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THE DEVELOPMENT OF MOTOR PROJECTION PATTERNS IN THE CHICK HIND LIMB

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SUMMARY

1. Retrograde transport of horseradish peroxidase was used to map the initial projection patterns of lumbosacral motoneurones to the embryonic chick hind limb.

2. The stage 28 segmental projection pattern to each of the four primary muscle masses was characteristic and indistinguishable from the stage 36 projection pattern to the sum of the muscles derived from that mass. In addition, the adductor motoneurone pool was found to be similar in position (both rostro-caudal and mediolateral) at stages 29, 30, 32, 33½ and 36.

3. Therefore axons from lumbosacral motoneurones project for the most part only to appropriate regions from early times shortly after they grow into the limb bud. Furthermore, the attainment of the segmental projection pattern occurs prior to the normal time of, and therefore without the aid of, cell death. This conclusion was supported by electrophysiological recordings made from muscle nerves.

4. A regionalization of the projection patterns within a single muscle mass could be shown both anatomically and physiologically prior to the cleavage of the mass into individual muscles and the projections were in a general way appropriate for the muscles derived from those regions.

5. Therefore the process of muscle cleavage does not in itself create the specific projection patterns observed, and motoneurone axons appear to grow to and to ramify and make synapses only within regions which correspond to their adult muscles.

6. Finally, the termination site of each motoneurone axon in the early limb was found to be tightly correlated in a somatotopic fashion with the position occupied by its soma in the cord. This suggests that some feature of the motoneurone related to its position may be of importance in achieving the specific projection patterns observed.

INTRODUCTION

A definite topological relationship between position of a motoneurone soma and its peripheral termination site in the limb has been shown for a variety of vertebrates and it would appear that the distinctness of such mapping is greater in higher (Romanes, 1951, 1964; Sharrard, 1955) than in lower vertebrates (Romanes, 1964; Cruce, 1974; Székely & Czéh, 1967). In the preceding paper (Landmesser, 1978) it was shown that in the chick also, motoneurones are clustered into coherent cell groupings whose position is tightly correlated with their peripheral termination in the

limb. However, little is known about how such a pattern is attained during development.

A previous electrophysiological study (Landmesser & Morris, 1975) provided evidence that a pattern that is indistinguishable from the mature one is attained early in development, at the time when the first functional connexions are made (stage 28–29; 5½–6 days) and prior to the massive spontaneous degeneration of motoneurones that occurs later (stage 30–36; 6½–10 days; Hamburger, 1975). This was taken as evidence that motoneurones send their axons to innervate only appropriate regions of the limb from the start, and that extensive projection errors which might be corrected during the cell death period were not made.

However, since this study was based on spinal nerve stimulation, it was not capable of distinguishing between motoneurones destined for different muscles whose axons were excited through a common spinal nerve. Further, it only provided evidence on the pattern of functional connexions, for it was still possible that axons might project wrongly or ramify throughout the limb but only form functional synapses within appropriate boundaries. The inappropriate projections would then be lost through cell death and/or retraction of axon branches. Some evidence of initially inappropriate projection patterns has recently been described by Lamb (1976) for *Xenopus* hind limb using a retrograde horseradish peroxidase labelling technique. The present study combines a similar technique with additional electrophysiological observations to determine in more detail the early projection patterns to the chick hind limb and to compare these with patterns to individual muscles after the cell death period (Landmesser, 1978). The early and late projection patterns were found to be essentially the same.

METHODS

General

Embryos used in these experiments were from White Leghorn eggs that had been incubated in a forced draft incubator. The embryos were staged according to Hamburger & Hamilton (1951) and placed into oxygenated Tyrode solution at 20–22 °C where they were decapitated, eviscerated, and the ventral half of the vertebral column removed from mid-thoracic to the end of the lumbosacral enlargement.

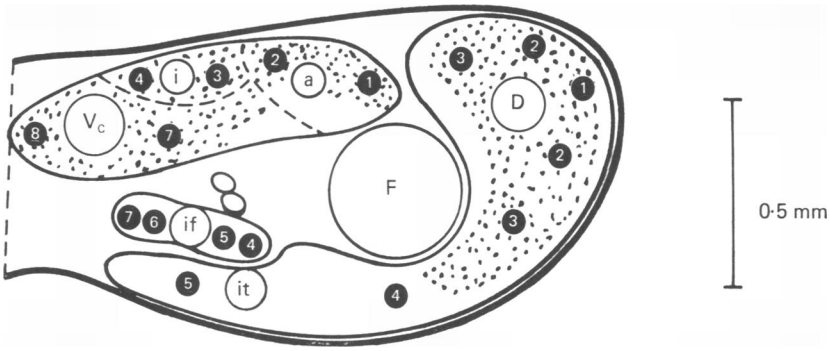
Retrograde labelling with HRP

Labelling with horseradish peroxidase (HRP) and subsequent treatment were carried out according to the same procedure described for older embryos (Landmesser, 1978) except that the injection pipette diameter (20–30 μm) and injection volumes were smaller and the skin was left in place.

At stage 28 (also early stage 29), the time at which the injections were made, the musculature consists basically of four primary muscle masses: dorsal thigh, ventral thigh, dorsal shank and ventral shank. The early arising iliofibularis had split from the dorsal thigh (see Text-fig. 1) but there had been no further individualization of muscles. (See also Romer, 1927; Wortham, 1948.) The initial intent was to inject each of the four primary muscle masses in its entirety. However, observation of injected limbs for histochemical reaction product showed that it was not possible to inject the iliofibularis and overlying prospective iliotibialis without leakage to the underlying ventral muscle mass. Therefore, in dorsal thigh injections, the injected region was confined to the medial pre-axial portion, which will give rise to the sartorius and femorotibialis muscles. The extent of injection was usually only visually controlled. In some cases where the extent of the injection was later checked histologically, it was found that the entire dorsal and ventral shanks had been injected as intended. At the injection site, presumably damaged muscle fibres were labelled diffusely, and in addition, small granules of HRP reaction product were distributed

over a much wider area. The usual extent of this labelling is shown on the camera-lucida diagram in Text-fig. 1. The anterior thigh injection was confined to the prospective sartorius and femorotibialis region; the ventral thigh region always labelled the entire ischio- and caudilioflexorius regions; however, while the anterior medial portion of the adductor area was labelled, generally the deeper portions were not.

Each of these four regions was therefore individually injected in a number of stage 28 embryos. In some cases a different region was injected in each leg. Labelled motoneurone cell bodies were subsequently localized and counted in 14 μm cresyl violet stained sections as described in detail previously (Landmesser, 1978). Almost all of the staining reaction was of the granular type (Oppenheim & Heaton, 1975) but an occasional diffusely stained cell was found (see Pl. 1). Since there was no evidence that diffusely stained cells resulted from damage to their cut axons (see also Heaton, 1977) they were included in the cell counts. However, since they represented less than 1 % of the labelled cells, their inclusion would not substantially alter the observed relationships in any case.



Text-fig. 1. Tracing from a photograph of a cross-section through a stage 28–29 hind limb bud at thigh level. The medial lying ventral muscle mass (V) is mostly labelled by HRP injection, extent indicated by stipling. Only pre-axial medial portion of dorsal muscle mass (D) is labelled, leaving the prospective iliotibialis (it) region and the iliofibularis (if) muscle which has already cleaved. The ventral muscle mass not yet sub-divided but the prospective regions of the adductor (a), ischioflexorius (i) and caudilioflexorius (c) are indicated. F = femur. In addition the general segmental projection pattern, based on the innervation of individual muscles (Landmesser, 1978) and the part of the muscle mass from which they are derived (Romer, 1927), is indicated by the numerals within the dark circles.

Histograms plotting the distribution of labelled cellular profiles along the ipsilateral spinal cord, as the number of labelled cells/28 μm (expressed as percentage of the total number of labelled cells for that muscle mass) were prepared. Histograms from different animals were combined, with values expressed as mean % of labelled cells $\pm$ S.E. The spinal cords from different animals were brought into register as previously described (Landmesser, 1978).

Partial injections were carried out in the ventral muscle mass of the thigh at late stage 28–early 29 before any subdivision of this muscle mass. In these animals a small portion of the muscle mass was injected in one leg, and a different portion in the contralateral leg. Cords were subsequently processed as described above. In most cases these were later verified by running the HRP reaction on the injected limbs. Counts for partial injections were only expressed as labelled cells/28 μm.

Electrophysiology

Electromyograms were recorded from different regions of the ventral muscle mass of the thigh at stage 26 $\frac{1}{2}$–29. Suction electrodes (20–30 μm tip diameter) of glass or polyethylene tubing were used to suck into the orifice in an *en passant* manner small groups of muscle fibres (5–10). The electrical activity resulting from spinal nerve stimulation was recorded with a Transidyne

MPA-6 differential amplifier and Tektronix 5030 Oscilloscope, and photographed with a Grass C4 camera. The recording site was noted on a scaled drawing of the limb. Different recording sites were sampled and their distance from each other noted with an ocular micrometer.

Suction electrodes were also used to record compound action potentials from various muscle nerves at stage 28. Only optimal recordings where the maximum response amplitude was at least 100 times the noise level of the recording were used. The spinal nerve eliciting maximum response was given a value of 1 and the contributions of other spinal nerves to the compound action potential expressed as a fraction of this.

In order to get an estimate of the minimum number of active axons that could be detected in a nerve, in one experiment a fine muscle nerve branch of a stage 30 embryo was stimulated antidromically while a recording was made from one half of the sciatic nerve. The recorded response was well above the level of detection. Later the areas of the nerves were determined histologically. The area of the nerve that was stimulated represented less than 2 % of the area of the half sciatic. Assuming that all fibres in the stimulated nerve were activated, this shows that one can detect activation of as few as 2 % of the fibres in an embryonic nerve at this stage. Such electrophysiological techniques are thus relatively sensitive indicators of axonal distribution.

RESULTS

Pathways taken by axons to the periphery

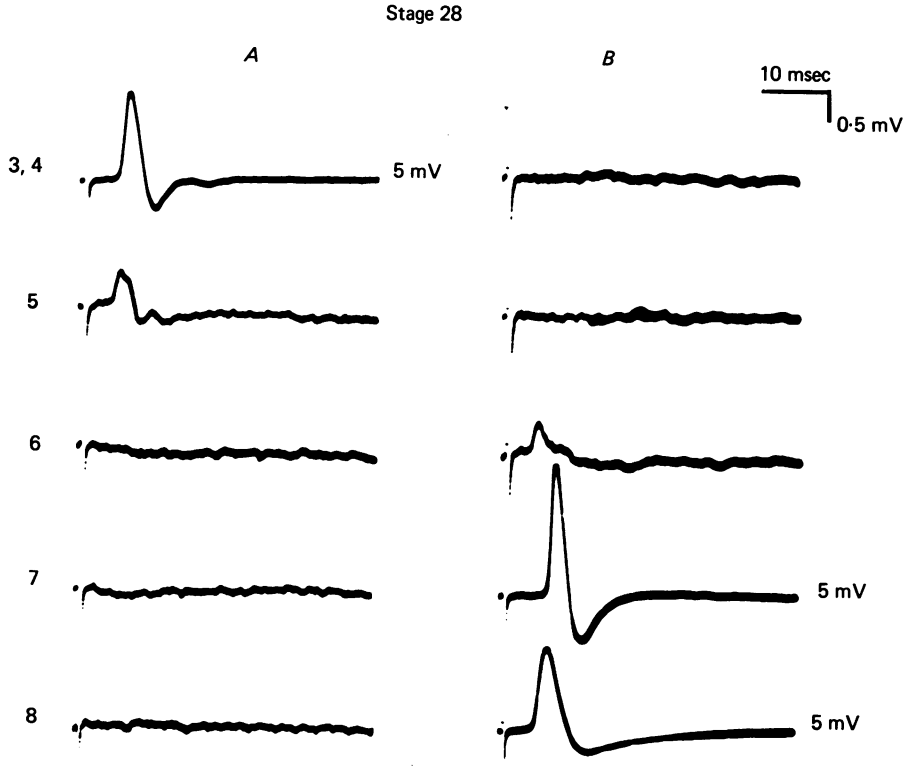
Previous results (Landmesser & Morris, 1975) had shown that the segmental contribution to various muscle nerves in the chick hind limb was similar to the mature pattern from early stages. This suggested that the majority of motor axons had grown along appropriate nerves before the major period of motoneurone death at stage 30–36 (Hamburger, 1975). However, only a few observations were made before stage 30, when some cell death was already under way.

Therefore the nerves to two posterior thigh muscles, the ischioflexorius and the caudilioflexorius, were selected for closer study. These nerves, which run parallel to each other for some distance in the thigh, were recorded from in a group of stage 28 embryos with fine suction electrodes. The individual spinal nerves were sequentially stimulated producing responses as seen in Text-fig. 2. Only cases with optimum recording (see Methods) were used. Generally stimulation of lumbosacral (LS) spinal nerves 7 and 8 elicited large responses in the caudilioflexorius nerve, and stimulation of LS spinal nerve 4 distal to where the ramus from LS spinal nerve 3 joins (this was necessary since the ramus from 3 is too fragile to stimulate consistently separately at this stage) elicited large responses in the ischioflexorius nerve. This is essentially the adult pattern.

Similar recordings were made in a number of embryos at stage 28 and at stage 36 following the period of cell death. In each case the spinal nerve eliciting a maximal response was given a value of 1, and the contribution of other spinal nerves expressed as a percentage of this (see Table 1). It can be appreciated that LS spinal nerves 7 and 8 make the major contribution to this nerve at both stages. The small contribution from 6, seen at both early and late stages and not detected in the previous study, is consistent with the localization of motoneurone somas following retrograde transport of HRP (Landmesser, 1978). The only obvious difference between the two stages is a small, variable contribution made by nerves 3 and 4 at stage 28 which is not seen following the period of cell death. Also labelled cells have not been found in segments 3 or 4 following HRP injection into the caudilioflexorius at stage 36–37 (Landmesser, 1978). These axons possibly represent 'ischioflexorius' axons that

have strayed into the neighbouring caudilioflexorius nerve and which are removed during the period of cell death. There is also a shift in the relative proportion of axons contributed by LS spinal nerves 7 and 8, again reflected in the HRP data (next section). However, it is not clear what this represents.

Likewise, the results for the ischioflexorius were similar for both stages, with a



Text-fig. 2. Segmental distribution to two muscle nerves at stage 28. Compound action potential recordings from ischioflexorius (*A*) and caudilioflexorius (*B*) muscle nerves elicited by stimulation of lumbosacral spinal nerves 3–8. In this case the ramus from spinal nerve 3 was stimulated together with spinal nerve 4. Calibration is 0·5 mV except where otherwise indicated.

TABLE 1. Percentage contribution of different spinal nerves to the ischioflexorius and caudilioflexorius nerves before (stage 28) and after (stage 36) the cell death period (mean ± S.D.)

Lumbosacral spinal nerve	3+4	5	6	7	8
Caudilioflexorius					
Stage 28 (n = 12)	0·7 ± 0·9	0	2·8 ± 0·4	74·6 ± 29·0	87·5 ± 22·0
Stage 36 (n = 10)	0	0	1·6 ± 1·9	97·3 ± 7·8	64·5 ± 22·4
Ischioflexorius					
Stage 28 (n = 6)	100	1·7 ± 2·9	2·5 ± 4·2	1·8 ± 4·5	0·8 ± 2·0
Stage 36 (n = 6)	100	2·0 ± 2·8	0·6 ± 1·3	3·6 ± 4·2	0

dominant contribution from LS nerves 3 and 4 and smaller contributions (none exceeding 10%) from LS nerves 5, 6 and 7. Since labelled motoneurones were not seen in these latter segments when HRP was injected into the muscles (Landmesser, 1978) it is possible that the responses represent a sensory or possibly even autonomic contribution to the nerve. The only difference between the two stages was a small contribution from LS spinal nerve 8 at the early but not the late stage, which may represent stray caudilioflexorius axons. LS spinal nerves 1 and 2 contributed to neither the ischioflexorius nor the caudilioflexorius at any stage studied.

Despite the small differences detected in these experiments, it is obvious that the pattern at stage 28 is essentially like that at stage 36, and that the basic projection pattern, at least with respect to segmental origin, is achieved prior to, and for the most part without the aid of, cell death.

Segmental projection patterns to the four primary muscle masses

The muscles of the tetrapod limb arise by the division of the two primary muscle masses, one dorsal and one ventral. In the stage 28 chick, a cleavage plane has separated each of these into thigh and shank. Except for the early arising iliofibularis which has already split off from the dorsal muscle mass there has been no further individualization into muscles (see Text-fig. 1), the other muscles of the thigh arising during the next few days (Romer, 1927; and by present observations made both on whole limbs under a dissecting microscope and on cross-sections of the limbs at various stages). The muscles of the shank also arise over the same general period (Wortham, 1948) but these were not examined in detail in the present study.

To assess the segmental projection pattern to the limb prior to the major period of cell death and muscle individualization, HRP injections of the following regions of the stage 28 limb were made: anterior pre-axial part of dorsal muscle mass thigh; whole ventral muscle mass thigh; whole dorsal muscle mass shank and whole ventral muscle mass shank. These regions were chosen because it was possible consistently to fill each with HRP (see Methods) and the muscles derived from each are known so that the initial and mature projection patterns could be reasonably compared.

Following histological processing as described in the Methods, the number of labelled cells in each section along the entire lumbosacral enlargement (from the last thoracic segment to LS 8) was determined for each of the four types of injections. The vast majority of labelled cells contained granular reaction product, but occasionally diffuse reaction product which filled cell processes and soma (Pl. 1) was observed. The labelled motoneurones were elongate, and granular reaction product was observed in the initial segments of dendritic processes. These processes, as can be appreciated from cells which were diffusely stained (Pl. 1), often stretched over considerable distances. In order to reduce the likelihood of counting such processes as cells, the preparations were lightly counter-stained with cresyl violet, and counts were only made of profiles where a rim of HRP containing cytoplasm was seen to surround a nucleus.

It should be stressed that these preparations were not optimal for determining the absolute numbers of cells. However, some estimation of the proportion of labelled cells was desirable. If one merely adds the mean number of cells labelled at stage 28 in each of the four muscle masses (without any correction for double counting) one

obtains a value of 6226. (It should be recalled that approximately half of the dorsal thigh was unlabelled, as was the deeper part of the ventral thigh, probably making this value low.) However, this does represent 31 % of the total uncorrected counts obtained by Hamburger (1975) for the entire lumbosacral lateral motor column at this stage. Careful observations of sequential sections made it clear that, with the criteria used for labelled soma identification above, double counting was probably minimal. However, clearly not all motoneurones at stage 28 were labelled by this procedure, one obvious reason being that only part of the thigh regions were injected. Further, while most motoneurone axons have reached the ventral root by stage 28 (Chu-Wang & Oppenheim, 1977), some may not have ramified within the muscle masses where their endings could pick up the HRP (but see Oppenheim & Chu-Wang, 1977). Alternatively exogenous application of HRP under the conditions used may not label all cells with axons present. However, these calculations do set a lower limit, and the initial projection patterns of at least one third of the cells can therefore be assessed before the major period of cell death.

Since there was some variation in the absolute number of labelled cells in different preparations, histograms of the raw numbers of labelled cells were replotted as the percentage of the total number of labelled cells per muscle mass that occurred in each 28 μm segment of cord. Then different preparations could be combined as shown in Text-fig. 3, giving the distribution of labelled motoneurones along the entire rostro-caudal extent of the cord expressed as mean % $\pm$ S.E.

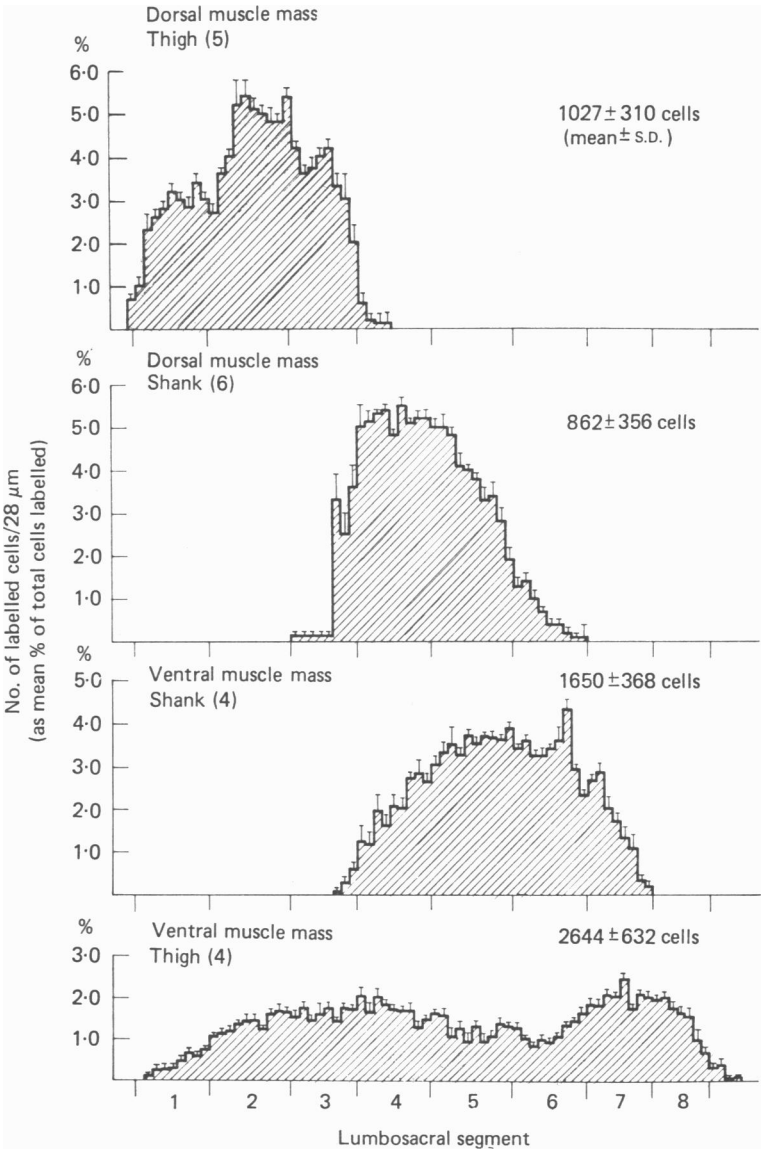
It can be seen that the spatial distribution of motoneurones for each muscle mass is quite consistent but differs one from another. For each muscle mass, certain segments were never found to project, and in general this is consistent with the known mature projections for the muscles derived from each (Landmesser & Morris, 1975; Landmesser, 1978). Thus even at this early stage, only the first three LS segments project to the pre-axial part of the dorsal muscle mass in the thigh; no labelled cells were observed in LS segments 5–8, and it should be kept in mind that every section was carefully observed. Similarly LS segments 1 and 2 do not contribute to either muscle mass in the shank, nor LS segments 7 and 8 to the dorsal muscle mass in the shank. Axons arising from LS segment 8 project to the ventral thigh but not the shank. Thus axons from the various segments do not ramify throughout the limb but appear to respect certain boundaries, the latter being consistent with mature projection patterns.

Comparison between projection patterns before and after motoneurone death

In order to evaluate more quantitatively these projection patterns, the distribution of motoneurones labelled by injection of each of the four muscle masses at stage 28 was compared with the distribution of motoneurons to the sum of the muscles derived from each mass. The comparison is most straightforward for shank musculature, since injections at stage 36–37 were comparable to early injections, filling all the muscles derived from either the dorsal or ventral mass. In Text-fig. 4*A*, *B* the data on stage 36–37 projections from the preceding paper have been replotted on the same graph to compare with stage 28 projections. Since the absolute length of the segments differed at early and late stages, each stage 36–37 segment was arbitrarily divided into as many increments as there were 28 μm sections at stage 28. The % of

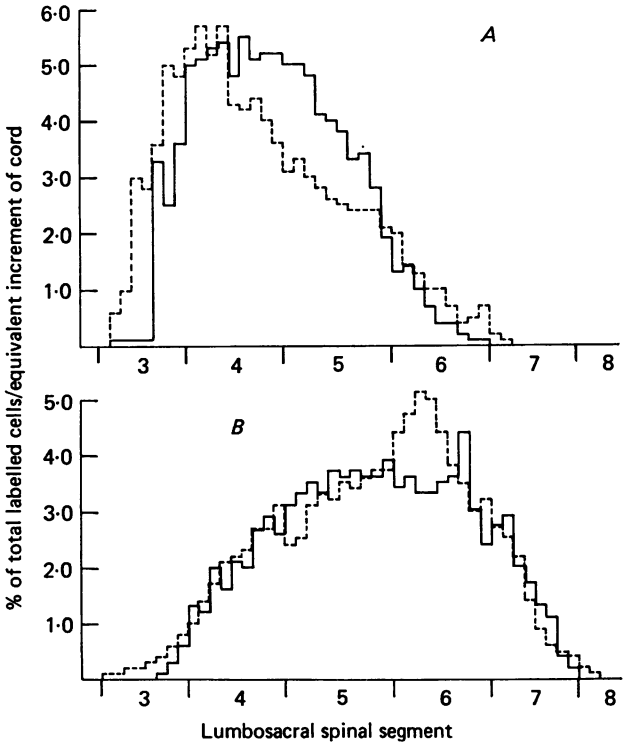
labelled cells per increment could then be superimposed on the stage 28 graphs, and a segment by segment comparison made at the two stages. At stages 28 and 36–37 the segmental distributions to dorsal and ventral shank musculature were found to be nearly identical. The only difference was a relative depletion in motoneurones from segments 4 and 5 to dorsal shank in later stages which was not statistically significant.

While the motoneurone distribution histograms could nearly be superimposed at



Text-fig. 3. Motoneurone projection patterns to the four primary muscle masses. The distribution of labelled motoneurones along the rostro-caudal axis of the spinal cord, following HRP injections into each of the muscle masses at stage 28. The mean number of labelled cells is given at the right and the number of embryos pooled for each histogram is given in parentheses at left. Error bars, drawn in one direction only, represent S.E.

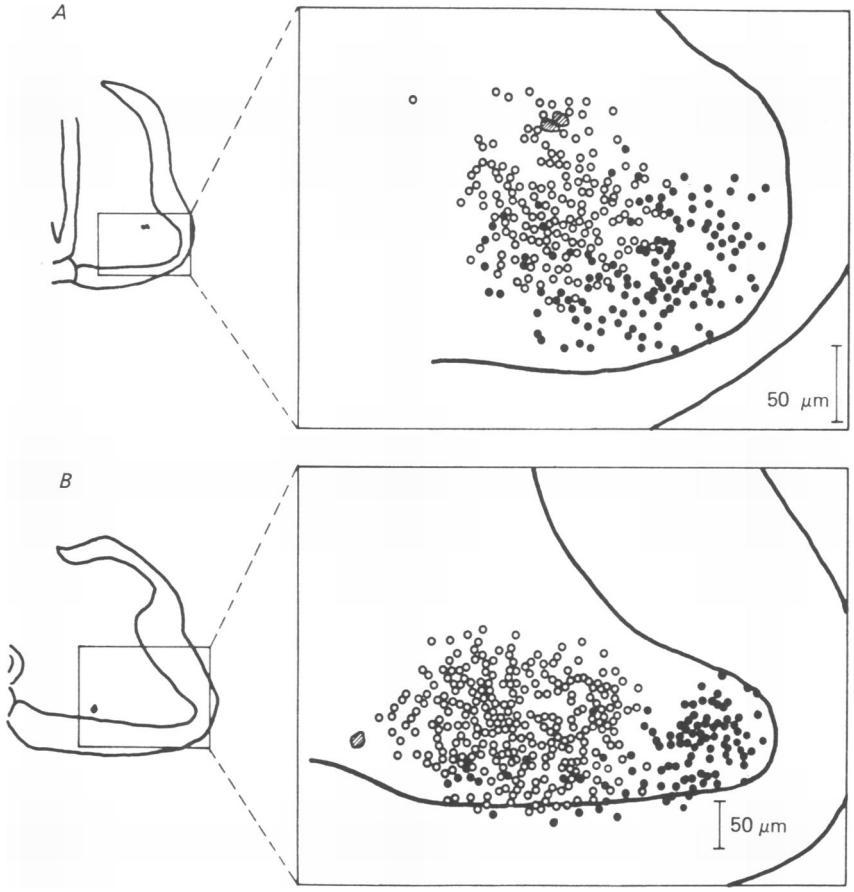
the two stages, there was a considerable overlap in the projections to the two muscle masses, especially those from segments 4, 5 and 6. Therefore it might be argued that many of the axons which project to the dorsal shank from segment 5 for example, were actually destined for ventral shank, and conversely. This can be tested because it has been shown (Landmesser, 1978) that muscles derived from the dorsal muscle mass were supplied by laterally situated motoneurones and those derived from the ventral muscle mass by medially situated ones. Therefore, in one embryo, for every



Text-fig. 4. Comparison of projection patterns to shank before and after cell death period. *A*, the distribution of labelled motoneurones (expressed as % of total labelled motoneurone pool per equivalent increment of cord) at stage 28 (continuous line) following HRP injection of dorsal shank muscle mass and at stage 36–37 following injection of all shank muscles derived from that mass (dashed line). *B*, similar treatment of ventral shank muscle mass at stage 28 (continuous line) and at stage 36–37 of muscles derived from that mass (dashed line).

third section in segment 5, this location of all motoneurones projecting to the dorsal shank was marked with the aid of a camera-lucida on a generalized spinal cord section from that segment, the central canal and white matter being used for alignment of individual sections. The segment 5 motoneurones projecting to the ventral shank were similarly noted. This was also done at stage 28, and the results can be seen in Text-fig. 5. At stage 28 the relative distribution of neurones projecting to the two regions is similar to that at stage 36–37; neurones innervating the ventral shank musculature lie for the most part medially, those innervating dorsal musculature laterally. One has the impression that there may be more overlap in the two

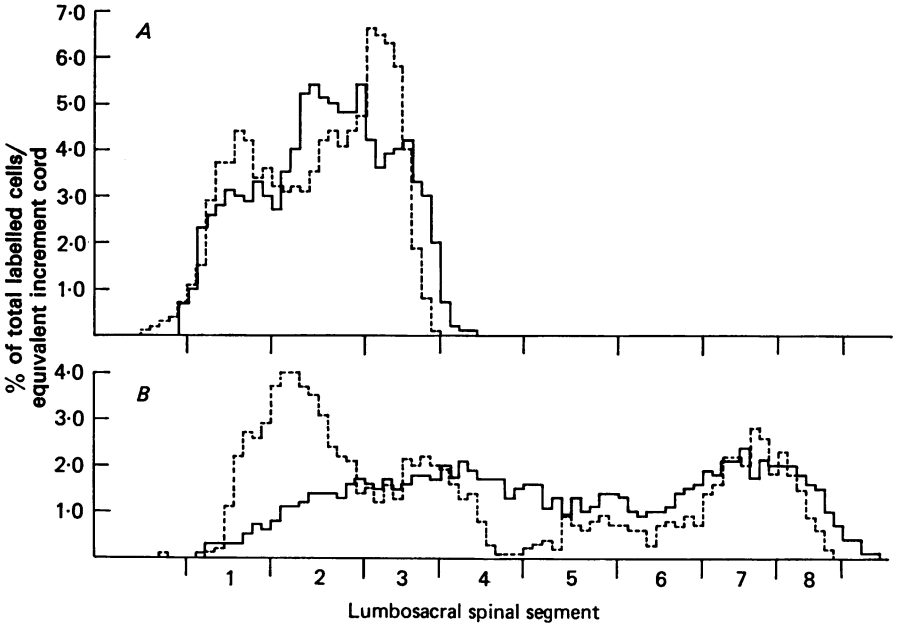
populations at stage 28. However, a more detailed analysis seems unwarranted since it is not certain that all motoneurones have migrated to their final position by stage 28, and it is not known to what extent the change in shape of the lateral horn could passively affect the neurone distribution. The least that can be said is that, as a population, the positions of dorsal and ventral shank neurones differ at stage 28, and that the difference is similar to that found at stage 36–37, after the period of cell death. Furthermore, none of the ventral shank motoneurones is situated in an extreme lateral position.



Text-fig. 5. The distribution of motoneurones to dorsal and ventral shank musculature in the transverse plane. *A*, the location of all stage 28 motoneurones projecting to either dorsal (filled circles) or ventral (open circles) shank musculature at the level of lumbosacral spinal segment 5. *B*, the distribution at stage 37 of all LS 5 motoneurones projecting to dorsal and ventral shank musculature (filled and open circles respectively). Diagrams at left show one half of the spinal cord for orientation, with area enlarged at right enclosed in box.

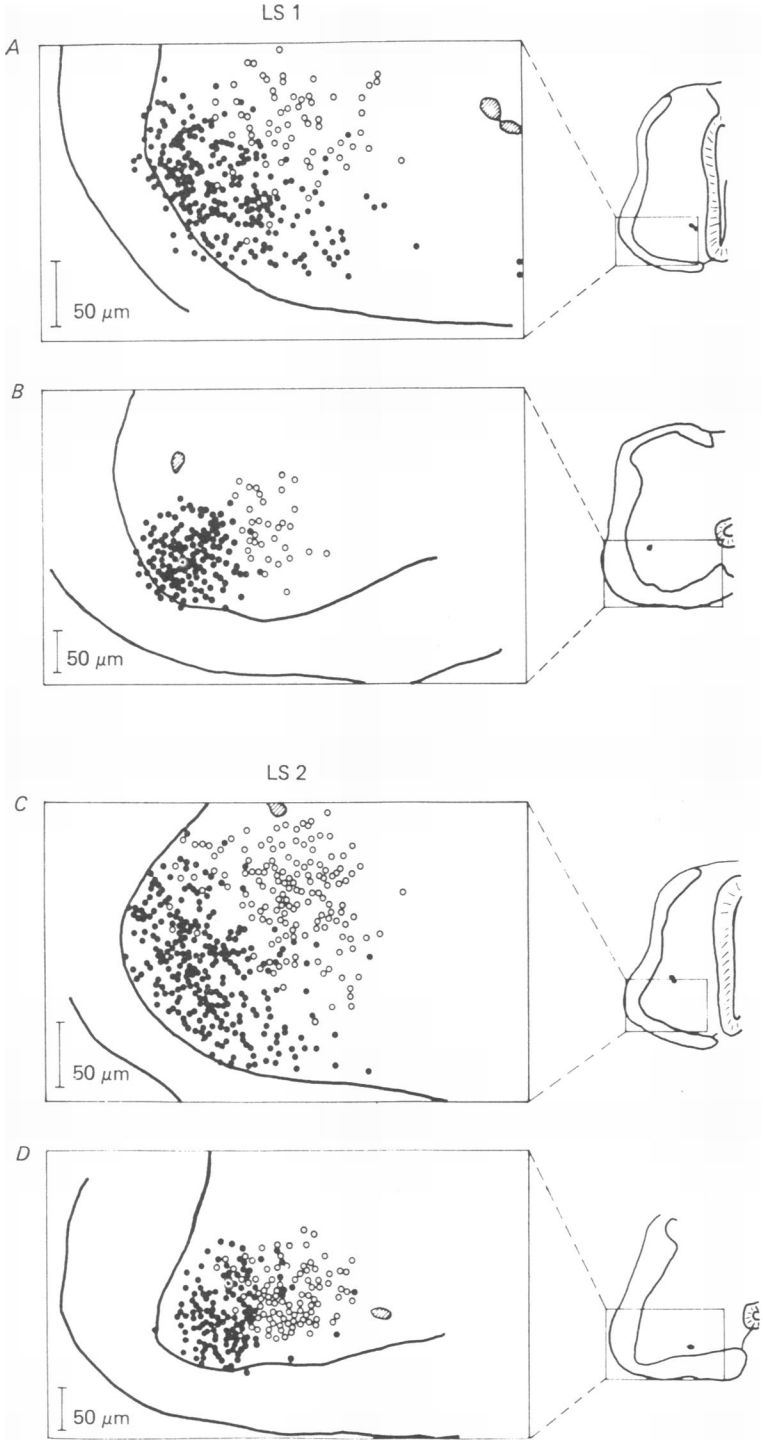
Somewhat similar results were found for thigh musculature. The distribution of motoneurones to the pre-axial portion of the dorsal muscle mass at stage 28 does not differ significantly from the distribution at stage 36½–37 to the muscles derived from that portion, the sartorius and femorotibialis (Text-fig. 6*A*).

For the ventral thigh musculature (Text-fig. 6*B*) the results for the two stages were also similar except for a paucity of motoneurones arising from the most rostral segments, 1 and 2, at stage 28. This was the only significant difference observed for any of the four primary muscle masses, and is not just a relative difference resulting from expressing the data as a percentage of the whole population. It remains when absolute number of cells are compared. It is probably due, at least partially, to the fact that ventral thigh injections at stage 28 did not include the deeper parts of the adductor region whose innervation is known to come from these segments. It may also reflect a late arrival of axons from motoneurones in the more rostral segments, which would have to grow further to reach the ventral thigh region.



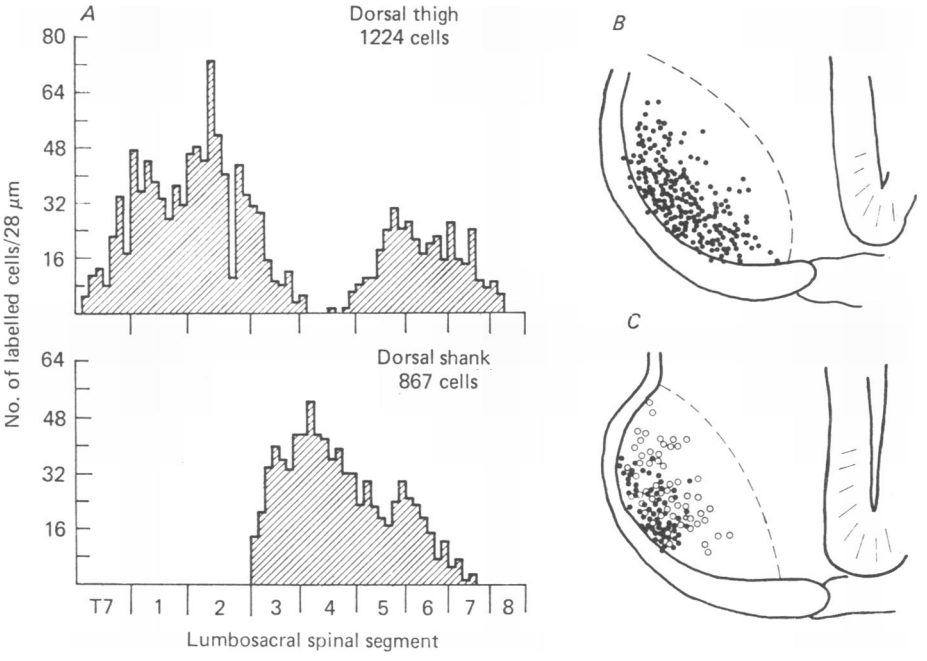
Text-fig. 6. Comparison of projection pattern to dorsal (*A*) and ventral (*B*) thigh before and after cell death period. In *A* the continuous line histogram shows distribution of motoneurones projecting to dorsal pre-axial thigh at stage 28 expressed as mean percentage of labelled population per 28 μm. Dashed line is histogram obtained by combining individual histograms of the two stage 36–37 muscles derived from this portion of the muscle mass, the sartorius and femorotibialis, and normalizing along longitudinal axis for differences in cord length. (See Methods for details.) *B*, similar comparison from motoneurones projecting to ventral thigh at stage 28 (continuous line) and to the combined stage 36–37 muscles that arise from that muscle mass: adductor, ischioflexorius, accessory and caudilioflexorius. Error bars not indicated for clarity; see text for further explanation. The only significant differences between the early and late histograms were in *B* in the first half of LS 2 (P = 0·01, Student's t test).

Nonetheless, the medio-lateral distribution of motoneurones at the two stages is remarkably similar, as seen in Text-fig. 7. Segments 1 and 2 are especially useful for comparison since they innervate only thigh musculature. Moreover, the only muscles which arise from that portion of the dorsal muscle mass injected at stage 28 are the sartorius and femorotibialis. The only muscle arising from the ventral muscle mass



Text-fig. 7. Comparison of medio-lateral position of motoneurones projecting to dorsal and ventral thigh at stages 28 and 37. The distribution of labelled stage 28 motoneurones occurring in LS 1 which project to ventral thigh (open circles) and dorsal thigh (filled circles) in *A* can be compared with projections to combined sartorius–femorotibialis (filled circles) and adductor (open circles) at stage 37 in *B*. Similar results for LS 2 at stages 28 (*C*) and 37 (*D*). Smaller diagrams of cross-sections through one half of the spinal cord are shown at right for orientation.

which receives a projection from segments 1 and 2 is the adductor. When camera-lucida projections were made of all labelled neurones in segments 1 and 2 at stage 28 it was found that neurones innervating the ventral muscle mass were primarily situated dorso-medially, and those innervating the dorsal muscle mass ventro-laterally (Fig. 8*A–C*). This is in essence similar to the stage 36½–37 distribution of motoneurones innervating the combined sartorius-femorotibialis (ventro-lateral) and of the adductor (dorso-medial) (Landmesser, 1978). In a few preparations, however, some laterally situated cells were found following ventral thigh injections. Their number was small, but it is possible that they represent inappropriately projecting cells. Yet with such small numbers of cells and the present technique it is difficult to distinguish between this possibility and slight leakage of HRP to the adjacent dorsal muscle mass.



Text-fig. 8. The distribution of motoneurones projecting to the dorsal muscle mass at stage 27. *A*, the rostro-caudal distribution of motoneurones projecting to the dorsal thigh (top) and dorsal shank (bottom). *B*, camera-lucida reconstruction of ventral quadrant of spinal cord showing that motoneurones in LS 1 and LS 2 that project to dorsal thigh occupy a lateral position. Dashed line indicates approximate limit of lateral motor column. *C*, similar camera-lucida reconstruction at level of LS 4–5, showing location of neurones projecting to dorsal thigh (filled circles) and dorsal shank (open circles).

In summary then, at stage 28 there is no evidence that significant numbers of motoneurones arising from inappropriate segments project to any of the four primary muscle masses and 40% of these cells will die during the subsequent cell death period. Furthermore, a consistent relationship has emerged for any segment: the ventral muscle mass is always innervated by medially situated motoneurone, the dorsal muscle mass by more laterally situated ones.

Earlier projection patterns

Stage 28 was chosen for the main part of this study because by this stage most motoneurones have apparently completed their migration to a final position (Hollyday & Hamburger, 1977) and it is prior to the major period of cell death (Hamburger, 1975). However, degenerating motoneurones, while considerably less frequent than after stage 28, were observed at stage 26–27 (see also Chu-Wang & Oppenheim, 1977). It is possible that these small numbers of degenerating cells could have projected inappropriately as has been described by Lamb (1976) for the earliest projecting cells in *Xenopus*. Therefore, a few observations were made at stage 27.

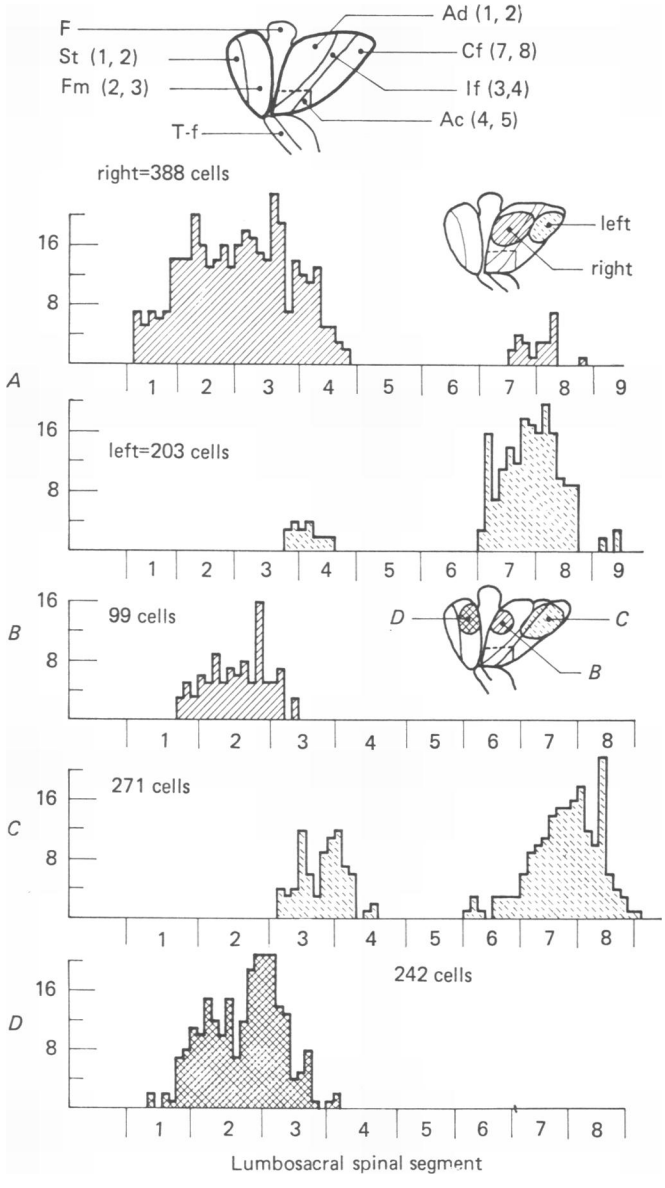
In three early stage 27 embryos, the dorsal thigh was injected in one leg and the dorsal shank in the other leg. In two, the rostro-caudal distribution of labelled cells was the same as at stage 28. In the third however (shown in Text-fig. 8), labelling also occurred in segments 5–7 following a dorsal thigh injection. It is thought that this resulted from labelling of the iliofibularis region before it cleaved from the primary muscle mass, a supposition supported by the fact that these neurones were situated laterally (Text-fig. 8*C*; circles) and not medially as would have been expected for neurones projecting to the ventral muscle mass. As can be seen in Text-fig. 8, all of the neurones labelled at stage 27 were relatively lateral. Furthermore, those in segments 5–6 which projected to the thigh were clustered more tightly laterally than those projecting to the shank, similar to the distributions for the iliofibularis and anterior shank motoneurones seen at later stages (Landmesser, 1978).

At earlier times (stage 26) following injection of dorsal and ventral muscle masses, distribution of labelled cells would be expected to be along the whole lumbosacral enlargement since no subdivision of the two primary muscle masses into thigh and shank has yet occurred, and this precludes distinguishing inappropriately projecting cells by their rostro-caudal distribution. Further, the medio-lateral distribution is difficult to interpret. First, the lateral motor column is very thin at this stage. Secondly presumably not all of the motoneurones have completed their migration from the proliferation zone in the ependymal layer. Most neurones appear to settle in the inside-out order, although there are apparent exceptions to this rule (e.g. the first born settling medially, the later born migrating through them to more lateral sites; Hollyday & Hamburger, 1977). These facts make the interpretation of lateral–medial position at early stages quite ambiguous. Thus this study can neither support nor rule out that small numbers of motoneurones project inappropriately at the earliest times.

Projection patterns within a muscle mass

It is next reasonable to wonder whether there is any evidence for a specific projection pattern within a muscle mass at stage 28. For example, the ventral muscle mass, which has been shown to receive innervation from the entire lumbosacral cord, has not yet been subdivided into any of its component muscles. Do the axons arising from different segments ramify widely within this mass, or are they restricted to certain regions perhaps corresponding to prospective muscle boundaries?

This was determined by confining the injected HRP to small regions of the muscle



Text-fig. 9. Distribution of labelled motoneurones following partial injections of different parts of the muscle masses of the thigh at stage 28. Diagram at top shows a medial view of stage 28–29 limb with approximate boundaries of prospective muscles delineated: F, femur; St, sartorius; Fm, femorotibialis; Ad, adductor; Cf, caudilioflexorius; If, ischioflexorius; Ac, accessory; T–f, tibia-fibula. Lumbosacral spinal segments making major contribution to mature muscles indicated in parentheses. *A*, partial injections to different regions of the ventral muscle mass, thigh (as indicated in limb diagram inset) in right and left legs of same stage 28 embryo resulted in the distribution of motoneurones shown in two top histograms. Injections of slightly different regions (shown in lower limb diagram inset) in two additional embryos resulted in distributions shown in *B* and *C*. Injection of posterior region of dorsal muscle mass of the thigh produced the distribution shown in *D*. Ordinate in all histograms shows number of labelled cells per 28 μm.

mass. The results for one such injection are shown in Text-fig. 9*A*. In the same animal the anterior region of the ventral thigh muscle mass was injected in the right leg and a more posterior region in the left leg, the approximate extent of HRP injected being indicated in the inset in Text-fig. 9*A*. Subsequent localization showed that most of the labelled cells on the right were located in the four most rostral segments, the same segments that innervate the adductor and ischioflexorius. These muscles are derived from the approximate region of the muscle mass injected. The injection into the more posterior 'caudilio-flexorius' region resulted in labelling of motoneurones located in predominantly lumbosacral segments 7 and 8. Thus the majority of axons seem to be restricted to definite regions of the primary muscle mass.

However, a small number of motoneurones did not follow this pattern; for example 6% of the cells labelled by the right leg injection were found in segments 7 and 8. These could represent caudilioflexorius motoneurones whose axons had overgrown the prospective muscle boundary and crossed into the anterior adductor-ischioflexorius region. Similar results were found for the left leg injection, where 10% of the labelled motoneurones were found in segments 3 and 4, and could represent ischioflexorius motoneurones which had crossed into the caudilioflexorius region. An alternative explanation is that the injection sites merely encroached on the prospective boundary between the ischio- and caudilioflexorius muscles. Two other animals, given similar injections, resulted in distribution of labelled cells similar to that in Text-fig. 9*A* and are not shown.

While the present technique does not allow one to distinguish unambiguously between these two possibilities, several observations can be made. The first is that if axons do cross over prospective muscle boundaries, they do so only in a limited way and do not ramify throughout the muscle mass. This can be deduced from the left injection illustrated in Text-fig. 9*A*, where axons of motoneurones located in segments 3 and 4 have possibly crossed into the 'caudilioflexorius region' whereas no axons from motoneurones located in segments 1 and 2 have done so. Furthermore, if the injection is confined to a smaller area which does not come close to the prospective boundary, as is the case in Text-fig. 9*B*, there is no labelling of cells located in the caudal LS segments 7 and 8, as occurred with the more extensive injection shown in the upper part of Text-fig. 9*A*. This latter injection was observed to extend across the adductor and ischioflexorius zones (as can also be deduced from the distribution of labelled cells) and thus may have encroached on the ischio-caudilioflexorius boundary. The former injection labelling primarily the adductor area did not reach the ischio-caudilioflexorius boundary. Thus the ischioflexorius motoneurones located in LS 4 and the caudal half of LS 3 were unlabelled, as were any motoneurones located in the more caudal LS 7 and 8.

In one additional injection (Text-fig. 9*C*) of the posterior thigh region, the injection site was observed at the time of injection, and later documented in cross-sections of the limb, to be more extensive than that shown in Text-fig. 9*A* and to include more of the 'ischioflexorius' region. This resulted in additional cells labelled in the ischioflexorius region of the cord (LS 3 and 4), but none in the adductor (LS 1 and 2) region.

Finally, in none of the ventral thigh injections was there extensive labelling of

cells in LS 6, 5 or the caudal half of LS 4. This is presumably because all of the injections were proximal to the region of the accessory muscle which receives predominantly from LS 5 and 6. Furthermore, virtually none of the large number of motoneurones located in segments 4, 5 and 6 which project to the ventral shank, apparently projected to the injected ventral thigh area.

In one additional animal an injection into the posterior 'femorotibialis region' of the anterior muscle mass resulted in extensive labelling of cells in LS 2 and 3, but a paucity of cells in LS 1 which projects predominantly to the sartorius (Text-fig. 9*D*). Comparison of this distribution with that resulting from injection of the combined sartorius-femorotibialis muscle regions (at the top of Text-fig. 3) suggests that a similar regionalization of the projection occurs in the dorsal muscle mass as well.

The technique used in these experiments cannot rule out the possibility that some motoneurones make projection errors by sending their axons across pre-muscle boundaries into regions that will become 'foreign' muscles. However, it does set some limit on the extent to which this occurs and suggests that motoneurones from a given segment do not project throughout a given muscle mass but have the ramification of their axons confined to regions that more or less correspond to the prospective muscles of their adult projections. One can further deduce that the final projection pattern is not the result of a relatively non-specific initial neural projection followed by the physical cleavage of the primary muscle mass into individual muscles. Cleavage planes during the latter process might be conceived physically to separate or 'axotomize' neurones projecting over boundaries, with the resultant death of their cell bodies.

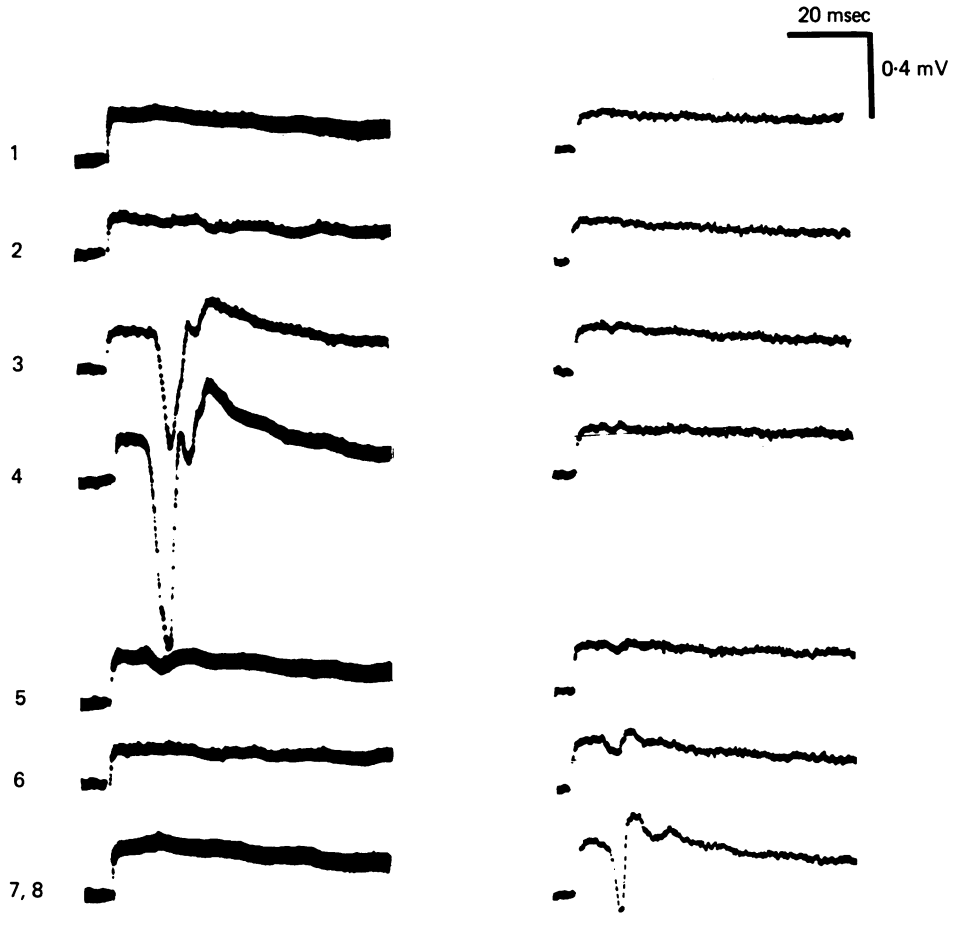
Functional innervation zones within a muscle mass

The retrograde HRP technique should label all neurones with axonal endings within a given region, whether or not such axons have made functional synapses (Heaton, 1977; L. Landmesser, unpublished observations). The functional projection pattern, deduced by evocation of post-synaptic activity in muscle cells should therefore lie within the boundaries of the anatomical, HRP-defined, projection pattern. Further, such physiological observations would provide an independent way of assessing the innervation pattern.

To this end eight stage 28–early 29 embryos were prepared as previously described (Landmesser & Morris, 1975), except that the skin was also removed from the thigh region. The medial surface of the ventral muscle mass was sampled by recording with a suction electrode in *en passant* fashion from a small number of muscle fibres. Stimulation of certain spinal nerves elicited electrical activity at the recording electrode which, as shown in Text-fig. 10, generally consisted of a large negative component with latencies of 5–10 msec although more complex wave forms were also observed. The responses were presumed to be due to synaptically activated activity in muscle fibres since they could be blocked by superfusion with (5×10^{-6} M) dTC (d-tubo-curarine) or repetitive stimulation at 8–10Hz. The latencies of 5–10 msec are compatible with observed muscle nerve conduction velocities of 0·1–0·5 m/sec and conduction distances of 0·5–1·0 mm.

Previous observations of innervation pattern based on observation of movements or tension measurements (Landmesser & Morris, 1975) had indicated that the nerve-

evoked response at stage 27–28 was extremely labile and unable to follow frequencies of 0·5–1·0 Hz. This was thought to be due to transmission block at immature synapses. However, the present observations showed that while the contractile response did block at such frequencies, the electrical response was capable of following stimulation rates of 1–5 Hz for prolonged periods (several minutes). It thus appeared



Text-fig. 10. E.m.g.s elicited from the ventral muscle mass by spinal nerve stimulation. Synaptically activated responses recorded with suction electrodes from a site corresponding to the 'ischioflexorius' region (left and from a slightly posterior site corresponding to the 'caudilioflexorius' region. Spinal nerves stimulated indicated on left. The lowermost right record has been retouched to show rapidly rising part of response.

possible that synapses were functioning even earlier than stage 27–28 when contractile responses could first be elicited. Indeed, evidence of such synaptically activated muscle activity was found as early as stage 26½. Thus there is a very short period between the time when nerves first grow into the limb (4 days) and that when synapses can be detected (4½ days).

The functional innervation pattern was mapped at stage 28 by recording at successive sites over the medial surface of the ventral muscle mass and sequentially

stimulating the eight spinal nerves for each recording site. Results similar to that shown in Text-fig. 10 were found in all cases. At recording position 1, roughly corresponding to the ischioflexorius region, large responses were only obtained from stimulation of spinal nerves 3 and 4, none resulting from stimulation of 1 and 2 (which contribute to the adult adductor) nor from 7 and 8 (which contribute to the adult caudilioflexorius). Moving the electrode a short distance (0·4 mm) posterior to position 2 caused these responses nearly to disappear, and responses could now be evoked by stimulation of 6, 7 and 8. Observations of similar recordings from a variety of sites in the eight embryos studied resulted in a functional innervation map similar to the one already deduced from HRP injections.

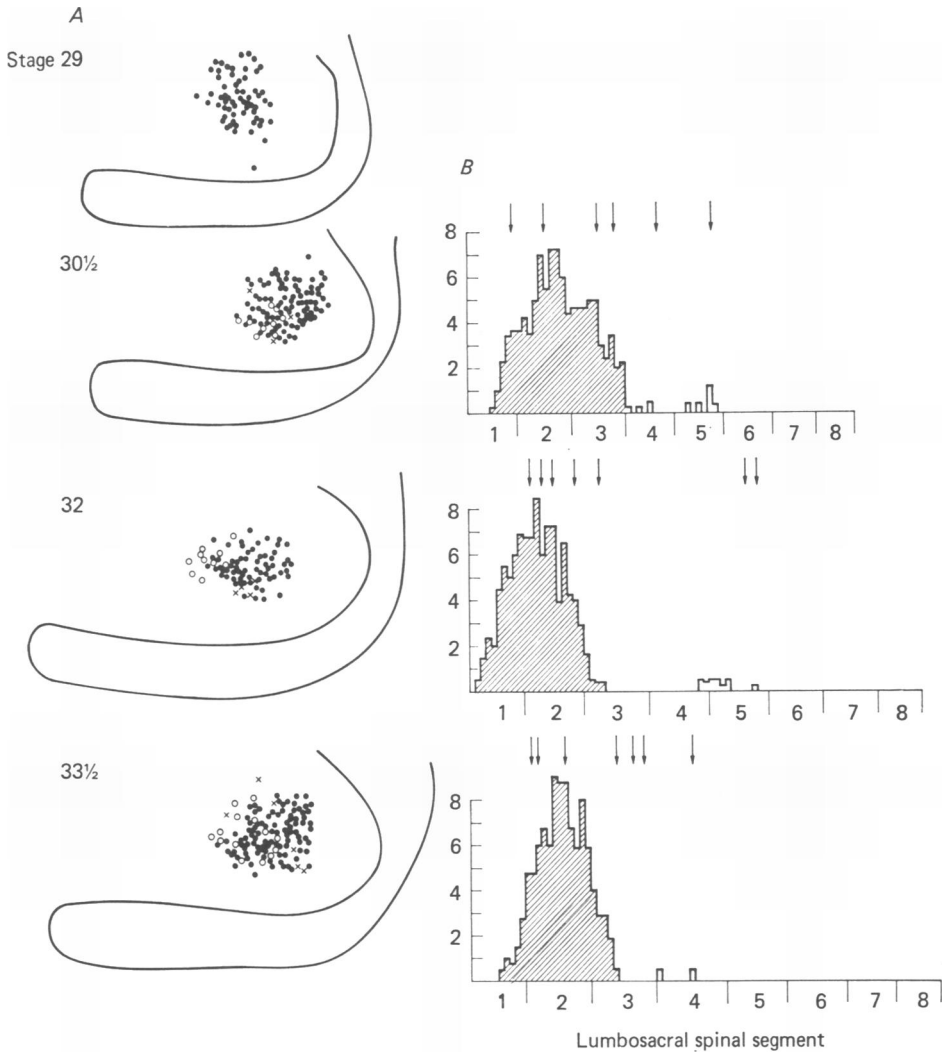
While it is clear that more extensive sampling of boundary regions by a combination of extra- and intracellular recording methods might detect weak subthreshold synapses missed in the present study, one can still conclude that the majority of effective synapses appear to be made within correct regions corresponding to adult projection patterns.

Projections to the adductor during the cell death period

Finally, a few observations were made on the adductor to determine if there were any transient changes in the projection pattern to a specific muscle during the period of cell death itself. Since the adductor first becomes individualized from the ventral muscle mass at late stage 30, HRP was injected into pairs of adductor muscles at stages 30, 32 and 33½, special care being taken to inject right up to the anterior boundary with the femorotibialis and up to the posterior boundary with the ischioflexorius. (This was later verified histologically.)

The distribution of labelled cells (mean of each pair) along the rostro-caudal axis does not change substantially during this period (Text-fig. 10*B*) and is similar to the final pattern achieved at stage 36 (compare with Text-fig. 3 in Landmesser, 1978). The small numbers of cells from clearly inappropriate segments (indicated by absence of cross-hatching) as well as larger projections from the caudal half of segment 3, may indeed represent axons from adjacent ischioflexorius and accessory regions that have grown into the adductor. Once again, however, it is difficult to exclude the possibility of slight leakage of HRP (not histologically detectable) over the adductor boundary.

Text-fig. 11*A* shows that there does not appear to be any transient projection from motoneurones that project to the anterior thigh (femorotibialis) region, for these cells would be situated laterally. The adductor motor nucleus is located medially in the same approximate position throughout the period studied. In this Figure, labelled cells in LS 3, which might represent accessory or ischioflexorius motoneurones are shown as open circles. They do tend to lie more medially, as do the motor nuclei for these two muscles. Labelled cells which appear to be degenerating are indcated by crosses. In general these lie within the confines of the main population of cells. Thus during the cell death period there do not appear to be any significant changes in the length or shape of the motor nucleus. Possible minor alterations due to ingrowth of axons projecting to adjacent regions will need to be confirmed by electrophysiological techniques.



Text-fig. 11. The location of the adductor motor nucleus at different developmental stages. In *A* the location of the adductor nucleus in the transverse plane (filled circles) is shown, pooling data from two embryos. Open circles indicate position of labelled motoneurones occurring in segments 3, 4 and 5. Crosses show positions of labelled cells which were degenerating. *B*, the location of the adductor nucleus in the rostro-caudal plane, plotting mean number of cells labelled per 28 μm section. Arrows above indicate sections where degenerating labelled cells were observed.

DISCUSSION

No obvious differences have been revealed between the early stage 28 projection pattern to the four primary muscle masses and the mature projection patterns to the muscles derived from these same masses. This study then corroborates the earlier electrophysiological study (Landmesser & Morris, 1975) in concluding that the massive spontaneous degeneration of motoneurones between stages 30 and 36 (during which 40 % of the neurones present at stage 28 die) does not alter the rostrocaudal distribution of motoneurones projecting to various sites in the limb. It goes

further in concluding that no substantial alteration in the medio-lateral distribution of motoneurones within the lateral motor column occurs either. These results are also generally consistent with Lamb's recent study (1976) for it appears that during the apparently comparable period of massive motoneurone death in *Xenopus* (Stage 54–58; Prestige, 1967) the projection patterns are not altered substantially.

However, the limitations of the retrograde labelling technique should be pointed out. Since this technique is sufficiently sensitive to detect all the neurones projecting to a given region early in development (Burstein, Landmesser & Pilar, 1977; Clarke & Cowan, 1976) there is little doubt that one should be able to distinguish even a small number of inappropriately projecting neurones, as long as these were sufficiently abnormal in position. This type of error was not seen (e.g. no neurones from segments 5–8 were ever found to project to the pre-axial dorsal muscle mass) and this clearly places limits on the sort of projection errors that might occur. However, there is normally a slight variability in both the rostro-caudal and the medio-lateral extent of motoneurone pools and a small number of wrongly projecting neurones along such boundary regions would be missed if they erred only slightly in projecting to regions immediately adjacent to their normal projection. Small actual increases in the extent of projections cannot easily be distinguished from possible diffusion of HRP to slight distances over a boundary region. Such errors, if they occur, will probably have to be detected by intracellular recording techniques.

While no substantial projection errors were detected prior to the major period of motoneurone cell death, a small amount of cell death seems to occur at earlier times (stages 25–27, L. Landmesser, unpublished observations; Chu-Wang & Oppenheim, 1977). Since some axons enter the limb at stage 24 (4½ days) (Oppenheim & Heaton, 1975) it is possible that some of the earliest cells could project wrongly and die (or retract their axons) prior to the earliest observations of the present study which were made at stage 27 or 5 days.

Such an observation has in fact been reported by Lamb (1976) in *Xenopus*. He speculated that a postulated guidance mechanism which allows later-arriving axons to reach their appropriate sites was not yet operative, so that the earliest axons merely projected to the site closest to their entry point in the limb, which in *Xenopus* was inappropriate. However, were these same events to occur in the chick hind limb, errors along the rostro-caudal axis would probably not be detectable, because projection to a site closest to entry in the limb would be generally appropriate. Errors in the medio-lateral distribution might be detected, but the results would only be interpretable with a detailed knowledge of HRP turnover rates in the motoneurone soma, and of the settling pattern of motoneurones as they migrate from the ependymal zone to the lateral motor column. Also it is not known to what extent motoneurone somas can migrate after sending axons to the limb. For these reasons observations were not made at stages 24–26 at the present time, and therefore the possibility of small numbers of motoneurones projecting wrongly at the earliest times cannot be excluded.

One interesting finding is that prior to cleavage of the primary muscle masses into individual muscles, there was a definite regionalization of the neuronal projection within a mass. Further, motoneurones anatomically projected to and made functional synapses only within regions corresponding to their mature projections; axons

therefore appeared to recognize and to respect pre-muscle boundaries. It can be concluded therefore that the process of muscle cleavage itself (about which very little appears to be known) does not physically create a relatively specific projection pattern from an earlier diffuse one. Some additional mechanism must therefore be proposed for allowing axons to grow to and to ramify only within appropriate regions.

In addition, once a muscle such as the adductor has individualized, the neuronal projection to that muscle remains relatively constant throughout the subsequent period of cell death and muscle maturation. A similar result has been reported recently for the gastrocnemius motoneurone pool (Hollyday, Hamburger & Farris, 1977). Motoneurone pools therefore do not appear to be shaped or refined by the process of cell death. These conclusions are of course subject to the limitations discussed above.

Also confirmed in the present study is the very early establishment of functioning synaptic contacts (Landmesser & Morris, 1975; Bekoff, 1976) which have been detected as early as stage 26½ (5 days) shortly after axons invade the limb bud. That these appear to be typical cholinergic chemically transmitting synapses is consistent with the rapid rise in cholineacetyl-transferase detected in hind limb buds between days 3 and 6 (Giacobini, 1972; Giacobini, Filagomo, Weber, Boquet & Changeux, 1973); the later fall in this enzyme (6–12 days) is temporally correlated with the period of motoneurone cell death (Hamburger, 1975). However, at this early time no definite ultrastructural synaptic contacts have been found (Kikuchi & Ashmore, 1976) and it appears that, following the early establishment of a relationship between a motor nucleus and a specific muscle, there is a prolonged period of possibly transient synaptic contacts during which growth cones actively expand, enter into relationship with developing muscle fibres and retract (Sisto Daneo & Filogamo, 1976). This may correspond to the period of multiple innervation and competition between axonal sprouts seen in mammalian (Brown, Jansen & van Essen, 1976) and chick wing (Bennett & Pettigrew, 1974) muscles, although no observations have yet been reported for chick hind limb. While the basic position of a motor nucleus does not appear to be altered during this period, substantial alterations within the nucleus may occur.

Finally the strong correlation between motoneurone soma position and early site of termination in the limb, suggests that some quality of the motoneurone associated with its position may be instrumental in allowing its axon to grow relatively directly to, and to ramify only within, specific pre-muscle regions of the limb. First, all motoneurones can be divided into two classes: medial ones which project solely to the ventral muscle mass and lateral ones which project to the dorsal muscle mass. The medio-lateral division possibly reflects differences in cell birthdays of the two classes (Hollyday & Hamburger, 1977), and it has previously been suggested that the specification of neurones may be related to the time at which they withdraw from the cell cycle (see Hunt, 1975 for discussion of this point).

A similar relationship has recently been noticed for *Xenopus* (Lamb, 1976). Further, within each muscle mass there is a spatially ordered relationship (see Text-fig. 1) such that the rostro-caudal position of a motoneurone cell body is correlated with the site of its axonal termination in the anterior–posterior axis of the muscle mass.

It is now necessary to determine the actual mechanisms by which this correspondence is achieved. Clearly it cannot be fully explained by diffuse outgrowth of axons followed by death of all which project wrongly. Something must also guide axons and allow them to recognize at least in a general way their proper termination sites. The present results would suggest that such a mechanism(s) operates relatively efficiently and that normally most motoneurones project to appropriate sites with few errors. This does not imply however that motoneurones are rigidly specified early in development. Various experimental manipulations (e.g. transplantation of supernumerary limbs) at about the time motoneurones are born show that they may be caused to project to (Hollyday *et al.* 1977) and to form functional synapses (Morris, 1976, 1978) with muscles inappropriate for their position. Further experimental manipulations of this sort (see also Stirling & Summerbell, 1977) will probably help to elucidate the mechanisms which normally operate. They will also be necessary to determine if the attainment of the projection pattern involves any interaction between the axons themselves (Hope, Hammond & Gaze, 1976; Prestige & Willishaw, 1975).

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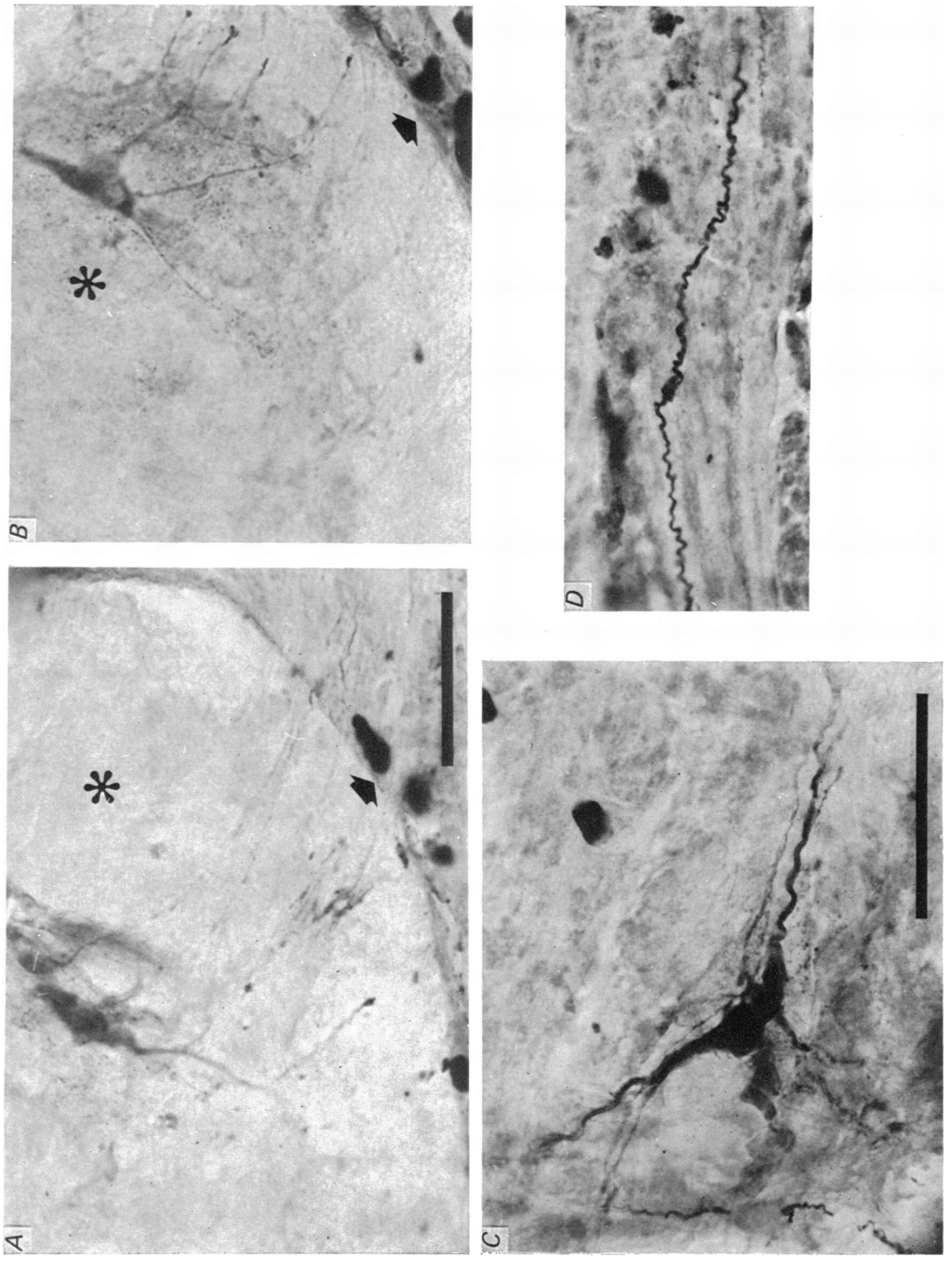
EXPLANATION OF PLATE

Labelled motoneurones in the lateral motor column at late stage 28. In *A* and *B* the ventrolateral edge of the white matter bordering the ventral horn is indicated by arrows; calibration for both = 50 μm. In *A*, injection of HRP into the ventral muscle mass of the thigh labelled a cluster of medial motoneurones, most of which show diffuse staining; no stained cells are found laterally (asterisk); section taken from level of LS 2. *B*, in another embryo, HRP injection into the dorsal muscle mass of the thigh labelled a cluster of lateral cells (right of asterisk) bordering the white matter; one cell is diffusely stained; the others, slightly out of the plane of focus show granular staining; no stained cells are found medially (to the left of asterisk) in the region occupied by the stained cells in *A*. In both *A* and *B*, processes from the stained cells can be seen to extend ventrally and laterally into the white matter. *C* shows several more diffusely stained motoneurones with processes extending ventrally toward the ventral funiculus and dorsolaterally toward the lateral funiculus; calibration bar for both *C* and *D* is 50 μm. *D* shows a stained motoneurone axon in the ventral root showing sinuous course of axons which is typical for this stage.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