Development of the segmental innervation of the chick forelimb

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SUMMARY

A number of recent studies have shown that during embryonic development the initial innervation of a target structure may be made up, in part, by axons which do not form part of the mature innervation of that structure. In the present study we have examined the motor innervation of the major muscles of the chick forelimb at different stages of development using HRP-uptake-labelling of motoneurons, electrophysiological recording and measurement of muscle contraction.

In the mature White Leghorn chick the major contribution to the motor innervation of the forelimb is from spinal segments 14, 15 and 16. Using the HRP-labelling technique we have shown that at stages 26–29 of development motoneurons in segments 12–17 have axon terminals in the presumptive biceps muscle. Between stages 30 and 35, however, the axon terminals arising from segments 12, 13, 16 and 17 are lost, leaving the mature innervation from segments 14 and 15. We have also observed the loss of innervation of the biceps muscle by segment 16 using electrophysiological recording of compound action potentials in the biceps nerve and by measurement of the local contraction of the biceps muscle in response to stimulation of the segmental nerves. Similar changes in the innervation of the triceps, extensor metacarpi radialis, flexor carpi ulnaris and flexor digitorum profundus muscles have also been observed.

These results are discussed in relation to the hypothesis that (i) the motoneuron pools and muscles in the developing spinal cord and forelimb are matched, (ii) that some axons which arrive in a particular muscle during early development are unable to form a stable connexion and (iii) that the inability of an axon terminal to form a stable connexion in a muscle results in the death of the motoneuron.

Intracellular recording from muscle cells at stage 35 shows that the synaptic site on each cell is innervated by about three separate axons. Over the next few stages, however, all but one of the innervating axons is lost. From our contraction studies it is clear that the removal of the excess axon terminals after stage 35 is not associated with the establishment of the mature segmental innervation pattern of the muscle.

INTRODUCTION

Several studies on the development of innervation patterns in amphibia, birds and mammals have indicated that some of the first contacts made between growing axons and target structures differ from the connexions observed in those...
structures in the mature animal (Clarke & Cowan 1975; Lamb, 1976, 1977; McGrath & Bennett, 1978). Hughes (1968) and McGrath & Bennett (1978), among others, have suggested that the establishment of the mature pattern of connexions is the result of a competition between axon terminals in the target structure and that the neurons which are the losers in this competition die. The latter authors have shown that early during the development of the axolotl, motoneurons in a number of spinal segments form synaptic connexions with limb muscle cells. Moreover, the synapses formed by the terminals of some motoneurons regress, while other terminals at the same site, from motoneurons which form the mature innervation, remain stable and increase in size. A brief report of similar observations in mammalian muscle development has also been presented (Harris & Dennis, 1977).

Studies on the development of the innervation pattern of the chick hindlimb by Landmesser & Morris (1975) and Landmesser (1977) have, however, shown no evidence for such a period of changing innervation of muscle cells. In the present work we have investigated the development of the innervation of different muscle cells in the chick forelimb. We present evidence that there is a very short period during the earliest stages of muscle development when the axons of motoneurons which do not contribute to the innervation of a particular muscle in the mature animal, can be found in that muscle. Moreover, we show that the loss of this part of a developing muscle's innervation occurs at about the same time as the reported period of motoneuron death in the chick.

METHODS

All experiments were performed on White Leghorn embryos which had been staged according to Hamburger & Hamilton (1951).

Histology: retrograde labelling of spinal motoneurons

The biceps muscle or proximal flexor muscle mass of the right forelimb of 62 embryos was injected with a 50 % solution of Sigma VI HRP (horseradish peroxidase) (Oppenheim & Heaton, 1975). Injections were made through a window in the shell using a 5 μl Hamilton syringe connected to a prefilled (HRP) glass capillary with a tip diameter of 10–20 μm. The glass capillary was held in a micromanipulator and advanced into the muscle to a depth of between 100 and 1000 μm depending on the stage of the embryo. Injections of 0.05–2 μl, also depending on the size of the embryo, were made on embryos of stages 25, 26, 28, 29, 31, 35 and 36.

Embryos which survived the HRP injection followed by 5 h incubation were then killed by decapitation. The brachial region of the spinal cord, together with the wing-buds, were fixed in 2 % glutaraldehyde and 5 % sucrose in phosphate buffer (pH 7.2) at 4 °C for 4 h. Specimens were washed overnight in running water and then placed in warmed phosphate buffer containing 0.08 % 3,3-
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Diaminobenzidine tetrahydrochloride. After 4 h, hydrogen peroxide was added to this solution to make a final peroxide concentration of 0.02%. Specimens were then rinsed in tap water, dehydrated and embedded in molten wax. After appropriate orientation, serial transverse sections of 15-100 μm were cut throughout the length of the brachial spinal cord and the injected limb, and each section was examined for HRP reaction product.

The distribution of HRP granules in labelled motoneurons was studied using phase microscopy. The ventral and lateral borders of the grey matter in the ventral horn were aligned with the edges of a 10 × 10 grid and each square on the grid was assigned an HRP density estimate between zero (no granules) and four (neurons in the square containing about 80 granules). Detailed granule counts on 10 squares at each density estimate showed that this estimation was linearly related to the actual number of granules in a square (20 granules per unit). In all embryos the contralateral side of the spinal cord served as the control.

A three-dimensional table of the HRP distribution in each spinal cord was made after examination of a hundred serial sections. The rostro-caudal axis of the reconstruction was related to the spinal segments using the vertebrae as an indication of the midpoint between adjacent segments. The segments were numbered according to their relationship to the first rib.

In order to combine the results from a number of embryos, the HRP densities for each embryo were first normalized to the respective maxima observed (see below) so that any variation in the volume of HRP injected into different embryos would not affect the analysis.

In Fig. 4 (rostro-caudal distribution of HRP densities), the granule densities in each section were totalled and expressed as a percentage of the maximum total observed along the length of the brachial cord. The normalized results for several adjacent sections at one level of the cord were then averaged and the values for each level of the cord in three embryos were meaned. Thus, each point in Fig. 4 shows the mean (± S.E.M.) normalized HRP density at each rostro-caudal level.

In Fig. 5 (medio-lateral and dorso-ventral HRP distribution), the granule densities at different levels of each axis were summed over each spinal segment and then normalized to the maximum value observed in each respective axis over all the spinal segments. The normalized results from three embryos were meaned and the variations in HRP density have been represented by three degrees of shading.

Electrophysiology

Each embryo was removed from the egg, decapitated and eviscerated, and pinned ventral side up in a perspex organ bath. The organ bath was perfused at room temperature with a modified Krebs solution containing (mm) Na 151, K 4.7, Ca 1.8, Mg 1.2, Cl 142, H₂PO₄ 1.3, SO₄ 1.2, HCO₃ 16.3, glucose 7.8 and
gassed with 95% O₂ and 5% CO₂. After the segmental nerves 13–17 and the brachial plexus had been exposed, nerves 14, 15 and 16 were cut at the spinal cord and stimulated using suction electrodes and pulses of 0.01–0.10 msec and 2–10 V.

Extracellular recordings of evoked action potentials were made with tungsten electrodes (Levick, 1972) placed in various muscles about 100 μm below the ectoderm. These recordings were made in embryos of stages 28–36. Intracellular recordings of synaptic potentials were made in embryos after stage 34 using glass microcapillary electrodes filled with 2 M-KCl and of 30–70 MΩ resistance. Both extracellular and intracellular signals were led through a high impedance unity gain preamplifier, displayed on an oscilloscope and photographed on moving film.

**Contraction**

Embryos were prepared as described above for electrophysiological recording. A differential strain gauge was used to measure the contraction of limb muscles in embryos younger than stage 34. The gauge consisted of two pins of 18 μm tip diameter and separated by 500 μm. One pin was attached to a stainless-steel wire and was used as a stable reference when placed in a limb joint. The other pin was attached to a watch spring and, when placed in the muscle region of interest, was displaced in relation to the other pin when the muscle contracted. Two semiconductor strain gauges (Keelite ADP-120-90) were cemented to the spring and these formed one half of a Wheatstone bridge, the other half of the bridge being two 120 Ω resistances. The whole bridge was connected to a Hewlett-Packard carrier amplifier and signals were displayed on an oscilloscope. A negative signal was recorded when the moving pin was displaced towards the reference pin. Extraneous movement from other muscles or distant parts of the same muscle mass always gave a positive signal and this was eliminated by careful pinning of the limb joints. Local contractions of less than 1 μm could be recorded with this device.

The contractions of individual muscles in embryos older than stage 34 were recorded with a Grass tension transducer. Muscles were freed from their distal insertions and the tendon was attached to the transducer by a suture thread. The rest of the limb was secured at the appropriate proximal joints.

**RESULTS**

In this study we have restricted our observation of muscle innervation by the brachial spinal nerves to the biceps, triceps, extensor metacarpi radialis, flexor carpi ulnaris and flexor digitorum profundus muscles (Fig. 1).
Fig. 1. Structure of the chick forelimb showing the arrangement of the bones, major muscles and nerves at two stages of development. Drawings of whole forelimbs were made from serial reconstructions at stages 29 (A) and 33 (B). The stippled area in A shows the condensation of chondroblasts which will form the bones of the limb; the latter are also stippled in B. Tracings of transverse sections at levels 1–2 and 3–4 shown in A and B were also made at stages 29 (C) and 33 (D). The muscles of the forelimb at stages 26–29 consist only of a flexor and extensor mass of myoblasts and myotubes. These masses then break up into the individual muscles of the limb at stage 30. The arrangement of bones and muscles shown in B and D for stage 33 is the mature anatomy of the limb.

Key to abbreviations: e, extensor muscle mass; f, flexor muscle mass; b, M. biceps brachii; b₁, b₂, b₃, separate heads of M. biceps brachii; t, M. triceps brachii; emr, M. extensor metacarpi radialis; fcu, M. flexor carpi ulnaris; fdp, M. flexor digitorum profundus; mn, radial nerve; mun, combined medial and ulnar nerves; mn, medial nerve; h, humerus; r, radius; u, ulnar.
Fig. 2. Distribution of horseradish peroxidase (HRP) reaction product in spinal segment 16 after injection of the peroxidase into the avian biceps muscle mass at stage 29 (6 days incubation). (A) Site of injection of HRP in the biceps (b) muscle mass at 6 days showing the extent of diffusion of the peroxidase (broken line) in the muscle from the site of injection; t, triceps; h, humerus. (B) High power view of the distribution of HRP granules in motoneuron somas (arrows) located in the ventral horn of spinal segment 16 after injection of HRP into the biceps muscle. Calibration is for A, 100 µm and B, 39 µm.
Histological identification of the segmental innervation of the biceps muscle during development

At stage 26 of development the musculature of the chick forelimb consists simply of a flexor and extensor sheet of myoblasts and myotubes (Fig. 1; Sullivan, 1962; Shellswell, 1977). The flexor muscle sheet is continuous over the chest (future pectoral muscle) and shoulder, the presumptive humerus (future biceps muscle) and presumptive ulnar (future flexor carpi ulnaris and flexor digitorum profundus). This continuity is retained until about stage 30 when the flexor sheet divides into its various distinct muscles with their tendinous insertions.

We have used the HRP uptake technique to identify the location in the spinal cord of those motoneurons with terminals in the presumptive biceps muscle during early development (see Methods). The site of injection in the biceps muscle and extent of diffusion of HRP from the site of injection was determined in each preparation by examination of serial sections of the whole limb (Fig. 2A). Preparations were rejected when the HRP had diffused more than 100 μm from the point of injection or when the endogenous peroxidase activity prevented clear identification of the injection site. Preparations were also rejected when the HRP reaction product in the motoneurons appeared as a diffuse brown stain, indicating uptake by injured axons (see La Vail & La Vail, 1974).

Prior to stage 25 no HRP reaction product could be detected in the spinal cord after microinjection of HRP into the proximal region of the forelimb, suggesting that the axons in the spinal nerves had not yet reached the limb. However, from stage 26 on, granules of HRP reaction product were easily detected in the motoneurons of the ventral horn of different spinal segments (Figs 2B and 3). These results are in agreement with those of Roncali (1970) on the timing of nerve arrival in the forelimb.

The rostro-caudal distribution of spinal motoneurons which contain HRP following injection of HRP into the proximal region of the flexor muscle sheet at later stages (presumptive biceps at stages 26–30 and the biceps muscle at stages 31–35) is shown in Fig. 4 (see Methods for details on the construction of Figs 4 and 5). Between stages 26 and 28 all the spinal segments which are known to contribute motor axons to the brachial plexus (S12–S17; Roncali, 1970) possessed motoneurons containing HRP following injection of HRP into the presumptive biceps muscle. However, after stage 28 there was a decline in the number of such motoneurons in segments 12, 13, 16 and 17 and by stage 31 HRP was only found in motoneurons in spinal segments 14 and 15.

We have also examined the medio-lateral and dorso-ventral distribution of motoneurons with axon terminals in the biceps muscle. At least 95% of the motoneurons in the brachial lateral motor column are produced by stage 23 (Hollyday & Hamburger, 1977) and as the spinal cord grows these motoneurons become dispersed by the establishment of glial cells between them. In
Fig. 3. Distribution of horseradish peroxidase (HRP) reaction product in a hemi-section of spinal segment 16 after injection of the peroxidase into the avian biceps muscle mass at stage 29. d, Dorsal; v, ventral. Arrow indicates the motoneurons labelled with HRP in the ventral horn. Calibration, 75 μm.
order to compare the distribution of motoneurons in the expanding spinal cord over the axes of interest we have normalized the width and length of the column at various ages. The distribution of biceps motoneurons at three different times during development is shown diagrammatically in Fig. 5. At early stages HRP reaction product was found throughout the medio-lateral and dorso-ventral extents of the lateral motor column in segments 12–17. By stages 32–35, however, the nucleus for the biceps muscle was confined to a region midway along both these axes.

The time course of the change in motoneuron distribution over these axes is the same as that shown above for the rostro-caudal axis. It can be seen in Fig. 5 that the most densely labelled part of the column at even the earliest stages corresponds to the location of the mature nucleus for the biceps muscle. This suggests that many axons growing to the limb reach their correct destination
Fig. 5. Diagrammatic representation of the dorso-ventral and medio-lateral distribution of motoneurons with axon terminals in the biceps muscle at different stages of development (see Methods for details). The spinal segments are numbered on the left and pooled results are presented for stages 26-28, 29-31 and 32-35. The dimensions of the spinal cord have been normalized and the results are the average from three preparations in each age group. Granule densities have been represented by three degrees of shading. Note that at all stages the highest granule densities in biceps motoneurons are in segments 14 and 15. In addition to the changes in the rostro-caudal distribution of biceps motoneurons there is considerable restriction of the biceps nucleus to a region midway along both the dorso-ventral and medio-lateral axes.
from the outset. The more sparsely labelled surround at early stages indicates motoneurons whose axons are in the biceps muscle but which do not form any part of its mature innervation.

In an attempt to ascertain that the present observations at stages 26–28 were not the result of undetected diffusion of HRP from the site of injection to other muscles in the limb we repeated the experiments described above using very small injection volumes (0.02 µl). In these experiments HRP was barely detectable at the injection site but granules of HRP reaction product could still be observed in motoneurons in segments 12–17. It is unlikely that this observation could be the result of undetected diffusion of HRP away from the injection site to other muscles in the limb.

**Extracellular recording of nerve activity**

The earliest in vitro recordings of electrical activity in the flexor muscle sheet were made at stage 28 with tungsten electrodes at about 100 µm beneath the ectoderm. It is most likely that the potentials recorded after stimulation of nerves S 14, S 15 and S 16 were compound action potentials from the axons within the muscle sheet, since they were both unaffected by the addition of (−)-tubocurarine (10⁻⁶ g ml⁻¹) to the Ringer solution and the muscle cells are incapable of generating action potentials until just before hatching (Masaakira, 1975).

Impalements of the presumptive biceps muscle at a proximal position at stage 29 gave recordings of compound action potentials of about 100 µV with stimulation of nerves S 14, S 15 and S 16 (Fig. 6). Impalements in this muscle at more distal positions (as far as the elbow region) gave lower amplitude recordings suggesting that the recordings arose from activity in the biceps nerve which enters the muscle in a proximal position (Sullivan, 1962). Similar compound action potentials were recorded at stage 29 in the presumptive flexor (flexor carpi ulnaris, flexor digitorum profundus) and extensor (extensor meta-carpi radialis) muscles of the distal forelimb at positions where the respective nerves enter each muscle. Again, potentials could be recorded in response to stimulation of S 14, S 15 and S 16 (Fig. 7).

A number of experiments were carried out on three separate preparations in order to check that the potentials observed were indeed arising from the axons within the muscle sheet and not from the major nerve trunks (containing S 14, S 15 and S 16 axons) that lie adjacent to the presumptive humerus and ulnar bones. In the case of the presumptive biceps muscle a series of recording and stimulation sites were chosen as shown in Fig. 6A. The normal response was first recorded at Position 1 with stimulation of S 14, S 15 and S 16 (Fig. 6B, i, ii and iii). The recording electrode was then repositioned near the medial and ulnar nerves at the elbow (Position 2) where responses could be recorded with stimulation of either S 14, S 15 or S 16 (Fig. 6B, iv and v). Next, the medial and ulnar nerves were stimulated at Position 2 and the resultant antidromic
Fig. 6. Compound action potentials recorded in the biceps muscle at stage 29 with stimulation of the spinal nerves from segments 14, 15 and 16. (A) Location of recording and stimulating electrodes when recording from the right forelimb. (B) Compound potentials recorded in the biceps muscle (1 in A) with stimulation of S 14 (i), S 15 (ii) and S 16 (iii). Compound action potentials recorded from the medial and ulnar nerves at the level of the elbow (2 in A) with stimulation of S 15 (iv) and S 16 (v). Compound action potential recorded at the brachial plexus (3 in A) with stimulation of the medial and ulnar nerves at the elbow (2 in A) (vi). No potentials could be recorded in the biceps muscle with stimulation of the medial and ulnar nerves at the elbow, indicating that activity in these nerves does not affect the recording of electrical activity in the biceps muscle.
Fig. 7. Changes in the amplitude of compound action potentials recorded from axons in various limb muscles between stages 28 and 36. Responses were recorded in the biceps (b), extensor metacarpi radialis (emr), and both the flexor carpi ulnaris (fcu) and flexor digitorum profundus (fdp) muscles with stimulation of segmental nerves S 14 (filled circles), S 15 (open circles) and S 16 (open triangles). At least six recordings were made from these muscles in four embryos; the error bars represent ± 1 S.E.M. The results suggest that between stages 28 and 32 the biceps and extensor metacarpi radialis nerves lose axons originating from S 16 and that the flexor carpi ulnaris and flexor digitorum profundus nerves lose axons from both S 14 and S 15.

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compound action potentials were recorded at S 14, S 15 and S 16 near the spinal cord, at the brachial plexus (Position 3, Fig. 6, vi) and finally at the original recording site in the presumptive biceps muscle. In the last experiment no activity could be recorded at Position 1, even though S 14, S 15 and S 16 axons in the medial and ulnar nerves were active. It seems likely therefore that the compound action potentials recorded in the presumptive biceps muscle with stimulation of S 14, S 15 and S 16 are action potentials in the axons within the muscle and not those in the nerve trunks coursing to the distal forelimb. These experiments also show that at stage 29 the motoneurons which have axons in the presumptive biceps muscle do not also have collateral branches in the medial and ulnar nerves at the level of the elbow.

The size of the compound action potentials recorded in different presumptive muscles in the flexor and extensor muscle sheets with stimulation of S 14, S 15 and S 16 changed during the period of development from stage 28 to stage 31 (Fig. 7). Although responses could be observed in the muscles with stimulation of each of S 14, S 15 and S 16 at stage 28, by stage 31 no compound action
potentials from S 16 axons could be recorded in either the biceps or extensor metacarpi radialis. Likewise at this stage no potentials from S 14 axons could be recorded in flexor carpi ulnaris and flexor digitorum profundus.

**Contraction**

The observations above suggest that there is a changing pattern of segmental innervation of presumptive muscles during the early development of the forelimb. The question still remains, however, whether all the axons in the muscles form functional connexions with the muscle cells. By using a specially constructed differential strain gauge (see Methods) we have quantified the local contractions of the proximal flexor muscle mass (presumptive biceps) of the forelimb with stimulation of S 14, S 15 and S 16. The contraction of this muscle mass in response to stimulation of S 14, S 15 and S 16 at stage 29 is shown in Fig. 8. It is clear that at this stage the terminals of axons from all three spinal segments have formed functional connexions with these muscle cells. After stage 31,
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Fig. 9. Percentage innervation of the major muscles of the forelimb by spinal nerves S 14, S 15 and S 16 at different times during the late development of the chick. Results are shown for stages 40–41 (filled triangles), 44–45 (open circles) and 0–1 week post-hatched (filled circles). Each point represents the mean of four observations from each muscle in each of four preparations; error bars give ±1 S.E.M. Note that there is little change in the percentage innervation of the different muscles by the spinal nerves from 14 days incubation to 7 days post-hatched, and that about 20% of the myofibres of each muscle are innervated by two spinal nerves. Key to the abbreviations for the muscles given in the abscissa are $b^1$ and $b^2$, biceps heads 1 and 2; emr, extensor metacarpi radialis; $t$, triceps; fcu, flexor carpi ulnaris; fdp, flexor digitorum profundus.

however, there was no contraction of this muscle with stimulation of S 16, while stimulation of S 14 and S 15 continued to cause the muscle to contract. The time course of this change in segmental innervation of biceps muscle cells is the same as that determined by electrical recording and HRP-labelling of motoneurons.

In order to study the segmental innervation of the forelimb muscles during later stages of development and during the first few weeks after hatching we have recorded the tetanic contraction of individual muscles using a conventional force transducer. All preparations studied after stage 35 showed a consistent pattern of segmental innervation of individual muscles (Fig. 9) and this pattern was the same as had been determined using the other techniques for stages 31–36. For convenience we have determined the percentage innervation of a muscle by a particular spinal nerve by expressing the amount of tetanic contraction in response to stimulation of that nerve as a percentage of the tetanic contraction with simultaneous stimulation of S 14, S 15 and S 16.
Fig. 10. Evoked potentials recorded intracellularly from muscle cells in the developing biceps muscle. (A) Endplate potentials recorded in a muscle cell at stage 35. Both slow and fast rise time endplate potentials were recorded with four stepwise increases in stimulus strength applied to the biceps nerve, indicating that the cell is innervated by terminals from at least four separate axons. (B) Endplate potentials and submaximal action potentials recorded in a biceps muscle cell at stage 44.
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Fig. 11. Changes in the mean number of nerve terminals per muscle cell in the biceps muscle between 9 days incubation (stage 35) and one week post-hatched. The means were determined from at least five muscle cells in each of three preparations at each age; error bars give ± 1 S.E.M.

Intracellular recording of nervous activity

We have not been able to impale biceps muscle cells with intracellular micro-electrodes prior to stage 35. However, at stage 35 evoked synaptic potentials were recorded in 18 muscle cells in the biceps muscle in response to stimulation of the biceps nerve. Some of the recordings from individual muscle cells were exclusively fast endplate potentials (e.p.p.'s) (rise time < 1.5 msec) and others were exclusively slow e.p.p.'s (rise time > 2.5 msec). Most cells, however, had e.p.p.'s of both the fast and slow types (Fig. 10A). Similar fast and slow rise time e.p.p.'s have previously been reported in the early innervation of the mammalian diaphragm (Bennett & Pettigrew, 1974).

Changes in stimulus strength to the biceps nerve led to changes in the latency and amplitude of the e.p.p.'s at all synapses studied, indicating that each of the synaptic sites received a multiple innervation from about three nerve terminals at this time (Fig. 10A). After stage 35 there was a gradual decline in the average number of terminals per synaptic site and by about stage 45 most synaptic sites were innervated by only one nerve terminal (Fig. 11). This loss of synapses follows a similar time course to that described for the development of synaptic sites in mammalian muscle (Bennett & Pettigrew, 1974).

Both before and after the period of loss of terminals from synaptic sites, most of the forelimb muscles show about 20% overlap in their innervation by two segmental nerves (Fig. 9). That is, about 20% of the myofibres appear to receive an innervation from two axons from different segmental nerves. We are uncertain at present whether this is the result of a continuing multiple innervation of synaptic sites on some myofibres which are deep in the muscles and relatively inaccessible to electrophysiological observations, or whether the synaptic sites are spaced more than a length constant apart on single myofibres and are unlikely to be detected by electrophysiological means. This last possibility
seems unlikely since single teased myofibres have only one ‘en plaque’ ChE deposit (personal observations).

In stage-44 embryos e.p.p.’s occasionally gave rise to fast potentials of varying amplitude (Fig. 10B). These potentials could not be evoked separately by changes in stimulus strength and they occurred alternately with the e.p.p. It is likely that these potentials are submaximal action potentials, since Masaakira (1975) has reported similar potentials evoked by current injection with a second microelectrode at this stage.

**DISCUSSION**

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The present results indicate that there is a period during the early development of the chick forelimb when the segmental innervation pattern of the presumptive muscles undergoes great change. For example, on histological criteria the presumptive biceps muscle contains axon terminals of segmental nerves 12–17 at stage 26, and using electrophysiological techniques we have found axons from segmental nerves 14, 15 and 16 in the same muscle at stages 28–30. By recording the contraction of the biceps muscle at stage 29 we have confirmed that the S 14, S 15 and S 16 axons in the muscle have formed functional synapses.

Furthermore, all three techniques demonstrate changes in the pattern of innervation of the biceps muscle such that after stage 32 the only segments which contribute to its innervation are segments 14 and 15. Our histological and electrophysiological techniques demonstrate that the loss of S 16 synapses from the muscle is accompanied by a loss of S 16 axons from the biceps nerve. The contraction studies on older embryos and chicks also indicate that the innervation of the biceps muscle by S 14 and S 15 remains constant, at least until 8 weeks post-hatched.

The HRP-labelling technique shows that in addition to the changes in the rostro-caudal distribution of spinal segments which contain motoneurons with axon terminals in the biceps muscle there is also considerable change in the medio-lateral distribution of biceps motoneurons within these spinal segments. Although the extent of HRP diffusion from the site of injection was checked in each embryo the level of endogenous peroxidase activity in the limb made it difficult to observe very low levels of injected HRP. Thus, one limitation of the HRP technique at early stages is that HRP may be present but undetectable in regions apart from the muscle of interest. However, we have also obtained consistent results in preparations where the volume of HRP injected was so small that the levels of HRP at even the injection site were barely detectable. It is unlikely that HRP has diffused to other parts of the limb in these preparations. Moreover, the consistency of the observations using three different techniques indicates that the undetected diffusion of HRP is not a severe limitation of the HRP technique.
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We have also observed a change in the segmental contribution to the nerves to the extensor metacarpi radialis, flexor carpi ulnaris and flexor digitorum profundus muscles of the lower forelimb between stages 28 and 30. Because these three muscles lie adjacent in the forelimb we have not been able to record their contractions separately prior to stage 32. However, from our observations on the biceps muscle it is possible that all axons in these muscles establish functional synapses for at least two developmental stages and that the subsequent loss of some axons from the muscle nerves reflects a loss of synapses. Studies on the identification of these synapses using electronmicroscopy are currently in progress.

These observations of a changing segmental innervation pattern during limb development are consistent with those reported by Lamb (1976, 1977) on the development of the amphibian hindlimb. However, it is not clear if the present observations are consistent with those of Landmesser & Morris (1975) on the development of the segmental innervation of the avian hindlimb. These authors found that after stages 30–31 there is no change in the number or distribution of segmental nerves which send axons to individual muscle nerves (their table 3) and this largely agrees with the present observations. However, these authors also show that for results pooled between stages 28 and 31 there is no change in the segmental contribution to (at least) the peroneous and gastrocnemius muscle nerves (their fig. 7). In the present work we have found evidence that the segmental innervation of the presumptive forelimb muscles has begun to change at stages 28 and 29 and that these changes are largely completed by stage 31. Thus it is important to know how many observations Landmesser & Morris made at stages 28 and 29 but included in their pooled data over stages 28–31. A similar question remains regarding their studies on individual muscle contractions using a force transducer attached to muscle tendons. Results are presented (their fig. 5 and table 2) for recorded contractions of individual muscles between stages 28 and 31, but is not clear how this was achieved since the muscles have ‘not fully separated from their respective primitive muscle masses’ at stages 28 and 29.

A more recent brief report by Landmesser (1977) using HRP labelling of motoneurons and EMG techniques appears to confirm the earlier study by Landmesser & Morris (1975). It is still unclear, therefore, why the development of the innervation of the chick hindlimb may be different to that of the forelimb, but the problem will best be resolved by intracellular recording of synaptic activity from myotubes in the primitive muscle masses. The observation of changes in the segmental innervation of developing muscle cells by intracellular recording of synaptic activity has already been reported in the rat (Harris & Dennis, 1977) and in the axolotl (McGrath & Bennett, 1978).

The present results, together with those of Lamb (1976, 1977), Harris & Dennis (1977) and McGrath & Bennett (1978) suggest that the development of the segmental innervation of the limb musculature involves, at first, a somewhat
diffuse pattern of motor connexions, followed by the elimination of some of these connexions to leave the mature pattern. The results are not consistent with the view that, from the outset, motor axons grow only to those destinations which constitute the mature pattern of innervation (Landmesser & Morris, 1975).

The elimination of some nerve terminals from a developing muscle may involve withdrawal and degeneration of some branches of an axon, withdrawal and relocation of an axon, or total degeneration of the axon and its motoneuron. The first of these possibilities seems unlikely since antidromic stimulation of the major limb nerves at the level of the elbow did not activate axons (branches) which innervate the more proximal biceps muscle. Moreover, Lamb (1977) has shown, using HRP labelling, that some cells responsible for the earliest motor projections to hindlimb muscles in *Xenopus laevis* do not selectively lose or relocate axon branches but die a short time later.

The period of maximum motoneuron death in lumbar regions of the chick spinal cord occurs from stage 26 to about stage 33 (Hamburger, 1975; Chu-Wang & Oppenheim, 1978). Hollyday & Hamburger (1977) have reported that the onset of production of motoneurons in the brachial lateral motor column precedes that in the lumbar column by 6 h (stages 17 and 19) but that there does not appear to be any difference between these regions in the termination of motoneuron production (about stage 23). It is probably reasonable to assume therefore that cell death in the brachial column occurs at about the same time as that in the lumbar column. (Note also that stage 26 covers a period of 12–14 h, see Hamburger & Hamilton, 1951; Hollyday & Hamburger, 1977, table 1). Such a period of cell death covers the time when we have observed changes in the segmental innervation of the developing limb. If our assumption is correct then the present observations are consistent with the hypothesis that the death of some neurons reflects a mechanism for the correction of developmental errors in axon terminal location (see Clarke & Cowan, 1975).

The contraction recordings in the present experiments and the intracellular recordings of Harris & Dennis (1977) and McGrath & Bennett (1978) have established that the axon terminals which are eliminated from a developing muscle have first made functional connexion with muscle cells. The latter authors have suggested that the establishment of the mature innervation pattern of a muscle involves a competition between nerve terminals at a synaptic site such that the terminal which is destined to remain ‘wins’ and grows, while the other terminals decrease in size and are presumably lost. Such a series of events has been suggested after experiments on the reinnervation of adult axolotl muscle which has been innervated by collateral sprouts from foreign nerves (see Bennett & Raftos, 1977).

Many authors have reported that abnormally high numbers of motoneurons die during development if they have been deprived of their normal peripheral target (Hamburger, 1958; Prestige, 1967; Prestige & Wilson, 1974; Chu-Wang & Oppenheim, 1978; Oppenheim, Chu-Wang & Maderdrot, 1978). These obser-
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Observations have led to the suggestion that the survival of a neuron is dependent on it making adequate peripheral contact (Hamburger, 1975; Prestige, 1976). Taken together, the observations of Lamb (1977) and McGrath & Bennett (1978) suggest that the motoneurons whose axons have lost in any competition in the periphery and have, therefore, not made adequate contact, may be the ones which die.

It has also been suggested that there is a system of matching between the motoneuron pools in the spinal cord and the various muscles of the developing limb which determines whether a motoneuron will win or lose in the periphery (Hughes, 1968; McGrath & Bennett, 1978). Thus, if the axon of a motoneuron in a particular pool succeeds in reaching its matched muscle then it will probably win and grow, while those axon terminals which arrive at a non-matched site will probably lose, resulting in death of those motoneurons. The results of the present study are consistent with all these suggestions.

It is important to note that the period of any matching between growing axons and muscle cells is probably limited to the early stages of muscle innervation, since reinnervation of the biceps muscle by the medial nerve at hatching does not reconstitute the original segmental innervation (personal observations).

Multiple innervation of synaptic sites

The multiple innervation of synaptic sites in developing muscle is now well established (e.g. Redfern, 1970; Bennett & Pettigrew, 1974, 1975; Brown, Jansen & Van Essen, 1976; Korneliussen & Jansen, 1976; Rosenthal & Taraskevich, 1977; Riley, 1977). It is not surprising therefore that similar observations were made in this study. The subsequent loss of this multiple innervation of synaptic sites took place between days 9 and 20 of incubation. During this time, however, there was no change in the pattern of segmental innervation of the limb muscles as determined by our contraction studies. Thus, at this time during the development of the chick, the removal of excess nerve terminals from a site does not involve changes in the segmental innervation of the muscle cell. It is possible, of course, that some terminals may have already been lost from the synaptic site before day 9 (stage 35) and that this did involve elimination of terminals from particular spinal segments. Such an observation has been made during the earliest stages of development of the rat intercostal muscle (Harris & Dennis, 1977).

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