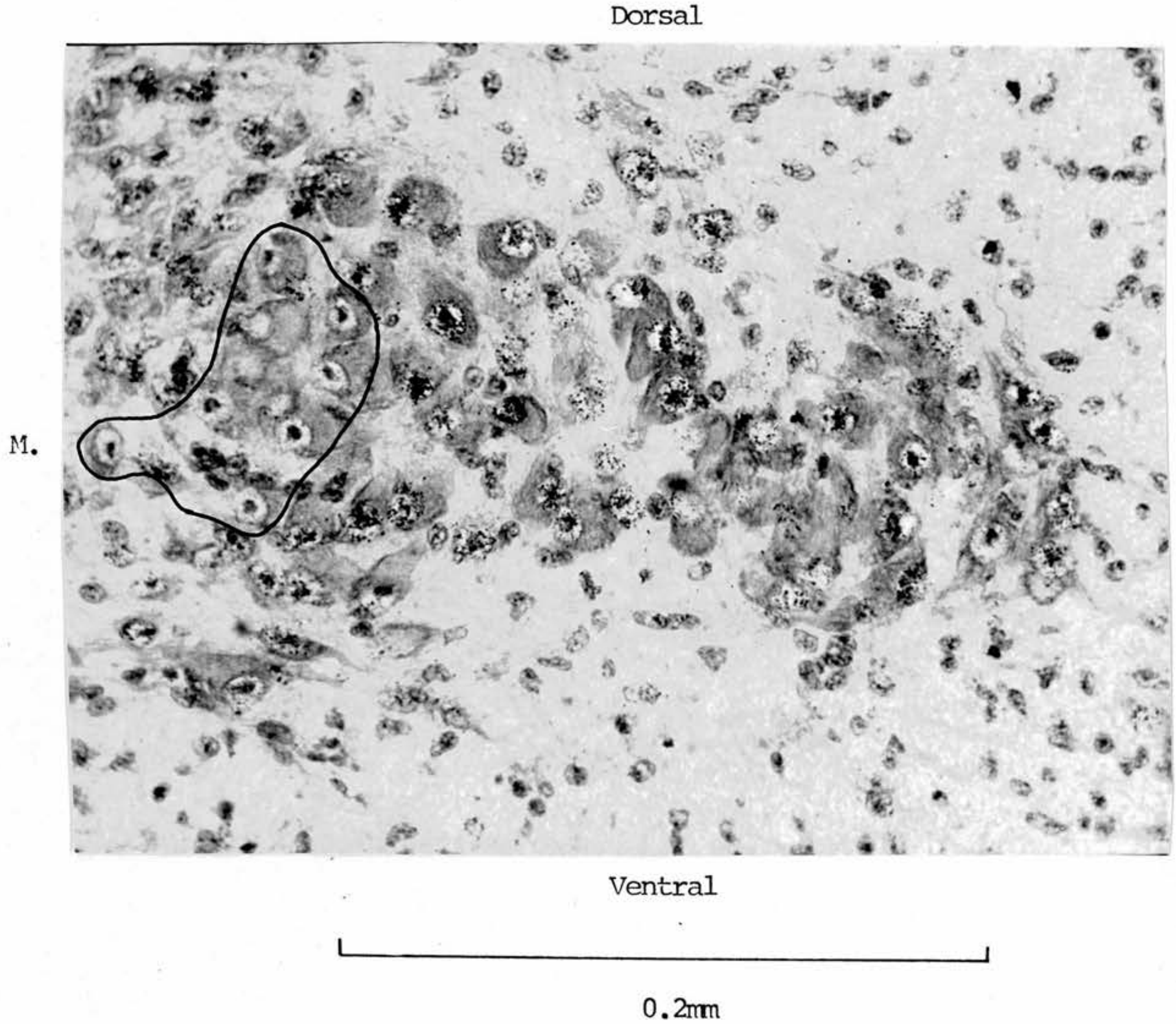


Figure 5.19a.

Autoradiograph of the control lateral motor column of an embryo on day 10 of incubation following dorsal amputation of the right hind limb bud on day 4 of incubation. 10μCi 3H-thymidine at stage 19 of development. The 'unlabelled' motoneurones lie in a cluster on the medial border of the lateral motor column, and are outlined in black. M- medial. L- lateral.

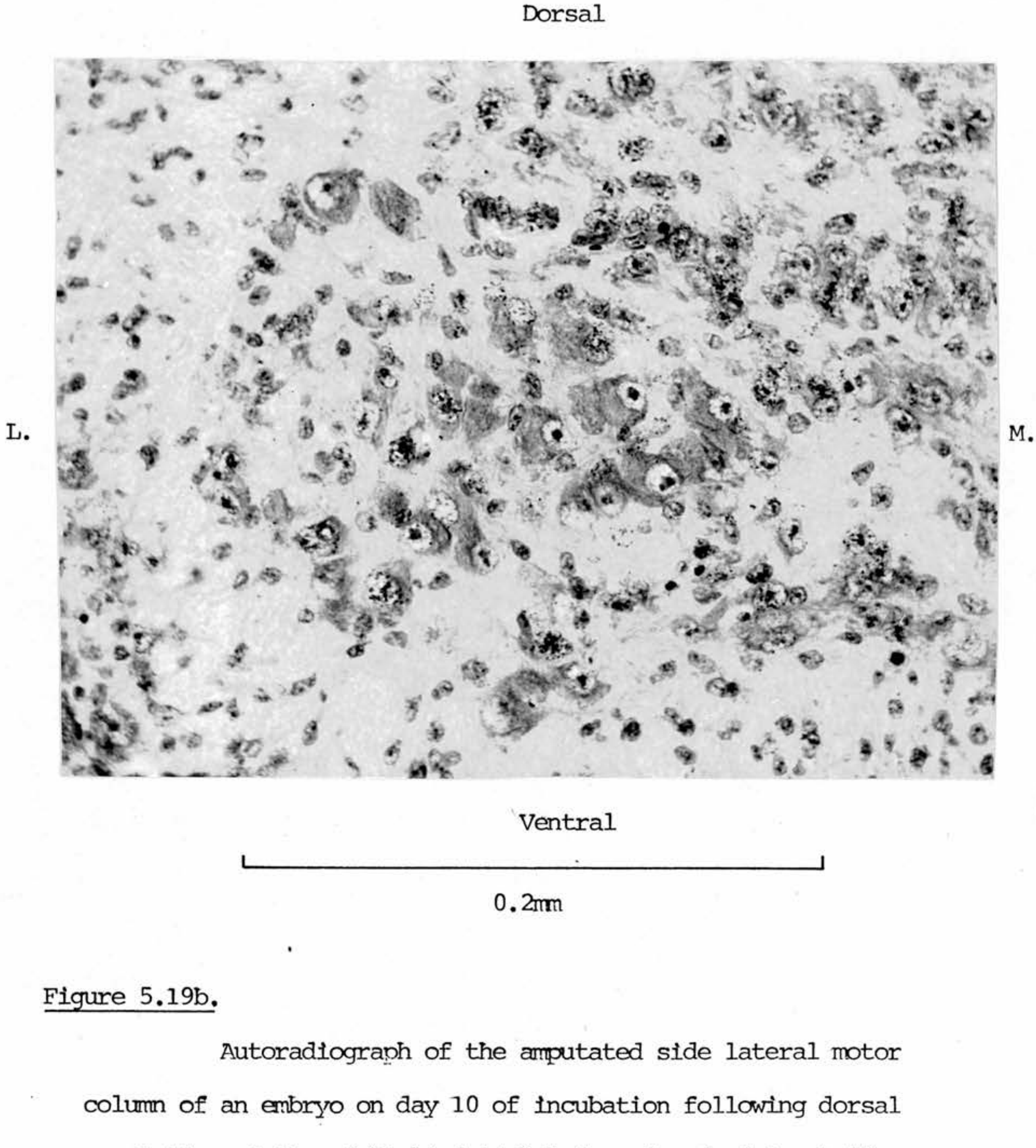


Figure 5.19b.

Autoradiograph of the amputated side lateral motor column of an embryo on day 10 of incubation following dorsal amputation of the right hind limb bud on day 4 of incubation. 10μCi 3H-thymidine at stage 19 of development. The 'labelled' motoneurone population is reduced compared to the control side. The 'unlabelled' motoneurone population is unaffected by the amputation. M- medial. L- lateral.

The distribution of 'unlabelled' and total motoneurones along the rostro-caudal axis of the lateral motor column for the control and amputated sides of embryos is shown in figs (5.20) (5.21). The distribution of 'unlabelled' motoneurones is similar between the control and amputated sides of embryos.

Amputation of the dorsal muscle mass on day 4 of incubation is therefore associated with exclusive loss of the 'labelled' motoneurone population on day 10 of incubation. The number and rostro-caudal distribution of 'unlabelled' motoneurones remains unaffected by the amputation.

5.3 Interpretation of Results

5.3.1. Rostro-Caudal Position

Transverse Amputations

Amputations of shank muscle on day 4 results in motoneurone loss from mid to caudal tenths of the lateral motor column on day 10 of incubation, and loss of thigh and shank muscle results in loss from rostral as well as caudal tenths. Motoneurone loss due to amputation of the thigh may be deduced to occur predominatly from rostral tenths, figs (5.7-5.12). These results confirm the findings of Laing (1979) who also noted a similar pattern of motoneurone loss in the lateral motor column on day 10 of incubation following transverse amputation on day 4.

Comparison of the above findings with the data obtained by HRP-uptake labelling of motoneurones on the distribution of motoneurones innervating thigh and shank muscle on day 10 of incubation fig (1.2), (redrawn from Landmesser (1978a)), shows that the position of loss of motoneurones following amputation of the shank is very similar to the position of motoneurones that normally innervate the shank. Both occur predominantly from mid

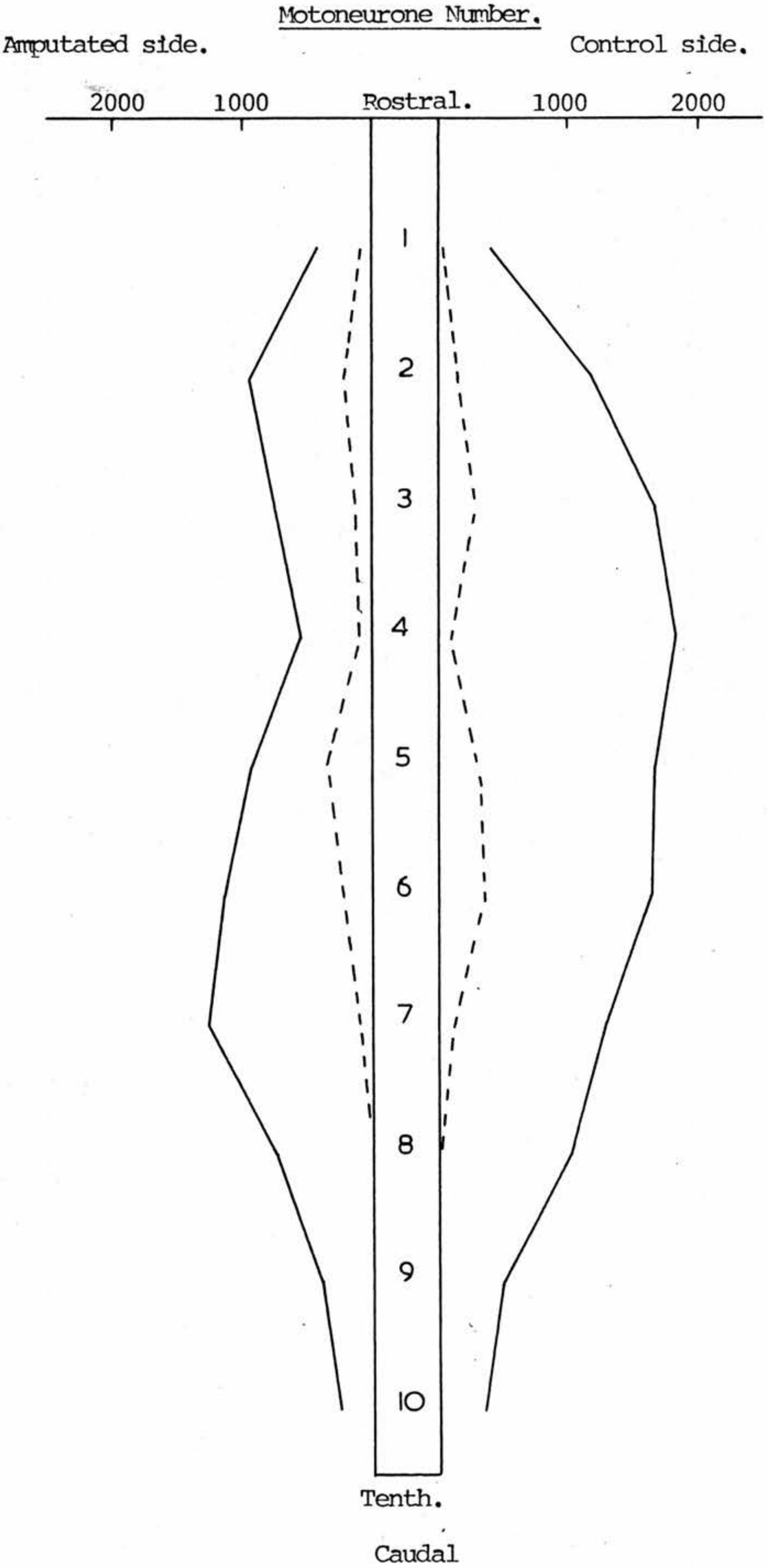


Figure 5.20.

Motoneurone number (solid line) and 'unlabelled' motoneurone number (dashed line) per rostro-caudal tenth in embryo T147 on day 10 of incubation. 10μCi 3H-thymidine at stage 19 of development. Dorsal amputation on day 4 of incubation.

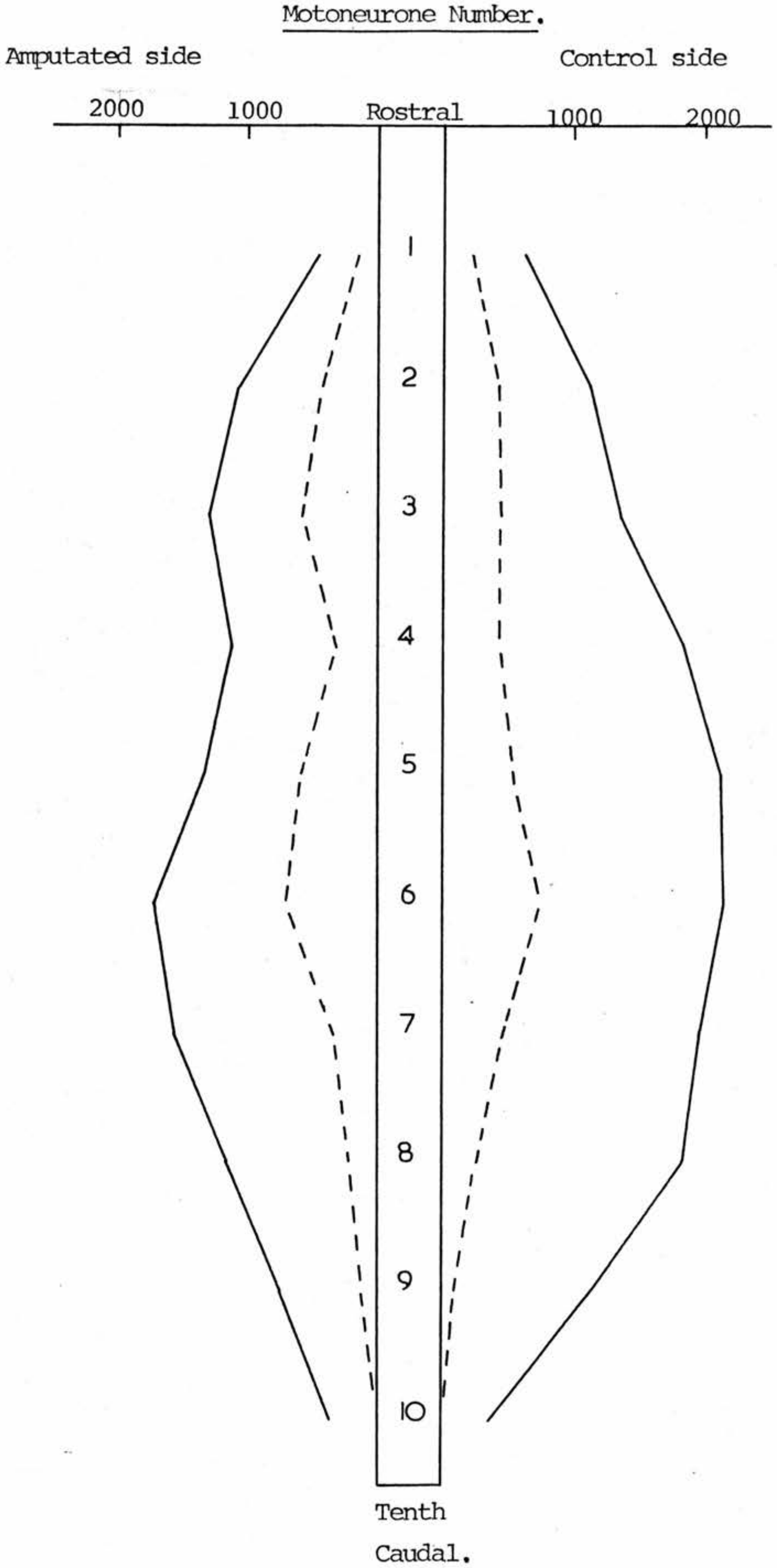


Figure 5.21. Motoneurone number (solid line) and 'unlabelled' motoneurone number (dashed line) per rostro-caudal tenth in embryo T73 on day 10 of incubation. 10μCi 3H-thymidine at stage 19 of development. Dorsal amputation on day 4 of incubation.

to caudal parts of the lateral motor column. Similarly the position of loss of motoneurones due to amputation of the thigh is very similar to the distribution of motoneurones that normally innervate the thigh. Both occur predominantly in rostral parts of the lateral motor column although some motoneurones and motoneurone loss lies more caudally.

Two reservations must be born in mind when comparing the HRP-uptake labelling data of Landmesser and the results presented here. One is that the rostro-caudal distribution of motoneurones of Landmesser is given in segments, whereas the rostro-caudal distribution is given in tenths in this thesis. Thus the two distributions are not directly comparable. The second is that the rostro-caudal distribution of motoneurones obtained by Landmesser can only be regarded as approximate. This is because not all muscles were injected with HRP and not all motoneurones innervating injected muscles become labelled with HRP reaction product.

However bearing in mind these reservations the results in this thesis suggest that the innervation to the remaining proximal muscle following transverse amputation is normal in the rostro-caudal axis of the lateral motor column, and that following loss of their distal muscle targets motoneurones do not innervate more proximal muscles.

Dorsal and Ventral Amputations

The rostro-caudal distribution of motoneurones innervating the remaining dorsal muscle following ventral amputation differs from that of motoneurones innervating the remaining ventral muscle following dorsal amputation, figs (5.1a,b - 5.6a,b).

Comparison of these distributions with the rostro-caudal distribution of motoneurones that innervate dorsal and ventral muscle obtained by HRP-uptake labelling of motoneurones on day 10 of incubation -[fig (1.3), redrawn from Landmesser(1978a)] shows a marked similarity between the distributions despite the reservations of direct comparisons outlined above.

This suggests that the position of motoneurones innervating the remaining dorsal or ventral muscle after ventral or dorsal amputation respectively is normal in the rostro-caudal axis of the lateral motor column. This is further suggested by the finding that adding together the rostro-caudal distribution of remaining motoneurones after dorsal amputation to the rostro-caudal distribution of remaining motoneurones after ventral amputation produces a distribution within the control range of rostro-caudal distributions figs (5.1c-5.6c).

One of the problems with dorsal and ventral amputations is that it is not possible to completely remove either the dorsal or the ventral muscle mass. All embryos had some muscle remnants remaining, albeit very small amounts ranging from 0.7% to 5.8% of control limb total muscle volume tables (4.2 and 4.3). These muscle remnants might be expected to distort the observed rostro-caudal distribution of motoneurones innervating the remaining ventral or dorsal muscle mass from the true rostro-caudal distribution. However the effect of adding together the observed rostro-caudal distributions show that these distortions are slight since they do not significantly alter the calculated distributions from the control distributions.

5.3.2. Medio-Lateral Position

Up to 25% of the first-born motoneurones that normally occupy an extreme medial position within the lateral motor columns and normally innervate ventral muscles were identified by an injection of [3H] - thymidine at stage 19 of development. Those motoneurones which appear 'unlabelled' on an autoradiograph are almost totally absent from the lateral motor column following amputation of the ventral muscle mass, their normal muscle targets, fig (5.14), but are unaffected by amputations of the dorsal muscle mass, targets for more laterally positioned motoneurones. Amputations of the dorsal muscle mass, result in exclusive loss of 'labelled' later-born, more laterally positioned motoneurones, fig (5.18).

These results suggest that following dorsal or ventral amputation the time of origin of motoneurones innervating the remaining muscle is normal and hence the position of the remaining motoneurones in the original medio-lateral axis of the lateral motor column is normal.

Two points must be raised concerning the interpretation of the results. One is that ventral amputation does not result in exclusive loss of 'unlabelled' motoneurones. However the injection schedule used only leaves 'unlabelled'a proportion of the motoneurone pool that normally innervates ventral muscle. The remainder plus the entire motoneurone pool that innervates dorsal muscle become 'labelled' by the injection. If, therefore, following amputation of the ventral muscle mass the innervation pattern to the remaining muscle is normal, then there will be loss of 'unlabelled' as well as 'labelled' motoneurones. Loss of motoneurones from both the 'labelled' and 'unlabelled' pools is to be expected.

The second is that partial or even almost total amputation of the ventral muscle mass does not remove the entire 'unlabelled' motoneurone population. The presence of 'unlabelled' motoneurones following partial amputation of the ventral muscle mass fig (5.14b) may be anticipated since they may innervate the remaining ventral muscle. However almost total amputation of the ventral muscle mass leaves some 2% of 'unlabelled' motoneurones remaining in the lateral motor column. Since about 1% of ventral muscle remains in this embryo, some of the 'unlabelled' motoneurones may innervate this muscle.

Another possibility is that they are motoneurones that innervate dorsal muscle and that have remained 'unlabelled' by the injection schedule. Although the lateral motor column is laid down in medio-lateral temporal sequence, there do appear to be exceptions to this. Occasionally 'unlabelled' motoneurones lie in a lateral position in the LMC surrounded by 'labelled' motoneurones. Similar 'ectopic' 'unlabelled' motoneurones were observed by Hollyday & Hamburger (1977). Their significance is unknown. These laterally positioned 'unlabelled' motoneurones may innervate dorsal muscle and would therefore remain unaffected by amputation of the ventral muscle mass. Another possibility is that since the LMC is laid down in rostro-caudal temporal sequence and the motoneurones innervating dorsal muscles are produced between stages 19.22 of development, the injection schedule used may leave 'unlabelled' a few motoneurones in the rostral segments that normally innervate dorsal muscle. Reference to fig (5.16) shows that the remaining 'unlabelled' motoneurones are indeed situated rostrally within the LMC.

CHAPTER 6

DISCUSSION AND CONCLUSIONS

6.1 General

The aim of the experiment was to examine to what extent motoneurones alter their pattern of connections when part of the limb is removed before the innervation has developed. The results show that motoneurones do not compensate for removal of part of the limb by altering their projection pattern to the remaining limb muscle. The motoneurones that normally innervate the missing muscles are unable to innervate the remaining muscles. Instead they degenerate and are absent from the lateral motor column on day 10 of incubation, leaving the innervation pattern to the remaining limb muscles normal.

A similar result has been described by Laing (1979) for the chick hind limb. Laing examined the position of motoneurones remaining in the lateral motor column on day 10 of incubation following amputations in the proximo-distal axis of the limb on day 4 of incubation. The position of the remaining motoneurones in the lateral motor column was normal. There was no alteration in the pattern of connections formed by motoneurones to compensate for the loss of limb muscle.

Stirling & Summerbell (1977) have also found that following early partial deletions of the chick wing in the proximo-distal axis, the pattern of functional connections to the remaining muscle is normal as judged by the results of spinal nerve stimulation. Similar results were found by Bennett et al (1979). However as the position of motoneurones in the lateral motor columns contributing to the spinal nerves was not determined in these experiments, possible distortions in the innervation pattern in terms of motoneurone position could not be ruled out.

In Xenopus, as well as in the chick, motoneurones do not compensate for removal of part of the developing hind limb bud by altering their projection pattern to the remaining muscle. (Lamb 1981b). Lamb mapped the position of the remaining motoneurones after the normal period of motoneurone death by injecting HRP into the amputated limb. The position of the remaining lateral motor column motoneurones was normal.

There are other reports of the effect of proximo-distal limb deletions on motoneurone survival and position (e.g. Romanes 1946; Hughes 1968). However these experiments are not directly comparable to the experiments of this thesis and to the experiments cited above, as the amputations were done after the limb bud was innervated, and the associated axotomy leads rapidly to motoneurone death.

The converse experiment to removal of part of the developing limb bud is removal of part of the developing lateral motor column. Lance-Jones & Landmesser (1980a) have found that following early removal of part of the prospective lumbar spinal cord in the chick the remaining motoneurones do not spread their connections to innervate the entire limb. The muscle normally innervated by the missing spinal segments remains uninnervated. Similar results were found by Castro (1963) following early removal of 2 or 3 segments of the chick brachial spinal cord and by Lamb (1979) in Xenopus following early removal of the rostral part of the lumbar spinal cord.

Thus following early removal of either part of the spinal cord or the limb there is no compensation such that the entire lumbar lateral motor column innervates the remaining limb tissue or that the remaining spinal cord innervates the entire limb. However some compensation may occur on a very minor level. Lance-Jones & Landmesser (1980a) found that following partial removal of some motoneurone pools, the motoneurones remaining in that pool in the lateral motor column were able to innervate adequately the target muscle. Similar results have been found for the chick wing. (Bennett et al 1979).

Nonetheless the results of partial limb and spinal cord deletions suggest that the rules that govern the formation of projections with the limb are rigid and do not allow for any major alterations in the projection pattern, or so-called 'connection plasticity' (Straznicky et al 1971). A similar rigidity is observed in the formation of connections onto lumbar motoneurones by chick sensory neurones. Following early removal of part of the neural crest at hind limb levels (Eide, Jansen & Ribchester 1982) there is very little replacement of synaptic connections onto motoneurones whose normal input has been removed.

There is however some evidence for 'connection plasticity' in the chick following certain embryonic manipulations of the limb and spinal cord. Its significance is discussed in the section on mechanisms of innervation pattern development.

6.2 Mechanisms of Innervation Pattern Development

Timing

One possible mechanism for the formation of innervation patterns is that of timed axonal outgrowth and target tissue differentiation. Jacobson (1970) suggested that the innervation pattern of the limb can be explained wholly by this mechanism. The spinal cord forms in rostro-caudal sequence (Hollyday & Hamburger 1977) and the limb in proximo-distal sequence (Saunders 1948). Motoneurones innervating thigh muscle tend to lie rostrally within the lateral motor column whereas motoneurones innervating shank muscle tend to lie more caudally (Landmesser 1978a; Hollyday 1980). These observations lend some support to the hypothesis. However if a timed outgrowth of axons with the limb were solely responsible for the development of the innervation pattern, then following partial amputation of the limb the first-born motoneurones should always innervate the remaining muscle because their axons will arrive in the limb first. Later arriving axons, finding all muscle sites occupied would degenerate once they become dependent on contact with muscle for survival.

This is not the case. The first-born motoneurones in the lateral motor column, which have been identified by injection of [3H]- thymidine at stage 19 of development, do not always innervate the remaining muscle irrespective of its dorsal or ventral origin. Their number is unaffected by amputation of the dorsal muscle mass but is almost totally reduced by amputation of the ventral muscle mass. A simple timed outgrowth of axons into the limb is therefore not sufficient to account for the innervation pattern.

However the possibility of a minor role for differences in the time of axon arrival and muscle mass differentiation remains. Bennett and his co-workers (Bennett et al 1980) have observed that the plexus pattern in the chick wing can be correlated with the position and state of development of the pre-muscle cell masses. At stage 23 of development segmental nerves converge towards the large ventral pre-muscle mass. By stage 24 of development the dorsal pre-muscle mass has increased in size and it is at this stage that nerves converge towards the dorsal pre-muscle mass, forming the plexus pattern. This correlates nicely with first-born motoneurones innervating ventral muscle and later-born motoneurones innervating dorsal muscle. (Hollyday & Hamburger 1977; Hollyday 1978). Nonetheless Bennett's observations only indicate that there is a correspondence in the timing of the plexus pattern formation and the appearance and development of the pre-muscle cell masses. They do not show a causal relationship between the two events.

Parallel Outgrowth and Contact Guidance of Axons

Horder (1978), based largely on the work of Roncali (1970), and Fouvet (1973) suggested that axons grow out from the spinal cord in parallel, maintaining their neighbourhood relations. Axon outgrowth is then directed passively by contact guidance within the limb. Axons from motoneurones in neighbouring parts of the spinal cord innervate the same muscle because of their similar position in the plexus and peripheral branching pattern.

Predictions from this hypothesis on the pattern of connections that will form following removal of part of the limb will vary depending on the type of amputation. Following transverse amputation, outgrowing axons encounter a morphologically normal limb until they reach the amputation site. Contact guidance of axons to the remaining proximal muscle should therefore be normal, and the innervation pattern will be unaltered. This prediction is compatible with the results.

Following amputation of the dorsal or ventral muscle mass axons will encounter a morphologically abnormal environment almost as soon as they enter the limb. Under these circumstances abnormal limb morphogenesis and contact guidance may bring axons into apposition with muscles which they would not normally contact. This would create distortions in the innervation pattern of the remaining muscle. However the results show that following amputation of the dorsal or ventral muscle mass the innervation pattern to the remaining muscle mass is normal. This could be taken as evidence against parallel outgrowth and contact guidance of axons forming the innervation pattern. However it is also possible to argue that the contact guidance points that separate motoneurones destined for ventral muscle from those destined for dorsal muscle lie proximal to the point of amputation. In this case, amputation of either the dorsal or ventral muscle mass would not affect the innervation pattern to the remaining muscle mass. The results of this thesis cannot therefore exclude the hypothesis of parallel outgrowth and contact guidance of axons.

Results of other experiments however throw doubt on the validity of this hypothesis in explaining the formation of the innervation pattern of the limb. For example Lance-Jones & Landmesser (1978; 1980b) have shown that following early rotations of small portions of the chick lumbar spinal cord through 180° in the anterior-posterior axis, motoneurones form connections 'appropriate' to their old position and not to their new position, and they form these connections from the onset. Axons alter their pathways in the limb albeit within the limits of the plexus and peripheral nerve branching pattern. This suggests that motoneurones can actively select between pathways in the limb leading to different target muscles. Evidence for active pathway selection has also come from work in <u>Xenopus</u>. Both sides of the spinal cord can be induced to innervate a single hind limb by surgical manipulation (Lamb 1981a). Motoneurones from the contra-lateral lateral motor column traverse very abnormal routes to the limb yet they form a normal pattern of connections, and if the operation is performed early enough, normal numbers of motoneurones survive on both sides of the cord. This strongly suggests that axons are actively guided to their muscle targets.

Evidence against parallel outgrowth of axons from the spinal cord into the chick hind limb has recently been produced by Lance-Jones & Landmesser (1981a). By injecting HRP into different regions of the lateral motor column they were able to trace the paths of axons into the limb. They found that axons from neighbouring motoneurones do not leave the spinal cord in parallel, maintaining their neighbourhood relations, but are mixed with other axons from non-neighbouring motoneurones. It is in the spinal nerves and plexus that axons achieve the same relative positions as their cell bodies with respect to one-another. Again this implies that there is some form of active pathway selection by axons.

The weight of available evidence then suggests that parallel outgrowth and contact guidance of axons is not a sufficient mechanism to explain the formation of connections in the limb.

Specificity Hypotheses

This heading covers a wide range of hypotheses. However they are all based on the concept that motoneurones are different from one another and that this difference then actively determines the pattern of connections formed with the limb. One possible way in which motoneurones may differ from one another is in the possession of individual cytochemical labels (Sperry 1963). Based on this idea Prestige & Willshaw (1975) divided possible mechanisms of innervation pattern development into two groups. They assume that the only significant property of the cytochemical label is its relative affinity with the sets of labels in the opposite set of cells, and that connections between the two cell sets are formed at random.

Group I Mechanisms - An axon in the presynaptic set has maximum affinity for its matched post-synaptic cell partner and less for all other members. The same is true for the affinity of a post-synaptic cell for axons. The peak in maximum affinity between an axon and a post-synaptic cell may be very sharp so that connections will only form between the two matched cells or it may be 'noisy' so that there is a number of cells any given cell may synapse with. Group I mechanisms are described as direct or rigid matching between neurone populations and are really a restatement of Sperry's (1963) original chemo-specificity hypothesis. Cells make and retain contact independently of each other and are not affected by removal of part of the set.

Group II Mechanisms - Synaptic affinities for members of the opposing set develop in a graded manner across the pre and post-synaptic sets. All cells have a 'preference' to form synapses with members at the high affinity end of the opposing set. However because there is a limit to the number of synapses a post-synaptic cell will accept, and a presynaptic cell will form, there is competition between cells to form synapses at the high end of the affinity gradient. This results in pre and post-synaptic cells at the high ends of the affinity gradients forming synapses with each other. Less highly placed cells form synapses not with high affinity sites as they would in isolation, but with correspondingly less highly placed ones. The lowest affinity cells make connections with the low affinity sites because they have no choice. The pattern of connections a cell will form and retain is thus dependant on the presence of other members in the set and will be influenced by removal of part of the set.

Group II mechanisms cannot explain the formation of connections in the chick hind limb. The model predicts that partial removal of the post-synaptic set of cells (assuming that the number of synapses a cell can make or receive is unaltered) results in cells from the high affinity end of the presynaptic set of cells always forming connections with the remaining postsynaptic cells. The low affinity members will be excluded by competition. The results show that this is not the case. One population of motoneurones does not always innervate the remaining muscle at the expense of the remaining population. Following partial amputation of the limb the innervation pattern to the remaining muscle is normal irrespective of whether dorsal, ventral, proximal or distal muscle is removed.

The results of this thesis can however be explained by a Group I mechanism, which predicts rigid or direct matching between neurones and targets. The connections a cell will form is independant of other cells and not affected by removal of part of the set. This is indeed what is observed following partial removal of the limb. The pattern of connections to the remaining muscle is normal. Similarly the results of partial spinal cord deletions (Castro 1963; Lance-Jones & Landmesser 1978, 1980a; Lamb 1979) can be explained by such a mechanism.

Although these results may be explained by a theory of rigid or direct matching between neurones and targets, based on the concept that cells are different from one-another, it is entirely possible that cell differences express themselves in other forms instead of, or as well as, direct matching between neurones and targets. Exactly what these expressions are remains to be elucidated. However some possibilities will be discussed.

One of the premises upon which group I mechanisms are based is that cells form connections at random and that these connections are constantly changing until stable connections form between 'matched' cells. However there is a considerable amount of evidence, that has already been discussed (p 163-164) to suggest that connections do not form at random and that outgrowing axons actively select between pathways leading to different target muscles. One possible expression of the difference between neurones may therefore be in pathway selection.

It has recently been suggested by Landmesser (1981b) that pathway selection by axons may also be a sufficient mechanism to account for the innervation pattern of the limb without the need to invoke any direct matching between neurones and targets. This has been based on the experiments that show that under certain experimental situations motoneurones form connections with muscles that they do not normally contact, and that these connections remain throughout the period of motoneurone death. These connections form following : supernumerary limb grafts (Hollyday et al 1977; Morris 1978; Lance-Jones & Landmesser 1981b); limb rotations through 180° in the anterior-posterior and dorso-ventral axes (Stirling & Summerbell 1979) and through 180° in the dorso-ventral axis only (Summerbell & Stirling 1981); large anterior posterior shifts of the developing bud (Lance-Jones & Landmesser 1981b); and rotations of the entire spinal cord through 180° (Lance-Jones & Landmesser 1981b).

However these experiments raise severe difficulties in the problem of interpretation because they introduce many variables. For instance it appears that the pattern of innervation that develops following limb bud rotations in the dorso-ventral axis depends on the proximo-distal level at which the limb is rotated. Summerbell and Stirling (1981) found that wing rotations result in the majority of motoneurones forming connections with muscles that they do not normally contact. However Ferguson (1978) has found that motoneurones form normal connections after dorso-ventral rotations. The level of rotation in Ferguson's experiments is more proximal than those of Summerbell and Stirling. (Stirling-personal communication).

Nonetheless there is no direct evidence to suggest that there is direct matching between motoneurones and muscle fibres in the chick although there is evidence for pathway selection.

However in Xenopus there is evidence for some kind of matching between motoneurones and muscle fibres. This has come from the work of Lamb (1981b) who studied the innervation pattern of partly amputated limbs at different developmental stages. Lamb found that when part of the limb is the missing the entire lateral motor column gains access to the remaining muscle prior to the period of motoneurone death. Despite this only those motoneurones that normally innervate the remaining muscle survive the period of motoneurone death. This indicates that there may be direct matching between motoneurones and muscle fibres in Xenopus. An alternative possibility is that motoneurones are unable to form connections in the presence of the normal innervation but would be able to do so in its absence. This indicates not an absolute matching between motoneurones and muscle fibres but a preference of synapse formation between the normal innervation and muscles excluding, by competition, any other innervation.

Whether such a preference of synapse formation exists in the chick has never been tested because in all experimental situations where 'abnormal' connections form the normal innervation has not been present. The partly amputated limb could prove to be an interesting test of this possibility. The question as to whether all motoneurones gain access to the remaining muscle following partial limb amputation and prior to motoneurone death could be investigated by injecting HRP into the limbs. If they do then the finding that after the period of motoneurone death the innervation pattern is normal would indicate preference of synapse formation between the normal innervation and the remaining muscle.

6.3 Summary.

The results of this thesis show that following partial amputation of the limb there is no alteration in the pattern of connections to the remaining muscle. These results confirm and extend the findings of other experiments on the innervation of partly amputated limbs. The results show that the formation of the pattern of connections during normal development cannot be explained by a simple timed outgrowth of axons into the limb or by mechanisms involving matching of motoneurones to muscles as whole populations. While the results connot exclude the mechanism of parallel outgrowth and contact guidance of axons the results of other experiments make this an extremely unlikely mechanism.

The results of this thesis suggest that motoneurones are different from one another and that these differences are important in determing the ordered pattern of connections that forms with the limb. Exactly how these differences express themselves remains to be determined.

CHAPTER 7

APPENDIX

7.1 Correction for Double Counting of Motoneurones

One possible source of error in estimating motoneurone number by the systematic sampling procedure outlined in the methods (2.6) is that of 'split cell' error. (Konigsmark 1970). This type of error occurs when the object being counted, in this case the motoneurone nucleolus(i) is split by the microtome knife into two and therefore appears in two adjacent sections. These split cells are then counted as two separate cells instead of one and this leads to an overestimation of motoneurone number.

Theoretically the magnitude of this type of error depends on the size of the nucleolus in relation to section thickness. If the nucleolar diameter is large relative to section thickness then the number of nucleoli that will be split by the microtome knife will be high and the overestimation of motoneurone number will be high. If however, the nucleolar diameter is small in relation to the section thickness then the number of nucleoli split by the microtome knife will be small. There will be little overestimation of motoneurone number.

In this thesis the spinal cords of embryos that received 10μCi [3H] - thymidine were sectioned at 5μm thickness. The spinal cords of all other embryos at 8μm thickness. Since the nucleolar diameter of the two groups is similar (3.86 ± 0.8μm (SD) and 3.62 ± 0.67 μm (SD) respectively), then theoretically motoneurone counts of embryos sectioned at 5μm thickness should be much higher than the counts of those sectioned at 8μm thickness due to the greater magnitude of 'split cell' error.

Reference to table (7.1) shows that this is not the case. Motoneurone number does not differ significantly between the two groups (p.70.40; student's t-test). There are three possible reasons why this is so. One is that 10μCi [3H] - thymidine injected onto embryos sectioned at 5μm thickness is neurotoxic and reduces actual motoneurone number in the lateral motor columns. The second is that the actual section thickness of the two groups does not differ. The third is that 'split cell' error does not affect motoneurone counts in the way predicted by theory. Each of these possibilities will now be discussed.

Neurotoxic Effect of [3H] - thymidine

The use of [3H] - thymidine in studying the developing nervous system is a well established technique (Sidman 1970) and a neurotoxic effect of [3H] - thymidine at the dosage used in this study has not been reported. In a study comparable to the one presented in this thesis, Hollyday and Hamburger (1977) injected 20μCi [3H] - thymidine dissolved sterile saline onto chick embryos at day 2 and 3 of incubation. The embryos were fixed on days 10 - 12 of incubation and the lumbar spinal cords sectioned at 8μm thickness. After correcting the motoneurone counts for 'split cell error' using the Abercrombie correction factor (Abercrombie 1946) they found motoneurone number to be 10,155 ± 922 which is in good agreement with other estimates of 'corrected' motoneurone number found by other workers at a comparable developmental stage, see table (7.2)

It seems unlikely therefore that 10μCi [3H] - thymidine has a neurotoxic effect on the developing motoneurones. However in order to check that this was so, two control embryos that had not

Table (7.1)

Motoneurone Number in Embryos sectioned at 8μm thickness. No [3H] - Thymidine				Motoneurone Number in Embryos sectioned at 5μm thickness. 10μCi [3H] - thymidine	
Embryo		Embryo		Embryo	
P23	17,000	P181	15,280	T33	13,950
P26	14,630	P182	13,160	T73	14,360
P34	13,600	P186	14,480	T88	13,390
P37	15,120	P189	12,200	T104	15,630
P38	14,440	P229	10,810	T147	11,920
P48	15,510	P230	13,360	T165	13,810
P49	15,080	P231	10,730	T173	11,620
P61	14,230	P232	12,240	T177	13,820
P71	14,660	A278	15,610	T296	10,890
P75	14,960			T318	11,650
P165	13,700				
P176	13,000				
		$\bar{X}$	13,910	$\bar{X}$	13,215
		SD	1,592	SD	1,474
		n	23	n	11

Motoneurone number in embroys at day 10 of incubation sectioned at 5μm and 8μm thichness. Embryos sectioned at 5μm thickness recieved 10μCi 3H thymidine at stage 19 of development.

Author	Corrected Motoneurone Number
Hamburger 1975	10,304
Hollyday & Hamburger 1977	10,155 ± 922
Laing 1979	10,229 ± 1,429
This study	9,570 ± 1,094

Table 7.2

Estimate for motoneurone number in the chick lumbar lateral motor column on days 10-12 of incubation, by different authors. All spinal cords sectioned at 8μm thickness and corrected for split cell error (Abercrombie 1946).

received [3H] - thymidine were sectioned at 5µm thickness. The results are shown in table (7.3). The motoneurone counts do not differ significantly from either the [3H] - thymidine treated group or the non-thymidine group (p 7.0.05, student's t-test)

Section Thickness

The two groups were sectioned at a knob setting of either 5µm or 8µm thickness. However if the section thickness as measured by the knob setting is inaccurate then there may not be a difference in actual section thickness between the two groups and a difference in uncorrected motoneurone counts would not be expected. However a check of the thickness of the section as measured by the knob setting was made according to the method outlined in section 2.7. The section thickness of embryos sectioned at a knob setting of 8µm was 7.90µm ± 0.55 (SD) n = 40; and of embryos sectioned at 5µm knob setting, 4.92µm ± 0.67 (SD) n = 49. There is therefore a difference in the section thickness of the two groups.

'Split cell' error does not affect motoneurone counts

The theoretical basis for 'split cell' error is that if the microtome knife hits a nucleolus it will be split in two and therefore appear in two adjacent sections. There is however evidence that this is not so. Jones (1937) took serial sections of the cat nodose ganglion, 6 to 12 µm in thickness and found very little difference between total neuronal estimates based on 6, 8 or 12µ thickness. He concluded that 'split nucleoli do not appreciably affect the nucleolar count'. Examination of 300 cells in adjacent paraffin sections showed that only 2.3% were in adjacent sections and split by the microtome knife. It was suggested that as the nucleoli are harder than the surrounding tissues they are pushed by

Embryo	Motoneurone Number
B206	13,010 12,010
P70	10,440
$\bar{X}$ SD	11,863 1,307

Table (7.3)

Motoneurone counts in control embryos sectioned at 5μm thickness.

the microtome knife into one or other of the adjacent sections thus resulting in far fewer 'split' nucleoli than would be expected theoretically. Similar results have been reported by Foley and Dubois (1937) in the cat nodose ganglion and by Cammermeyer (1967) in rabbit and cat brain.

The results of this thesis also support this conclusion. 'Empty' neuronal profiles, that is nucleii that contained no nucleoli were more frequently observed in embryos sectioned at 5µm thickness than at 8µm thickness. Although no quantitation of this was carried out it suggests that nucleoli may be displaced into one or other of two adjacent sections rather than being split by the microtome knife.

The value of correcting for split cell error in neurone number estimates is therefore dubious. For this reason uncorrected motoneurone counts were used in this thesis.

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