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# THE FORMATION OF SYNAPSES IN STRIATED MUSCLE DURING DEVELOPMENT

BY M. R. BENNETT AND A. G. PETTIGREW
*From the Neurobiology Laboratory, Department of Physiology, University of Sydney, Sydney 2006, Australia*

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## SUMMARY

1. A study has been made of the formation of synapses in developing striated muscles which receive either a focal (the rat hemidiaphragm) or a distributed (the avian anterior latissimus dorsi) innervation using histological, ultrastructural and electrophysiological techniques.

2. In the developing diaphragm only a single synaptic contact was initially established at random along the length of the short (300 $\mu$m) myotubes by a single axon; in the developing ALD more than one synaptic contact could be established initially along the length of the long (2500 $\mu$m) myotubes by axons, but the distance between these was never less than 170 $\mu$m.

3. Each synapse established by the initial axonal contact in either the diaphragm or the ALD subsequently received a multiple innervation from further exploring axons in the muscles, and all such additional innervation of muscle cells was constrained to the sites of the initial synaptic contacts; this multiple innervation of synaptic sites was lost in the subsequent 4 weeks.

4. It is suggested that the axon forming the initial synaptic contact on myotubes induces a property over an adjacent length of myotube which makes its membrane refractory to synapse formation over this length; this characteristic length is longer for axons forming a focal innervation than it is for those forming distributed innervation.

## INTRODUCTION

During the reinnervation or cross-reinnervation of adult muscles which receive either a focal (Bennett, Pettigrew & Taylor, 1973; Bennett, McLachlan & Taylor, 1973*a*, *b*) or distributed (Miledi, 1960; Landmesser, 1972; Bennett *et al.* 1973) innervation, the pattern of synapses on many individual muscle fibres is restored, as a result of nerves returning exclusively to the original synaptic sites (Bennett *et al.* 1973*a*). This is due

to some property of the muscle fibre membrane at the position of the original synapse or synapses, which makes them a region of preferred innervation (Bennett *et al.* 1973*a*, *b*). The present work describes the initial formation of these synapses during the development of muscles which receive either focal or distributed innervation, whilst the subsequent paper (Bennett & Pettigrew, 1974) enquires into the factors which determine the pattern of these synapses over the surface of muscle fibres during development.

## METHODS

The formation of synapses in the focally innervated mammalian hemidiaphragm was studied in foetal and post-natal albino rats. The age of the foetuses was determined from the appearance of sperm at oestrus in the vaginal smear and the crown rump length of the embryos according to Angulo y Gonzales (1932). The day of birth determined the post-natal age.

### *Light microscopy*

Histological studies were made on whole mounts of foetal and 1- to 2-day post-natal hemidiaphragms. Foetuses were removed from the mother under ether anaesthesia and killed by decapitation. The hemidiaphragms were removed during fixation and kept in 15% sucrose–10% formalin until the total fixation time was 1 hr. Post-natal hemidiaphragms were removed under ether anaesthesia and fixed as above. All hemidiaphragms were stained for cholinesterase (ChE) activity according to Karnovsky & Roots (1964) for 30–40 min, at room temperature and using acetylthiocholine as the substrate. Frozen sections were cut (30–50 $\mu$m) from two 19-day gestation hemidiaphragms and these were also stained for ChE activity. About half of all hemidiaphragms stained for ChE activity were post-fixed in 10% formalin for 1–2 hr and then impregnated with silver using Weddell & Glees' (1941) modification of the Bielchowsky-Gros method for staining of nerve fibres.

### *Ultrastructure*

Small pieces of a diaphragm from a 2-day-old and a 4-day-old rat were prepared for electron microscopy by fixing for 1 hr in cold 6·5% glutaraldehyde in cacodylate buffer, washing overnight, post-fixing in osmium tetroxide, staining in block with uranyl acetate and embedding in Araldite (CIBA). Sections were cut on a LKB ultramicrotome, stained with lead hydroxide and examined using a Phillips EM 201 or EM 300 electron microscope.

### *Electrophysiology*

Electrophysiological recordings were made from hemidiaphragms from 15 days gestation to 4 weeks post-natal. Foetuses were removed under ether anaesthesia, killed by decapitation and the diaphragm region placed in a Perspex organ bath which was perfused with a prewarmed modified Krebs solution (Bennett *et al.* 1973) at 34–35° C and gassed continually with 95% $O_2$ and 5% $CO_2$. Post-natal diaphragms were removed under ether anaesthesia and placed in the organ bath in the above conditions. The diaphragms were further dissected in the organ bath and pinned out, the insertions of the muscles on the ribs being left intact. Conventional electrophysiological techniques previously described by Bennett & McLachlan (1972) were used for the stimulation of the phrenic nerve and for the recording of intracellular potentials. D-tubocurarine ($5 \times 10^{-7}$–$1 \times 10^{-6}$ g ml.$^{-1}$) was added to the

organ bath with preparations older than 17 days gestation in order to render the evoked end-plate potentials subthreshold. The pattern of transmitter release from nerve terminals was examined by applying a conditioning-test sequence of stimuli of different intervals to the phrenic nerve. The amplitudes of the two responses were measured and analysed for facilitation ($f$) by substitution in the equation $f = (V - V_0)/V_0$ (Mallart & Martin, 1967) where $V_0$ is the amplitude of the conditioning response and $V$ that of the test response. Negative $f$ values were taken as a measure of depression of transmitter release.

The formation of synapses in a muscle which receives a distributed innervation was studied in the anterior latissimus dorsi (ALD) muscle of the chick. The age of chick embryos was determined from the number of days incubation and the date of hatching determined the post-hatched age. All animals were killed by decapitation.

*Light microscopy*

All ALD muscles were fixed *in situ* in 15% sucrose – 10% formalin for 2–3 min, removed and then kept in fixative so that the total fixation time was 1 hr. ALD muscles up to 12 days incubation were stained for ChE activity and impregnated with silver as whole mounts. From 13 days incubation to the adults frozen sections (30–50 $\mu$m) were cut and stained. All staining procedures were the same as described above for the rat hemidiaphragm although incubation times in the ChE staining were considerably longer (1½–2 hr).

*Ultrastructure*

ALD muscles in embryos from 6 days incubation to hatching and during the first 6 weeks post-hatched were fixed in 6·5% glutaraldehyde in cacodylate buffer *in situ* for 3–5 min after decapitation, dissected into small pieces and placed in fresh fixative so that the total fixation time was 1 hr. Subsequent procedures were identical to those described above.

*Electrophysiology*

The techniques used in the preparation of all ALD muscles, the recording of intracellular potentials, the examination of the pattern of transmitter release and the determination of the muscle fibre sensitivity to iontophoretically applied acetylcholine were the same as described previously (Bennett *et al.* 1973).

In the electrophysiological study of the development of both the mammalian hemidiaphragm and the avian ALD muscle the minimum number of nerve terminals innervating a muscle cell in the vicinity of the recording electrode was estimated by gradually increasing the strength of stimulation to the primary nerve trunk (thereby recruiting additional axons in the nerve trunk) and noting consistent changes in the time course and amplitude of the end-plate potential (e.p.p.) response measured in the muscle cell. Each consistent change in the e.p.p. response was assumed to be due to the activation of an additional nerve terminal synapsing on the muscle cell.

## RESULTS

### *The formation of synapses in a muscle which receives a focal innervation: the mammalian hemidiaphragm*

*Muscle growth*

At 15 days gestation the rat hemidiaphragm consists of a band of transversely orientated myotubes which extend about 1 mm (932 $\mu$m

$\pm 46$, $n = 25$ diaphragms) either side of the point of nerve entry into the muscle (Text-fig. 1, Pl. 1*a*). The band also contains individual myoblasts, as well as myoblasts undergoing fusion with other myoblasts and with myotubes (Kelly & Zacks, 1969*a*); at the ends of the band is a pool of muscle cells which consist only of myoblasts (Text-fig. 1, Pl. 1). Over the next 24 hr gestation the diaphragm grows in length primarily by the fusion of these myoblasts at the ends of the diaphragm into myotubes and in width by both the elaboration of cytoplasm and the addition of myoblasts at the ends of the myotubes (Williams & Goldspink, 1971). At 17–19 days gestation the myotubes at 1 mm from the point of nerve entry show sarcomere alignment, indicating their differentiation to myofibres which increase in length by the addition of sarcomeres at their ends (Williams & Goldspink, 1971).

*The location of nerves and synapses in the developing diaphragm*

The location of nerves and synapses in the muscle during early development was determined from the distribution of silver impregnated axons, of ultrastructurally identified axons and of cholinesterase (ChE) deposits.

At 15 days gestation the primary nerve trunk (50–60 axons) (the phrenic nerve) divides at its point of entry in the centre of the hemidiaphragm into two secondary nerve trunks (20–30 axons) which traverse the hemidiaphragm at right angles to the myotubes (Text-fig. 1, Pl. 1*a*). Smaller tertiary nerve bundles (2–5 axons) branch from the secondary nerve trunks and pass between and parallel to the long axis of the myotubes, as well as into the pools of myoblasts at the ends of the hemidiaphragm (Text-fig. 1, Pl. 1*b*). At 1 mm from the point of nerve entry, where the myotubes are only about 300 $\mu$m long (Text-fig. 2), some of the tertiary nerves run almost the entire length of the myotubes (Text-figs. 1 and 2). These tertiary nerves give rise to single axons which either run for varying distances between the myotubes or obliquely into the adjacent pool of myoblasts (Text-fig. 1, Pl. 1*c*).

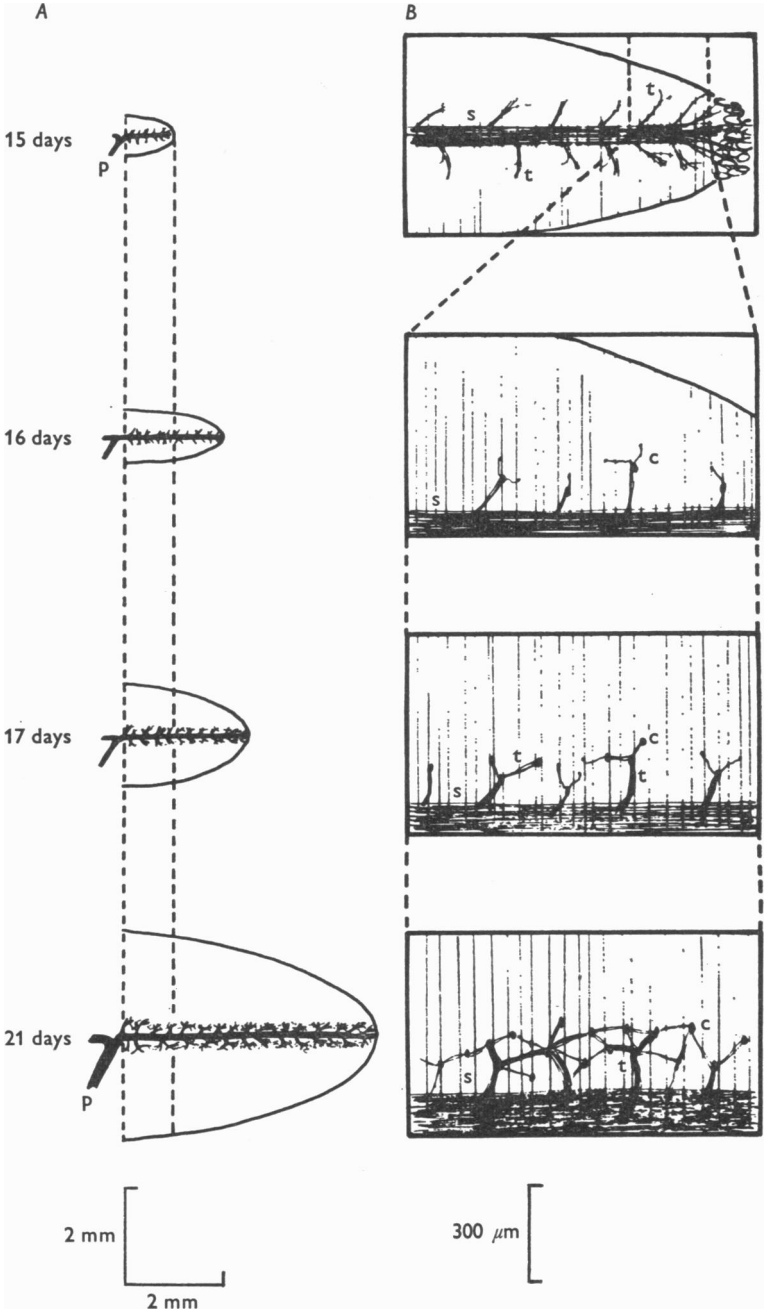
By 16 days gestation both the secondary (now 30–50 axons) and tertiary (now 2–8 axons) nerve bundles had increased in size at 1 mm from the point of nerve entry, and the latter nerve bundles now innervated the myotubes at points of localized ChE activity (Text-fig. 1, Pl. 2*a*). In this region of the muscle about 30% of the myotubes possessed a localized ChE deposit and only one such deposit was observed on any myotube. In addition, the deposits were scattered at random throughout that length of the muscle which was occupied by the exploring axons at 15 days gestation. Single exploring axons were sometimes observed leaving the cholinesterase deposits and growing without any preferred orientation amongst the muscle cells (Text-fig. 1, Pl. 2*b*). These axons extended for

only short distances along the length of the myotubes, so that all nerves were still confined within a narrow band only slightly wider than that observed at 15 days gestation (Text-figs. 1 and 2). Since at this time the myotubes in this region of the muscle had increased over three times their length at 15 days (Text-fig. 2), the percentage lengths of myotubes occupied by nerves had dropped to 31 % in the 24 hr period (Text-figs. 1 and 3).

From 17 days gestation to 1 day post-natal the innervation band at 1 mm from the point of nerve entry into the muscle, consists of secondary nerve trunks running across the myofibres, and large (10–15 axons) tertiary nerve branches which give rise to a number of smaller nerve bundles (2–5 axons). These small bundles innervate each of the muscle cells at one point of localized ChE (Pl. 2*c*), as well as divide and anastomose to form a complex network of axons between the synapses (Text-fig. 1; Pl. 2*d*). Examination of these synapses with the electron microscope revealed the presence of many axon profiles in synaptic contact with a muscle cell within the same synaptic fold (Pl. 4*a*). In this region of the muscle about 80 % of the muscle cells possessed a localized ChE deposit at 17 days and this increased to 100 % by 1 day post-natal. Although a few single axons can be observed leaving the small nerve bundles and terminating in ChE deposits of very low density at 17–19 days gestation (Text-fig. 1; Pl. 2*e*), no single axons forming new synapses were present at one day post-natal (Text-fig. 1).

Synapse formation appears to be complete in this region of the muscle by 18–19 days gestation. Up to this time the small increase in the width of the end-plate zone over that at 15 days gestation (Text-fig. 2) is probably partly the result of some outgrowth of single axons during the formation of synapses on uninnervated myotubes within the region. After 17 days gestation the fraction of the increasing muscle fibre length occupied by the end-plate zone remains fairly constant at 15 % (Text-fig. 3). This suggests that the increase in the end-plate zone width, especially at later times, may also be due to uneven growth at the ends of the myofibres.

In summary the sequence of events leading to the formation of synapses in a given region of the diaphragm appears as follows. Single exploratory axons enter a region of the muscle which contains myoblasts and these subsequently fuse to form myotubes. A single synapse is then formed by an exploratory axon at random along the length of each of the newly formed myotubes. Thus synapses occur along the entire length of the newly formed pool of myotubes. Other axons grow preferentially along the pathway of the exploratory axon into this region of the muscle, thereby forming a tertiary size nerve branch and innervating each synaptic site on




Text-fig. 1. For legend see facing page.

a myotube. Single axons sometimes leave these multiply innervated synaptic sites to become exploratory axons, some of which enter the adjacent pool of myoblasts together with the main bulk of the exploratory axons from the secondary nerve, and the cycle of synapse formation is repeated. Other exploratory axons grow out from established synapses and form synapses on the newly formed myotubes within the region. During the early post-natal period some axons are removed, so that synaptic sites cease to be multiply innervated, and the adult condition of one axon terminal per synapse is attained.

*Spontaneous and evoked electrical activity in the developing diaphragm*

Early during development the electrical activity of muscle cells was recorded at about 1 mm either side of the point of nerve entry into the hemidiaphragm.

At 15 days gestation, when single axons are observed running for

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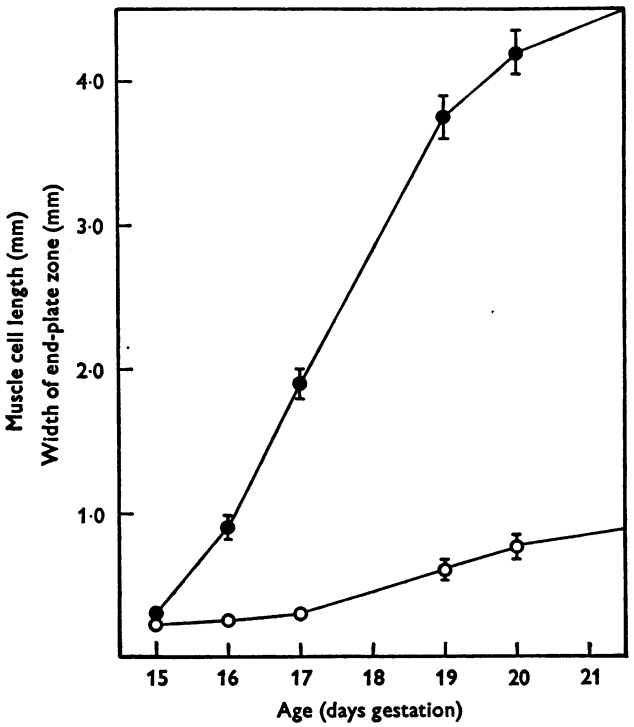
Text-fig. 1. Diagram of the pattern of innervation in one half of the rat hemidiaphragm over the last six days gestation.

*A*, the primary (phrenic) nerve (p) enters the hemidiaphragm and divides into two secondary nerve trunks which traverse the hemidiaphragm along its long axis. Smaller tertiary nerve bundles leave the secondary branches and extend along the myotubes for a short distance corresponding to the length of the myotubes at the time of their differentiation. This low power diagram also shows the large increase in dimensions of the muscle during this period and the small increase in width of the area occupied by nerves. Vertical dashed lines show the point of phrenic nerve entry into the muscle and the position 1 mm lateral to this point.

*B*, high power diagram showing the distribution of nerves in the area of the muscle 1 mm from the point of nerve entry (bounded by dashed lines). The vertical lines represent the orientation of the myotubes and the small dots at and after 16 days gestation represent cholinesterase deposits (c). The secondary nerve trunks (s) traverse the diaphragm at right angles to the myotubes and give rise to tertiary nerve bundles (t) which extend along the myotubes for a short distance corresponding to the length of the myotubes at the time of their differentiation. At 15 days gestation the nerves at 1 mm from the point of nerve entry occupy almost the entire length of the myotubes and extend into the adjacent pool of myoblasts at the end of the muscle. From electrophysiological evidence some of the cells at this stage are innervated at a single point by a single axon. At 16 and 17 days gestation cholinesterase deposits appear in the middle of the myotubes and these are innervated by at first one and then later by a number of silver impregnated axons. At this stage evoked end-plate potentials and spontaneous miniature end-plate potentials of the fastest rise time are recorded in the middle of the muscle cells. From electrophysiological criteria the muscle cells are innervated at one site by up to four nerve terminals. By 21 days gestation there is a complex network of axons which extend between the synapses and no newly forming synapses are observed.

varying distances between the newly formed myotubes, no spontaneous electrical activity was recorded in the muscle cells (over twenty cells impaled for 1 min in four diaphragms). Mechanical fibrillation of different parts of the muscle was observed, however, suggesting that spontaneous action potential firing had occurred. Evoked electrical activity was recorded from low resting potentials (range $-30$ to $-40$ mV) in a few muscle cells (three out of thirty cells impaled in four diaphragms): two of these cells had fast end-plate potentials (e.p.p.s) (rise time $\leqslant 3$ msec) whilst the

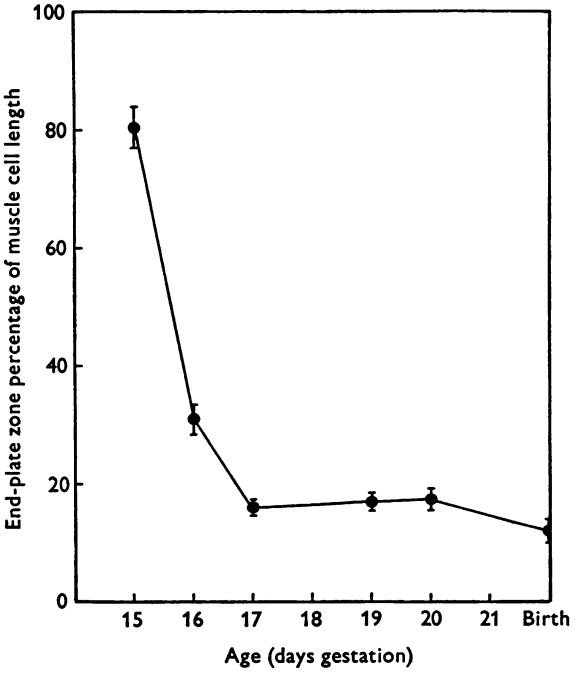



Text-fig. 2. Changes in the length of muscle cells (filled circles) and width of the end-plate zone (open circles) in the rat hemidiaphragm over the 6 days before birth, measured at a position 1 mm from the point of nerve entry into the muscle. Error bars represent the S.E. of the mean ($n > 12$) where this error is larger than the symbols used.

remaining cell had very slow e.p.p.s (rise time $>$ 40 msec). As the amplitude of the e.p.p. in all three cells was invariant to changes in stimulus strength, each of them was probably innervated by only a single nerve terminal (Text-fig. 4).

By 16 days gestation, when the first signs of localized ChE appears on

some of the myotubes and these are innervated by up to three axons, spontaneous miniature end-plate potentials (m.e.p.p.s) (rise time $\leqslant 3$ msec) were recorded in only one cell (out of sixteen cells impaled for 1 min in two diaphragms). However, evoked electrical activity was recorded in many muscle cells (fourteen out of twenty cells impaled): four of these cells had exclusively fast e.p.p.s, five exclusively slow e.p.p.s (rise time, 4–10 msec) (Text-fig. 5*A*), whilst the remaining cells possessed e.p.p.s

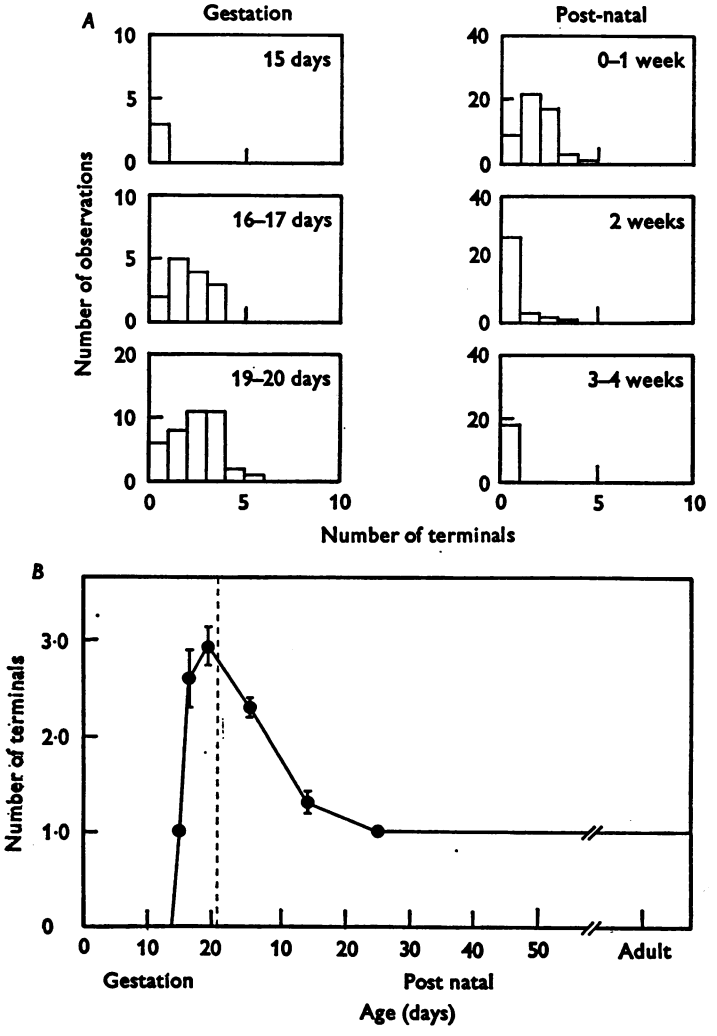



Text-fig. 3. Changes in the percentage of the muscle cell length which is occupied by the end-plate zone in the rat hemidiaphragm over the last 6 days gestation and at birth. Error bars represent the S.E. of the mean ($n \geqslant 12$).

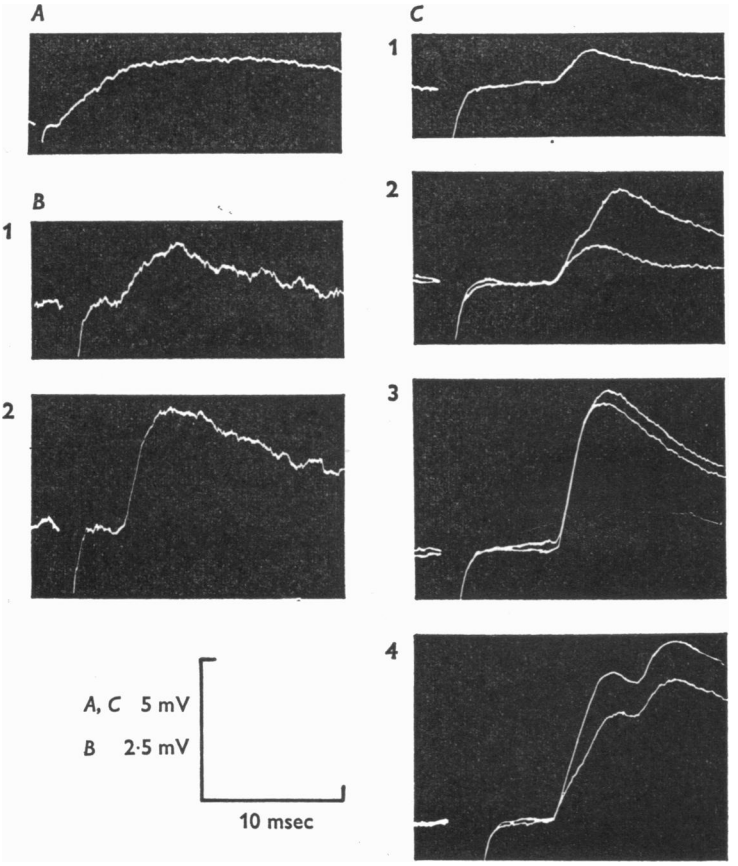
of both these fast and slow types (Text-fig. 5*B*). Changes in stimulus strength led to changes in the latency and amplitude of the e.p.p.s at seven of the fourteen synapses studied, indicating that each of these now received a multiple innervation from about three nerve terminals (Text-fig. 4).

During the period from 17 days gestation to 1 day post-natal, when the myofibres are rapidly increasing in length (Text-fig. 2) and secondary synaptic folds begin to appear at the multiply innervated motor end-plates, fast m.e.p.p.s (rise time 2–3 msec) were recorded in many cells

(thirty-four out of fifty cells impaled in the middle of six diaphragms) from a resting membrane potential which at 1 day post-natal had reached the adult value of $-80$ to $-90$ mV. The frequency of m.e.p.p.s had increased considerably by this time (from 2 to 5 $min^{-1}$ to $0{\cdot}723 \pm 0{\cdot}300$ $sec^{-1}$, $n = 20$), and although their amplitude-frequency histogram was




Text-fig. 4. *A*, frequency histograms of the number of nerve terminals per muscle cell of the rat hemidiaphragm, determined by electrophysiological means, during the last 6 days gestation and the first 4 weeks post-natal. *B*, changes with age in the mean numbers of terminals per muscle cell of the rat hemidiaphragm. Error bars represent the S.E. of the mean ($n \geqslant 14$).



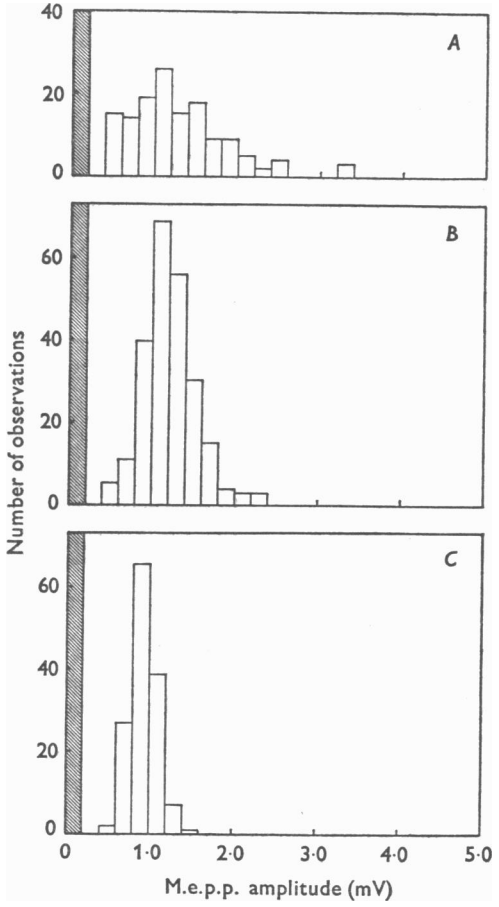

Text-fig. 5. End-plate potentials (e.p.p.s) recorded in the developing rat hemidiaphragm.

*A*, slow time course e.p.p. recorded in a muscle cell at 16 days gestation. This potential was invariant to increases in stimulus strength applied to the phrenic nerve indicating that there is only one nerve terminal in the vicinity of the recording electrode.

*B*, slow and fast rise time e.p.p.s recorded in a muscle cell at 16 days gestation with low (1) and high (2) stimulus strengths applied to the phrenic nerve.

*C*, e.p.p.s recorded in a muscle cell at 19 days gestation showing changes in the evoked response with three stepwise increases in stimulus strengths applied to the phrenic nerve (1–4). A number of traces at each stimulus strength have been superimposed and it is apparent that this cell is innervated by at least four nerve terminals. All potentials have a similar rise time which suggests that they arise at the same point on the muscle cell.

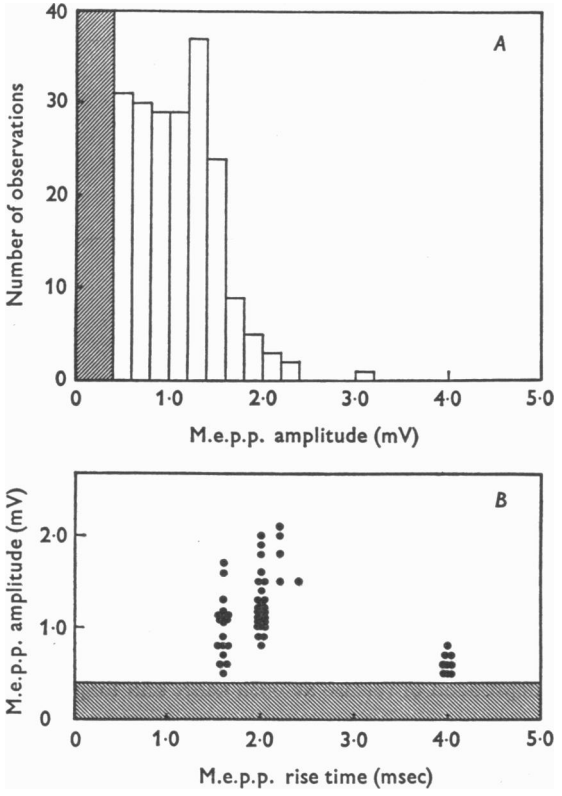
usually unimodal it was always skewed to the right (Text-fig. 6*A*) indicating the occurrence of large amplitude m.e.p.p.s. In the one cell which had a true skewed amplitude frequency distribution of m.e.p.p.s (Text-fig. 7*A*), the larger m.e.p.p.s had faster rise times than the smaller ones (Text-fig. 7*B*), suggesting that this cell had a distributed innervation. Evoked potentials were recorded in most cells (forty out of fifty-eight cells impaled in the middle of the muscle) and, since twitching of the muscle accompanied stimulation, D-tubocurarine ($5 \times 10^{-7}$–$1 \times 10^{-6}$ g ml.$^{-1}$) was




Text-fig. 6. Frequency distributions of miniature end-plate potential (m.e.p.p.) amplitudes recorded in muscle fibres of the rat hemidiaphragm at 19 days gestation (*A*), 2 days post-natal (*B*) and 3·5 weeks post-natal (*C*). Shaded columns represent the noise level. At young ages these distributions are unimodal but skewed to the right and as development proceeds this skewness is lost.

added to the Ringer solution. Most of the e.p.p.s now had fast rise times (thirty-seven out of the forty cells with evoked transmission) whilst the remainder had slow rise times. The complexity of the e.p.p. varied with stimulus strength (Text-fig. 5*C*), such that each synapse was still multiply innervated by about three nerve terminals (Text-fig. 4).

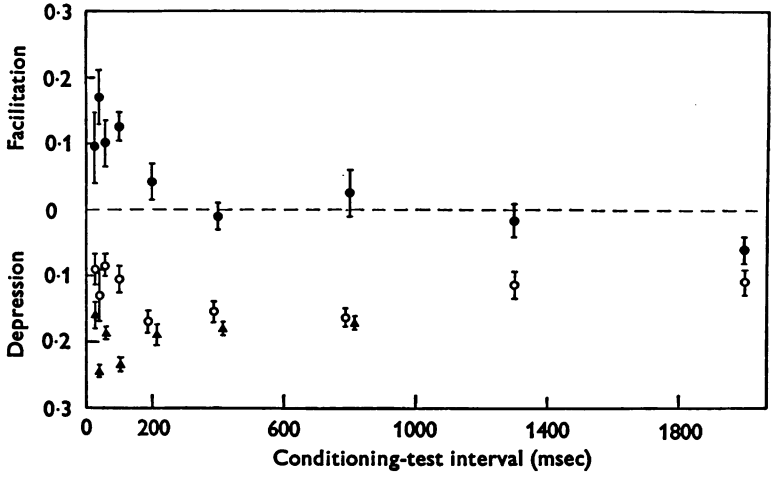
In the first post-natal week two different time course m.e.p.p.s were




Text-fig. 7. *A*, skewed miniature end-plate potential (m.e.p.p.) amplitude distribution for a cell in the rat hemidiaphragm at 19 days gestation. *B*, m.e.p.p. amplitudes (ordinate) plotted against their rise time (abscissa) for the frequency distribution shown in *A*. The larger m.e.p.p.s had faster rise times than the smaller m.e.p.p.s, suggesting that this cell had a distributed innervation.

recorded in the middle of nine of forty-four cells impaled in five diaphragms: large slow m.e.p.p.s (rise time 2·5–6 msec), resembling those observed early during the reinnervation of adult diaphragms (Bennett, McLachlan & Taylor, 1973*a*), together with the normal fast time course m.e.p.p.s. In the subsequent 4 weeks the amplitude frequency distribution

of m.e.p.p.s ceased to be skewed (Text-fig. 6 *B* and *C*), and slow m.e.p.p.s were no longer observed. Evoked potentials with exclusively fast rise times were recorded in the centre of all myofibres impaled (fifty-four cells), during post-natal life. The multiple innervation of synapses, as determined by electrophysiological means, decreased during early post-natal life, until at 3·5 weeks all synapses were innervated by only one nerve terminal (Text-fig. 4).




Text-fig. 8. Pattern of transmitter release from nerve terminals in the developing rat hemidiaphragm at 0–1 week (filled circles), 2 weeks (open circles) and 3–4 weeks (filled triangles) post-natal for different conditioning-test intervals. Error bars represent the S.E. of the mean ($n \geqslant 4$ measurements). Early after birth there is a facilitation of transmitter release at short conditioning-test intervals but as development proceeds the pattern changes to a depression of release at both short and long conditioning-test intervals.

The effect of a conditioning impulse on the size of the e.p.p. evoked by a subsequent test impulse was studied during the early post-natal period. Soon after birth there is a considerable facilitation of transmitter release which gradually changes with increasing age to a depression of release as is observed at adult motor nerve terminals (Text-fig. 8).

### *The formation of synapses in a muscle which receives a distributed innervation: the avian anterior latissimus dorsi (ALD) muscle*

#### *Muscle growth*

At 9 days incubation the avian ALD consists of a band of longitudinally orientated myotubes which extends about 1 mm on the humerus side of the nerve entry and about 1·5 mm on the spinal cord side of nerve entry

(Text-fig. 9). From electron microscopical examination the band also contains individual myoblasts, as well as myoblasts undergoing fusion with other myoblasts and with myotubes (see also Shimada, 1971). During the next few days of hatching the ALD grows in length by both the elaboration of cytoplasm and the fusion of myoblasts at the ends of the myotubes (Williams & Goldspink, 1971), and in width by the formation of new myotubes within the band. By 11–13 days incubation many myotubes have differentiated to myofibres which are now about 50 % of all the muscle cells present, and by 17 days incubation nearly all (90 %) of the muscle cells have differentiated to myofibres, which increase in length by the addition of sarcomeres (Williams & Goldspink, 1971).

*The location of nerves and synapses in the developing ALD*

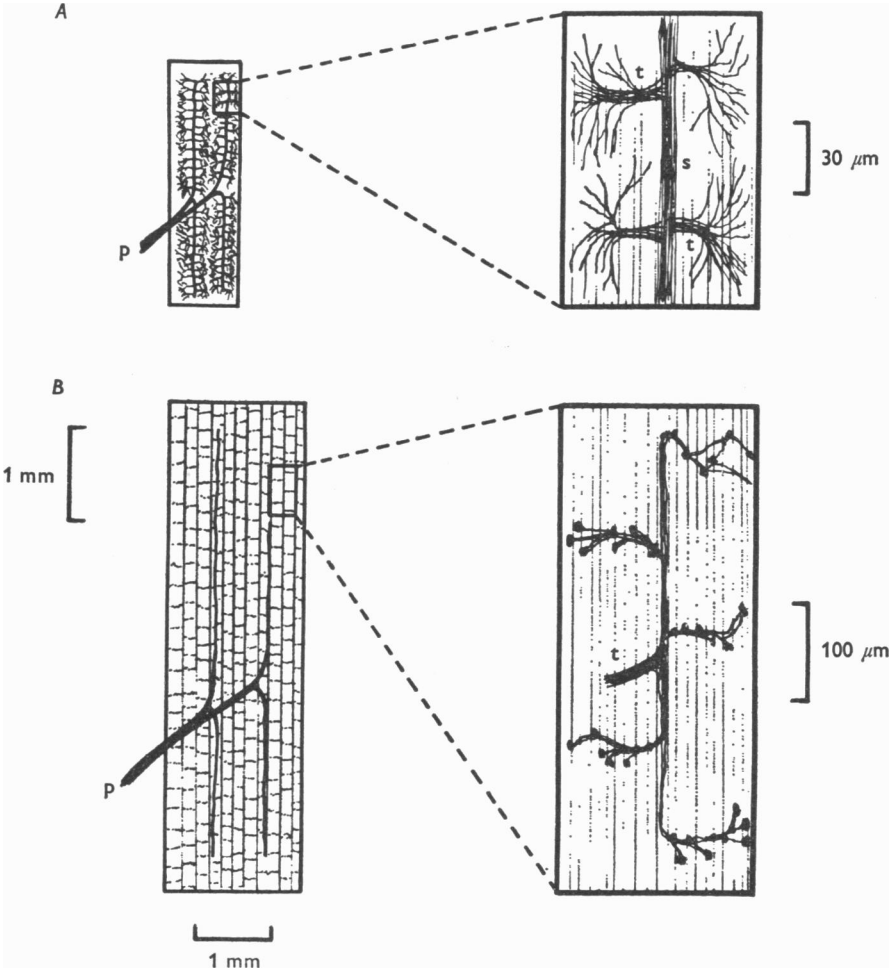
The location of nerves and synapses in the muscle during early development was determined from the distribution of silver impregnated axons, of ultrastructurally identified axons and of ChE deposits.

At 9 days incubation each of the two primary nerve trunks of the ALD nerve divide at their point of entry into the muscle into two secondary nerve trunks which traverse the ALD muscle parallel to the myotubes (Text-fig. 9*A*). Smaller tertiary nerve branches (5–10 axons) leave the secondary nerve branches at right angles and at regular intervals of about 60 $\mu$m ($61{\cdot}8 \pm 6{\cdot}5$, $n = 16$), traversing the myotubes for distances up to 30 $\mu$m (Text-fig. 9*A*). Single unmyelinated axons leave these tertiary nerves and pass for varying distances up to 60 $\mu$m mostly parallel and along the surfaces of the myotubes, on which they terminate (Text-fig. 9*A*; Pls. 3*a*, *b*, 4*b*); these terminals contain a few small agranular vesicles (50 nm diameter), coated vesicles, and large dense-cored vesicles (90 nm diameter) (Pl. 4*b*).

At 11–13 days incubation the muscle had increased in length by 2·2 times its size at 9 days (Text-fig. 10*A*). Localized ChE appears on both myotubes and myofibres at this time, the deposits occurring in register across individual fasicles of muscle cells at regular intervals of 164 $\mu$m $\pm 8$ ($n = 11$) (Text-fig. 9*B*; Pl. 3*c*). These ChE spots at regular intervals along the lengths of the muscle cells were innervated by one or two silver impregnated nerves (Pl. 3*d*), whilst the electron microscopy revealed between one and four axon profiles at each synaptic site; these profiles contained the same complement of synaptic vesicles as previously described (Pl. 4*c*) (see also Zelena & Sobotkova, 1973).

During the period from 14 days incubation to 4 weeks post-hatched the nerve branches continue to grow into the new muscle as it increases to about ten times its size at 9 days incubation (Text-fig. 10*A*). At the ends of the muscle where the muscle cells grow in length, the last group

of ChE deposits across individual fasicles was still maintained at about 170–200 $\mu$m from its adjacent and more centrally located group of ChE deposits (Pl. 3*e*). The 'en grappe' configuration of the ChE deposits on individual cells was first observed between the fourteenth and sixteenth incubation day (Pl. 3*f*), and electron microscopy revealed that after this time each synapse consisted of from one to three varicose axon profiles lying in a shallow synaptic cleft on the surface of the myofibres. These varicosities contained variable numbers of agranular synaptic vesicles (Pl. 4*d*). The distance between the 'en grappe' ChE deposits on individual fasicles and muscle cells was maintained about 200–300 $\mu$m




Text-fig. 9. For legend see facing page.

during the first 4–5 weeks of post-hatched life (Text-fig. 10 *B*), but thereafter increased, until at 15–20 weeks post-hatched the distance between adjacent ChE deposits was about 1000 $\mu$m (Text-fig. 10 *B*) (cf. Ginsborg & Mackay, 1961). This distance between adjacent synapses is maintained throughout adult life and is presumably reached by the selective elimination of nerve terminals.

*Acetylcholine sensitivity distribution of muscle fibres in the developing ALD*

In the adult ALD the distance between peaks of ACh sensitivity on individual myofibres corresponds reasonably well with the measured distance between ChE deposits on the myofibres of 600–1000 $\mu$m (Bennett, Pettigrew & Taylor, 1973). It was of interest to see if the peaks of ACh sensitivity on muscle fibres early during development corresponds to the much smaller distance between ChE deposits of about 200 $\mu$m observed at this time. The ACh sensitivity distributions of thirteen fibres was determined over the first 5 weeks post-hatched and a typical example is shown in Text-fig. 11, in which the distance between the peaks of ACh sensitivity is about 200 $\mu$m. There was no significant difference in this distance for individual fibres over this period, and the average peak to

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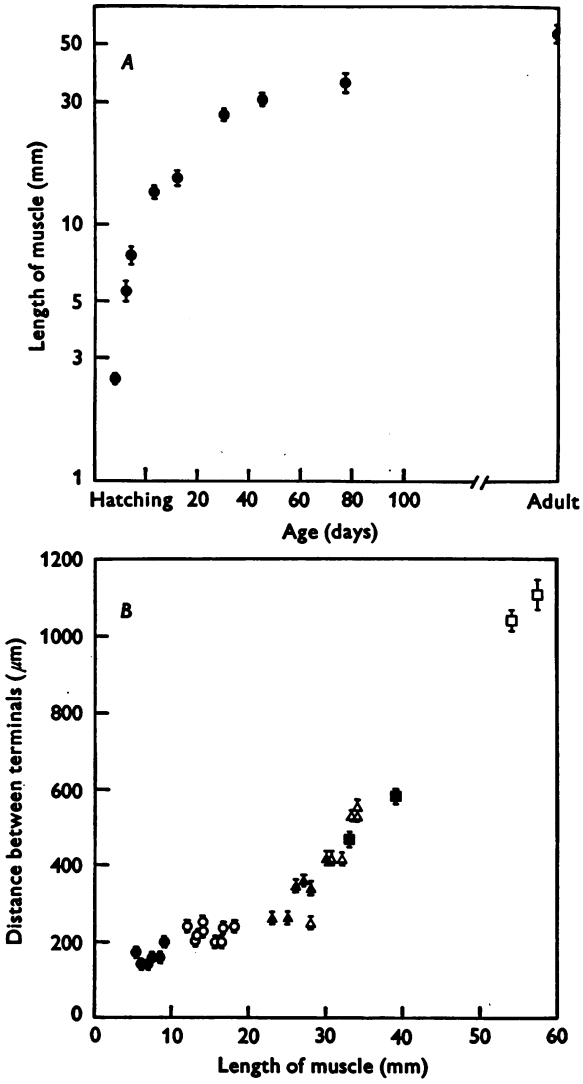
Text-fig. 9. *A*, low and high power diagrams showing the pattern of innervation of the chick anterior latissimus dorsi (ALD) muscle at 9 days incubation. The myotubes making up the muscle are orientated vertically as shown in the high power diagram. The two primary nerve trunks (p) of the ALD nerve divide at their point of entry into the muscle into two secondary trunks (s) which traverse the ALD muscle parallel to the myotubes. At regular intervals of about 60 $\mu$m smaller tertiary nerve branches (t) leave the secondary branches and traverse the myotubes for distances up to 30 $\mu$m. Single axons then radiate out from these tertiary branches and pass mostly along the myotubes for varying distances. At this stage of development spontaneous miniature end-plate potentials (m.e.p.p.s.) could be recorded in a few cells. In some of these cells the m.e.p.p.s. appeared to have arisen from several positions on each cell while other cells appeared to have a focal innervation near the point of impalement. From electrophysiological criteria the cells receive a multiple and distributed innervation.

*B*, low and high power diagrams showing the regular appearance of cholinesterase (ChE) deposits along fasicles of fibres in the ALD muscle at 11–13 days incubation. Each group of ChE deposits in a fasicle is innervated by one or two silver impregnated axons which arise as branches from the tertiary nerve trunks (t). At this stage of development m.e.p.p.s were recorded in many cells and in all of these cells the rise times of the m.e.p.p.s was correlated with their amplitudes in such a way as to suggest that these cells received a distributed innervation. Changes in the complexity of the e.p.p.s recorded in these cells with changes in the strength of stimulation to the ALD nerve indicated that the cells were innervated by about five terminals in the vicinity of the impalement.

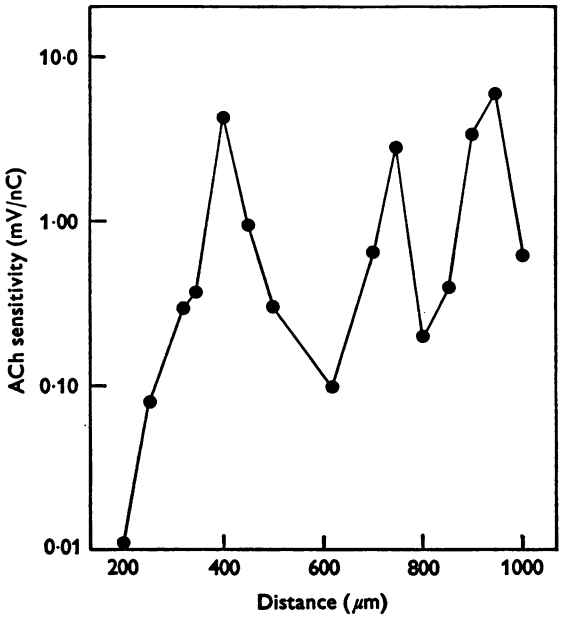



Text-fig. 10. *A*, changes in the length of the chick ALD muscle during development from 9 days incubation to the adult. Error bars represent the S.E. of the mean ($n \geqslant 2$ muscles). *B*, changes in the distance between adjacent groups of terminals on fasicles of ALD muscle fibres and between adjacent terminals on individual fibres with growth in length of the ALD muscle. Each symbol represents the mean of twenty or more measurements from one muscle, the error bars being the standard error of that mean. The different symbols represent the following age groups of animals: 15–16 days incubation (filled circles); 1–12 days post-hatched (open circles); 30–40 days post-hatched (filled triangles); 50 days post-hatched (open triangles); 75–80 days post-hatched (filled squares) and adult (open squares).

peak distance was 270 $\mu$m $\pm$ 40 ($n$ = 15), which agrees favourably with the distance between synapses determined by the ChE technique at this stage of development.

*Spontaneous and evoked electrical activity in the developing ALD*

At 9 days incubation, when nerve terminals were observed synapsing on myotubes, m.e.p.p.s were recorded in only a few cells at different points in the muscle (three out of fourteen cells impaled for at least 1 min in two muscles), and these arose from low resting potentials ($-30$ to $-40$ mV):

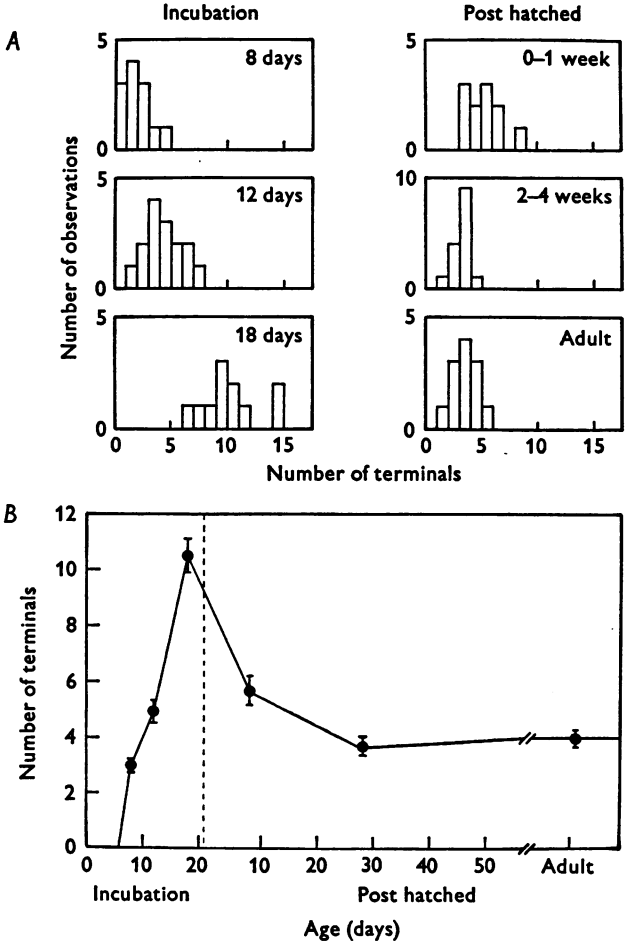



Text-fig. 11. Acetylcholine sensitivity distribution for a chick ALD muscle fibre at 4 weeks post-hatched. The distribution shows three peaks of sensitivity about 200 $\mu$m apart. This distance corresponds with the histologically observed distance between terminals on these fibres at this stage of development. Abscissa, distance from the recording electrode.

in one of these cells the amplitude of the m.e.p.p.s varied considerably and was correlated with their rise time in such a way as to suggest that the cell had a distributed innervation near the point of impalement; the remaining cells had large amplitude m.e.p.p.s ($>2$ mV) with fast rise times ($<2$ msec), suggesting that these cells received a focal innervation near the point of impalement. Evoked potentials were recorded in most myotubes impaled throughout the muscle (fourteen out of twenty cells in two muscles), and their rise times varied greatly from 2 to 12 msec.

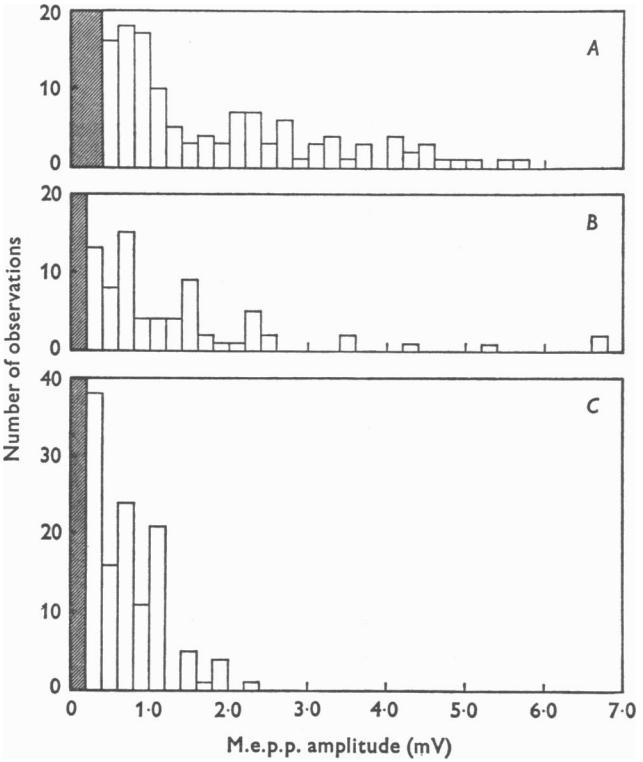
Changes in stimulus strength lead to changes in the latency, rise time and amplitude of the e.p.p.s in most cells (nine out of twelve cells), indicating that they received a multiple and distributed innervation. An analysis of these e.p.p.s showed that each cell was innervated by about three nerve terminals in the vicinity of the impalement (Text-fig. 12).

At 11–13 days incubation when localized ChE is first observed at regular intervals along the length of muscle cells, and each synapse is innervated




Text-fig. 12. *A*, frequency histograms of the number of nerve terminals in the vicinity of the recording electrode, determined by electrophysiological means, for muscle cells of the developing ALD muscle in the chick. *B*, changes with age in the mean numbers of nerve terminals in the vicinity of the recording electrode per muscle cell of the chick ALD muscle. Error bars represent the S.E. of the mean ($n \geqslant 11$).

by a number of nerves, m.e.p.p.s were recorded in many cells (ten out of twenty-seven cells in three muscles) at low frequency (2–3 $min^{-1}$). The amplitude-frequency distribution of these m.e.p.p.s was always skewed (Text-fig. 13*A*), and their rise time was correlated with their amplitude in

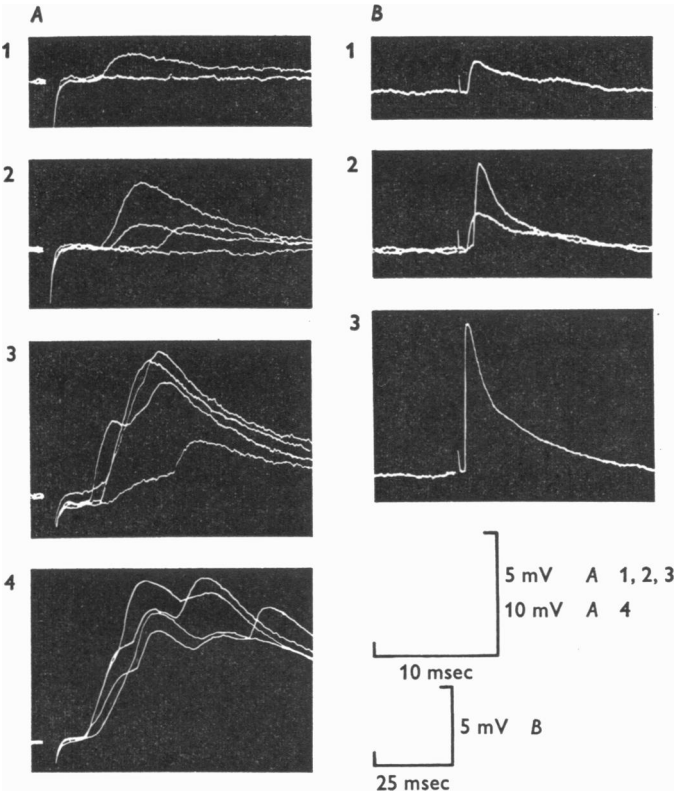



Text-fig. 13. Frequency distributions of miniature end-plate potential (m.e.p.p.) amplitudes recorded in muscle cells of the chick ALD at 12 days incubation (*A*), 6 days post-hatched (*B*) and adult (*C*). Shaded columns represent the noise level. All distributions are skewed but the number of large amplitude m.e.p.p.s decreases as development proceeds.

such a way as to suggest that these cells received a distributed innervation. Evoked potentials were recorded in nearly all cells (twenty-five out of twenty-seven in three muscles) throughout the muscle, and the changes in complexity of these e.p.p.s with changes in stimulus strength indicated that these cells received a multiple and distributed innervation from about five nerve terminals in the vicinity of the impalement (Text-fig. 12).

From 14 days incubation to 5 weeks post-hatched the resting membrane potential of the muscle cells increases from about $-50$ to $-70$ mV,

whilst the frequency of m.e.p.p.s increases from 2 to 3 $min^{-1}$ towards the adult value of 1·5 $sec^{-1}$; the amplitude-frequency distribution of m.e.p.p.s recorded at any point in the muscles is skewed at this time (Text-fig. 13 *B*), and there is a progressive elimination of very large amplitude m.e.p.p.s (Text-fig. 13*C*). Evoked potentials can be recorded in all cells and the changes in complexity of the e.p.p. with changes in stimulus strength (Text-fig. 14) indicates that each cell is innervated by about ten nerve



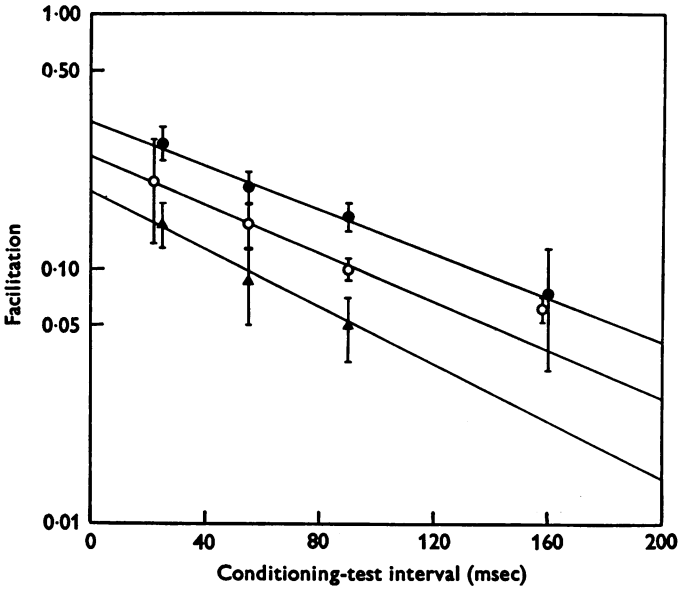

Text-fig. 14. End-plate potentials (e.p.p.s) recorded in the developing chick ALD muscle.

*A*, changes in the complexity of the e.p.p. with three of five stepwise increases in stimulus strength applied to the ALD nerve (1–4) recorded in a muscle cell at 18 days incubation. A number of traces at each stimulus strength have been superimposed. From a study of the consistent changes in amplitude and time course of the potentials with increases in stimulus strength it is apparent that this fibre has at least ten nerve terminals in the vicinity of the recording electrode.

*B*, changes in the e.p.p. recorded in an adult ALD muscle fibre with two changes in stimulus strength applied to the ALD nerve (1–3). The fibre has four terminals in the vicinity of the recording electrode.

terminals in the vicinity of the impalements at 14–18 days incubation (Text-fig. 12). This density of innervation drops to the adult value of four terminals by 4 weeks post-hatched (Text-figs. 12 and 14), confirming the anatomical observations of a transient multiple innervation of synaptic sites.

The effect of a conditioning impulse on the size of the evoked response due to a subsequent test impulse was studied during the early post-hatched period. The degree of facilitation is very high at hatching (Text-fig. 15) and then subsequently decreases over the following 4 weeks until the adult facilitatory value is reached (Text-fig. 15).




Text-fig. 15. Pattern of transmitter release from nerve terminals in the developing chick ALD muscle at 0–1 week (filled circles) and 2–3 weeks (open circles) post-hatched for different conditioning-test intervals. The adult pattern of release is represented by the filled triangles. Error bars represent the S.E. of the mean ($n \geqslant 11$ measurements). Early after hatching there is a relatively large facilitation of transmitter release at the conditioning-test intervals shown but as development proceeds this facilitation gradually decreases to the adult value.

## DISCUSSION

### *The sites of synapse formation on myotubes*

The increase in length of the developing hemidiaphragm occurs by the fusion of myoblasts to form myotubes at the ends of the muscle. This differentiation occurs at least 24 hr after the invasion of the myoblasts

by exploring axons from the secondary nerve trunk, which grows at right angles to the newly formed myotubes. The growth of muscle in the axial myotomes during ontogeny is very similar: exploring axons enter the myotomes when only myoblasts are present (Tello, 1917; Filogamo & Gabella, 1967; Tennyson, Brzin & Slotwiner, 1971), and within a few hours these fuse to form myotubes (Mumenthaler & Engel, 1961; Filogamo & Gabella, 1967). In contrast, the increase in length of the developing ALD occurs in the direction of growth of the already formed myotubes, so that exploring axons from the secondary nerve trunks which grow parallel to the myotubes do not enter pools of myoblasts, but simply follow the growing ends of the muscle cells. Nerve terminals are therefore observed throughout the length of newly formed myotubes in both the diaphragm and the ALD, as has been observed in other muscles: chick leg, paravertebral and intercostal muscles (Tello, 1917; Mumenthaler & Engel, 1961; Atsumi, 1971); mammalian myotomes (Filogamo & Gabella, 1967); fish myotomes (Filogamo & Gabella, 1967); lizard tail muscles (Liu & Maneely, 1968).

The first signs of close apposition between nerve terminals and myotubes had occurred within one day after myotube formation was first observed, at a time when evoked transmission from single nerve terminals could be recorded in a few myotubes (at 15 days gestation 1 mm from the point of phrenic nerve entry in the diaphragm and at 9 days incubation in the ALD). A similar interval elapses between the formation of myotubes and that of synapses in the axial myotomes of birds and mammals (Filogamo & Gabella, 1967; Mumenthaler & Engel, 1961; Kelly & Zacks, 1969*b*; Teräväinen, 1968). Up to this time the myotubes of the ALD have grown to about 2500 $\mu$m whilst those of the diaphragm at 1 mm from the point of nerve entry have grown to only 300 $\mu$m, and nerve terminals are found throughout these respective distances at the time of synapse formation.

The deposition of ChE at synapses was first observed about 1–2 days after synapse formation commenced (at 16 days gestation one mm from the point of phrenic nerve entry in the diaphragm and at 11 days incubation in the ALD), as has already been shown for regenerating (Lentz, 1969; Bennett, Florin & Woog, 1974) and developing (Kelly & Zacks, 1969*b*; Teräväinen, 1967, 1968) muscle. At this time only one ChE deposit is observed on any one myotube in the diaphragm, and there is a random distribution of these confined to the now small area of the diaphragm originally occupied by the exploring axons at 15 days gestation. This confirms that the initial site of synapse formation occurs at random along the length of each myotube. In contrast, ChE deposits are observed at regular intervals along the whole length of the ALD myotubes, indicating that the initial formation of synapses occurs at regular intervals along

the length of each myotube at 9 days incubation, and that the synapses formed on the new myotube membrane laid down in the subsequent 2 days also occurs at these regular intervals.

As there are several points of high cholinergic receptor density and ACh sensitivity along the length of myotubes and myofibres grown in tissue culture (Vogel, Sytkowski & Nirenberg, 1973; Fischbach & Cohen, 1973; but see Hartzell & Fambrough, 1973), which seem in the case of myofibres to occur on the membrane where hyperlemmal nuclei occur (Fischbach & Cohen, 1973), and such points also occur at sites of synapse formation in tissue culture (Fischbach & Cohen, 1973), it has been proposed that these sites may be points of preferred innervation (Fischbach & Cohen, 1973). The distance between the sites is of the order of 100 $\mu$m on myotubes (Sytkowski, Vogel & Nirenberg, 1973) and 1000 $\mu$m on myofibres (Fischbach & Cohen, 1973), and it is suggested that once a synapse has formed on one of these the remaining sites are removed (Sytkowski, Vogel & Nirenberg, 1973). We have not been able to determine unequivocally if the initial site of synapse formation on a myotube does occur opposite one of its nuclei, although this has been claimed (Tello, 1917; Atsumi, 1971). Nevertheless, even if such a mechanism should exist it would not alone explain how the different innervation patterns of the myotubes in the diaphragm and the ALD are established.

*The transitory multiple innervation of synaptic sites*

The single synaptic site first formed on myotubes one mm from the point of phrenic nerve entry by a single axon in the diaphragm at 15 days gestation is multiply innervated 24 hr later, and the extent of this innervation increases to about three axons at birth, thereafter decreasing to one axon early in post-natal life (see also Redfern, 1970). The fact that this multiple innervation develops at a single synaptic site is indicated by the following observations: only one ChE deposit was observed along the length of any one myotube and this was transitorily innervated by two or more silver stained axons, confirming the large number of axon profiles revealed at each site by the electron microscope early during development (see also Teräväinen, 1968; Kelly & Zacks, 1969*b*; Hirano, 1967; James & Tresman, 1968, 1969); after 17 days gestation the different components of the e.p.p. all had the same fast time course when recorded from the middle of the muscle fibres, and the fastest time course m.e.p.p.s were recorded at this position (Diamond & Miledi, 1962).

It is also likely that each of the distributed synaptic sites on the ALD muscle cells is first established by a single axon and that these sites then receive a transitory multiple innervation during development: the ChE deposits on muscle cells are innervated by more than one silver stained

axon early during development and electron microscopy reveals a transitory increase in the number of axon profiles at single synaptic sites; furthermore the complexity of the evoked potential increases greatly after the initial formation of synapses at 9 days incubation and then returns to its initial form by 4 weeks post-hatched.

It seems that each of the single synaptic sites on the muscle cells of the diaphragm and the very regularly distributed sites on the muscle cells of the ALD are first established by single axons, and that newly invading axons subsequently hyperinnervate these areas. The fact that newly arriving axons only innervate the sites already established and not other points along the length of the muscle cells, indicates that the synaptic region of the muscle cells is a site of preferred innervation, as it is in adult muscle (Bennett, McLachlan & Taylor, 1973*a*, *b*; Bennett, Pettigrew & Taylor, 1973). The subsequent removal of the multiple innervation of the synapses is possibly associated with the loss of ventral horn cells (Prestige & Wilson, 1972) and their axons (Reier & Hughes, 1972) which is known to occur in vertebrates during development.

*The statistics of transmitter release at developing synapses*

During the formation of synapses in reinnervated adult striated muscle (Bennett, McLachlan & Taylor, 1973*a*; Bennett, Pettigrew & Taylor, 1973; Bennett & Florin, 1974) the quantal content of the e.p.p. is initially low and increases in size as the synapse matures. A similar correlation between quantal content and size of the nerve terminal has been observed at adult end-plates (Kuno, Turkanis & Weakly, 1971) in tissue culture (Robbins & Yonezawa, 1971; Fischbach, 1972) and during development (Redfern, 1970; the present study). Robbins & Yonezawa (1971) suggested that this increase in quantal content was due to an increase in the number of quanta available for release with growth of the nerve terminal ($n$), rather than due to an increase in the average probability of release of quanta ($p$). This has now shown to be the case at newly formed synapses in adult muscle (Bennett & Florin, 1974), in which $p$ increases to 1·0 in only a few days after the beginning of synapse formation whereas $n$ continues to increase as the terminal grows.

In the first few days of synapse formation in reinnervated adult muscle, there is a facilitation of transmitter release while $p$ is relatively small ($p < 0{\cdot}5$; Bennett & Florin, 1974), and the e.p.p. is subthreshold for the initiation of the action potential; thereafter there is a depression of transmitter release, $p$ approaches 1·0, and the e.p.p. becomes suprathreshold as the quantal content increases with growth of the terminal. A similar series of events is observed during ontogeny: there is initially a low quantal content and facilitation for the first few days after the initial synaptic

contact is established, and this changes to a depression of transmitter release as the quantal content increase with age. This suggests that the changes in the statistical parameters $n$ and $p$ which govern transmitter release are similar during synapse formation in either developing or adult material, and that changes in $n$ are simply related to the size of the terminals.

*Factors determining the pattern of synaptic sites on muscle cells*

The short myotubes of the diaphragm are innervated at a random point along their length by a single axon, and later by a number of axon terminals which are eventually lost. The observations that no further synaptic points occur on the myotubes, even though there are a large number of terminal axons in the intercellular spaces, and that further innervation of the myotubes is constrained to occur at the initial synaptic point, suggests that the axon making the first synaptic contact induces a property over the remaining length of the myotube which makes its membrane refractory to synapse formation. Thus as the muscle cells of the diaphragm grow in length this refractory property includes the new membrane laid down, so that further innervation of the muscle cells can occur only at the point of initial synaptic contact. In the case of the ALD muscle, the longer myotubes are each innervated at regular intervals of about 170 $\mu$m, and during the transitory period of hyperinnervation of the muscle cells which occurs, the extra nerves terminate only at these regularly spaced synaptic spots. These observations can be explained by the present suggestion if the small 'en grappe' ALD nerve terminals can only make the myotubes refractory to further innervation for a distance of $\pm$ 170 $\mu$m, thus allowing new synapse formation at this distance along the muscle cells. According to the present explanation then, the adult pro perty of muscle cells which makes their surfaces refractory to synapse formation by either reinnervating or cross-reinnervating axons, except at the point or points occupied by the original synapses (Bennett *et al.* 1973*a*,*b*; Bennett *et al.* 1973), is established by the nerves during ontogeny. In the following paper (Bennett & Pettigrew, 1974) experiments are presented which have been designed to test this idea.

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## EXPLANATION OF PLATES

### PLATE 1

Distribution of nerves in the rat hemidiaphragm at 15 days gestation. *a*, the primary (phrenic) nerve (p) enters near the centre of the hemidiaphragm and divides into two secondary nerve branches (s) which traverse the myotubes (my) at right angles and extend to the pools of myoblasts (mb) at the ends of the hemidiaphragm. Smaller tertiary nerve bundles (t) leave the secondary nerve trunks and run in general parallel to the myotubes and obliquely into the pool of myoblasts. The tertiary nerve branches extend almost the entire length of those myotubes which are adjacent to the pool of myoblasts. Calibration 100 $\mu$m. *b*, tertiary nerve bundles at the end of the secondary nerve trunk radiating into the pool of myoblasts (mb) at the end of one hemidiaphragm. Calibration 50 $\mu$m. *c*, single axons leaving the tertiary nerve branches at a position 1 mm from the point of nerve entry into the hemidiaphragm and growing out between and along the myotubes (my) in this region. The tertiary nerve bundles arise from the secondary nerve trunk (s) at the bottom of the plate. Some axons have been retouched since they pass out of the plane of focus of the three photomicrographs used to make up the plate. Calibration 30 $\mu$m.

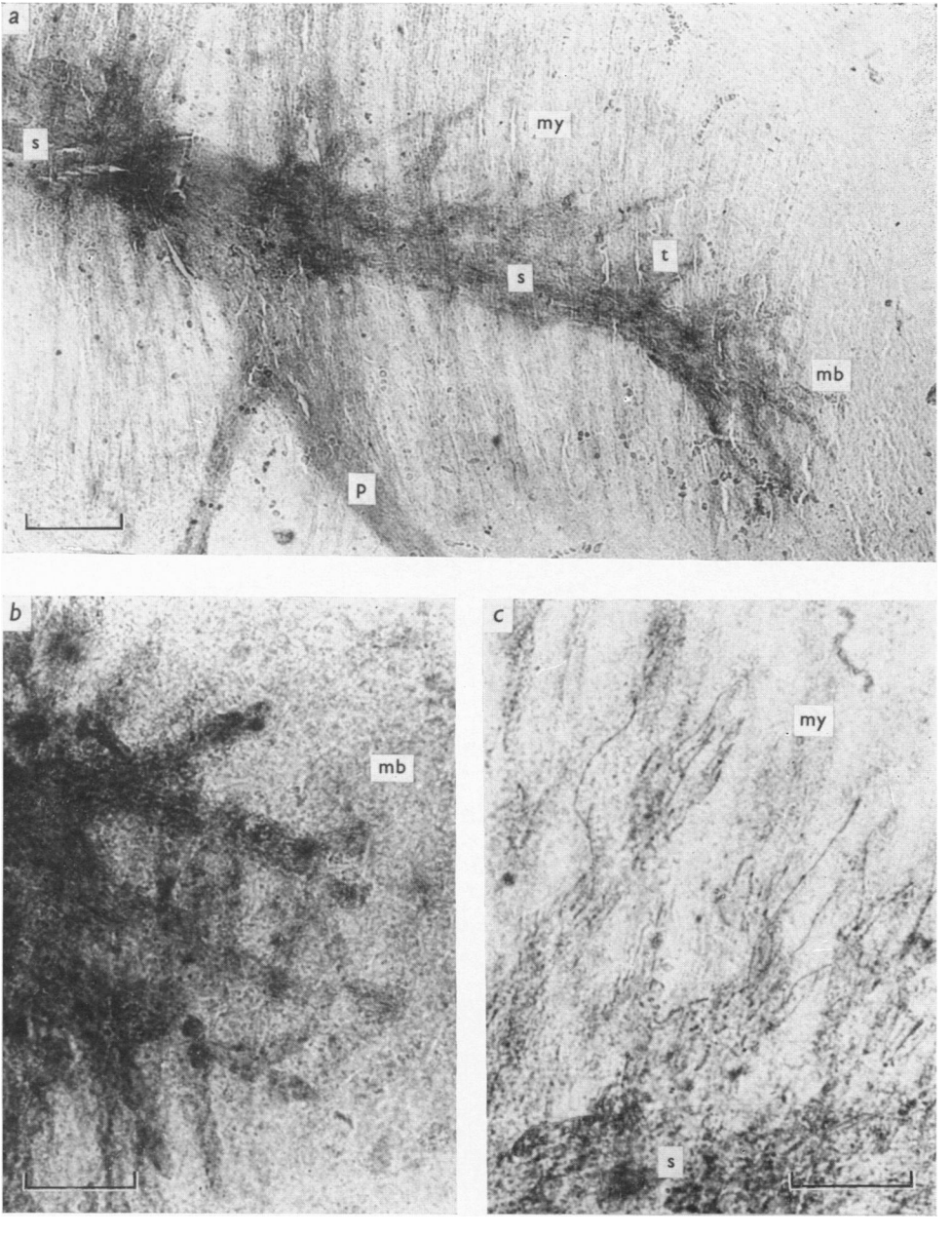
### PLATE 2

Distribution of nerves and synapses one mm from the point of phrenic nerve entry in the developing rat hemidiaphragm as shown by combined silver impregnation and cholinesterase (ChE) staining. In records *a*, *c*, *d* and *e* the muscle cells are orientated horizontally, while in *b* they are orientated vertically.

*a*, at 16 days gestation small, primitive ChE deposits (arrows) appear and are innervated by tertiary nerve bundles of 2–5 axons. These tertiary nerve bundles arise from the secondary nerve trunk on the left of the plate. Calibration 25 $\mu$m. *b*, single axons (arrows) leave a large tertiary nerve bundle at 16 days gestation and cross the muscle cells in the region without any preferred direction of growth. Calibration 15 $\mu$m. *c*, ChE staining only of a section from a 19 day gestation hemidiaphragm; the ChE deposits are confined to a narrow region in the centre of the muscle and there is only a single deposit per muscle cell. Calibration 200 $\mu$m. *d*, at 20 days gestation the ChE stained synapses of the end-plate zone are innervated by a complex network of dividing and anastomosing tertiary nerve branches. The large secondary nerve trunk runs down the centre of the plate. Calibration 100 $\mu$m. *e*, between 17 and 19 days gestation single axons (arrows) can be seen leaving small nerve bundles which already innervate a muscle cell. These small exploratory axons soon terminate in small areas of very low ChE activity. Calibration 25 $\mu$m.

### PLATE 3

Distribution of nerves and synapses in the developing chick ALD muscle as shown by combined silver impregnation and cholinesterase (ChE) staining. In all plates the muscle cells are orientated vertically. *a*, at 9 days incubation small tertiary nerve branches (arrows) leave the secondary nerve trunk and cross the myotubes at right angles for distances up to 30 $\mu$m. The branches then break up into single axons which grow throughout the region. Calibration 25 $\mu$m. *b*, single axons growing mostly parallel to and along the surfaces of ALD myotubes at 9 days incubation. Calibration 25 $\mu$m. *c*, localized ChE deposits on muscle cells at 13 days incubation. The deposits occur in register across a fascicle of muscle cells and at regular intervals along the length of the fasicle. Calibration 100 $\mu$m. *d*, group of ChE deposits on ALD myofibres at 13 days incubation. These deposits are innervated by one or two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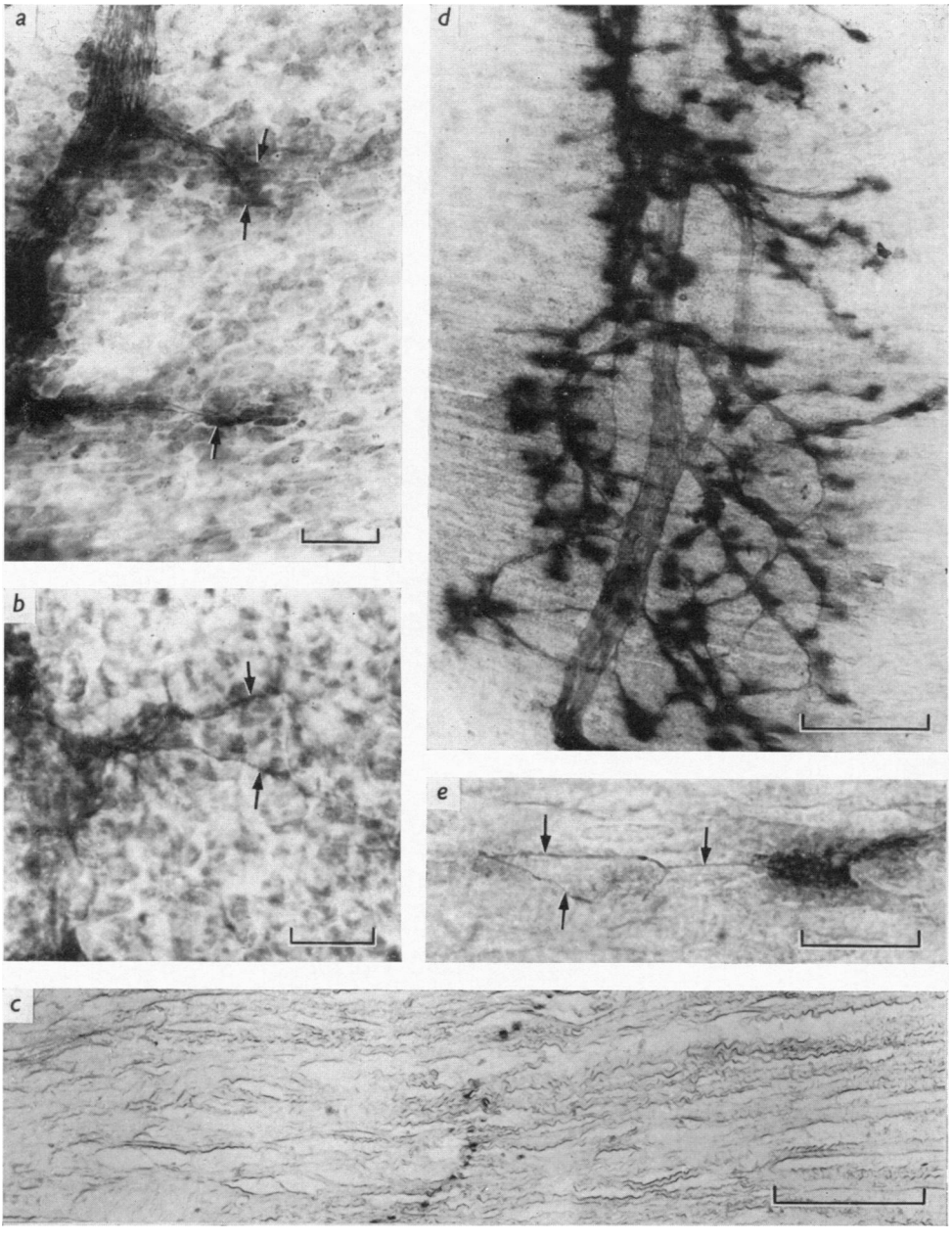
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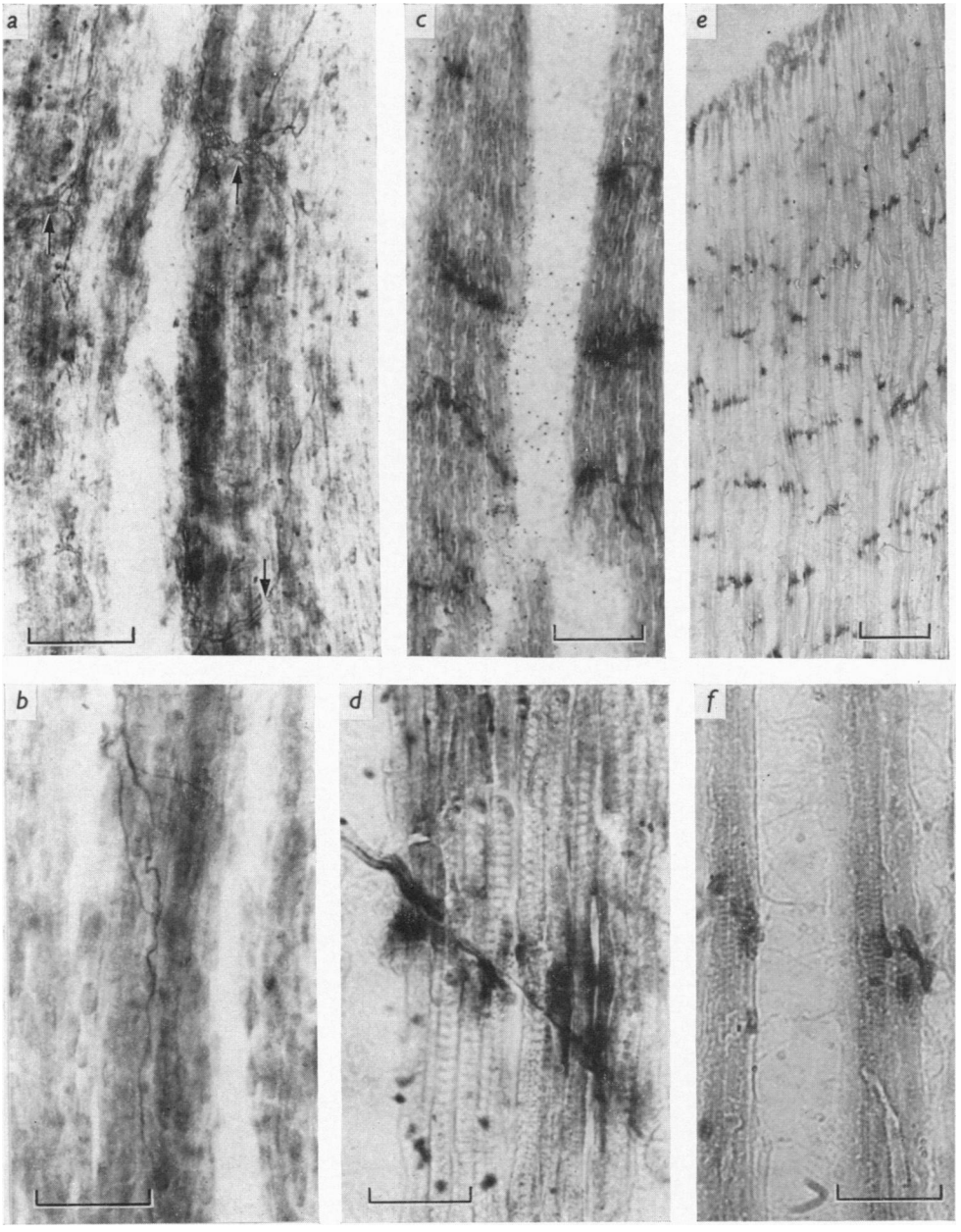
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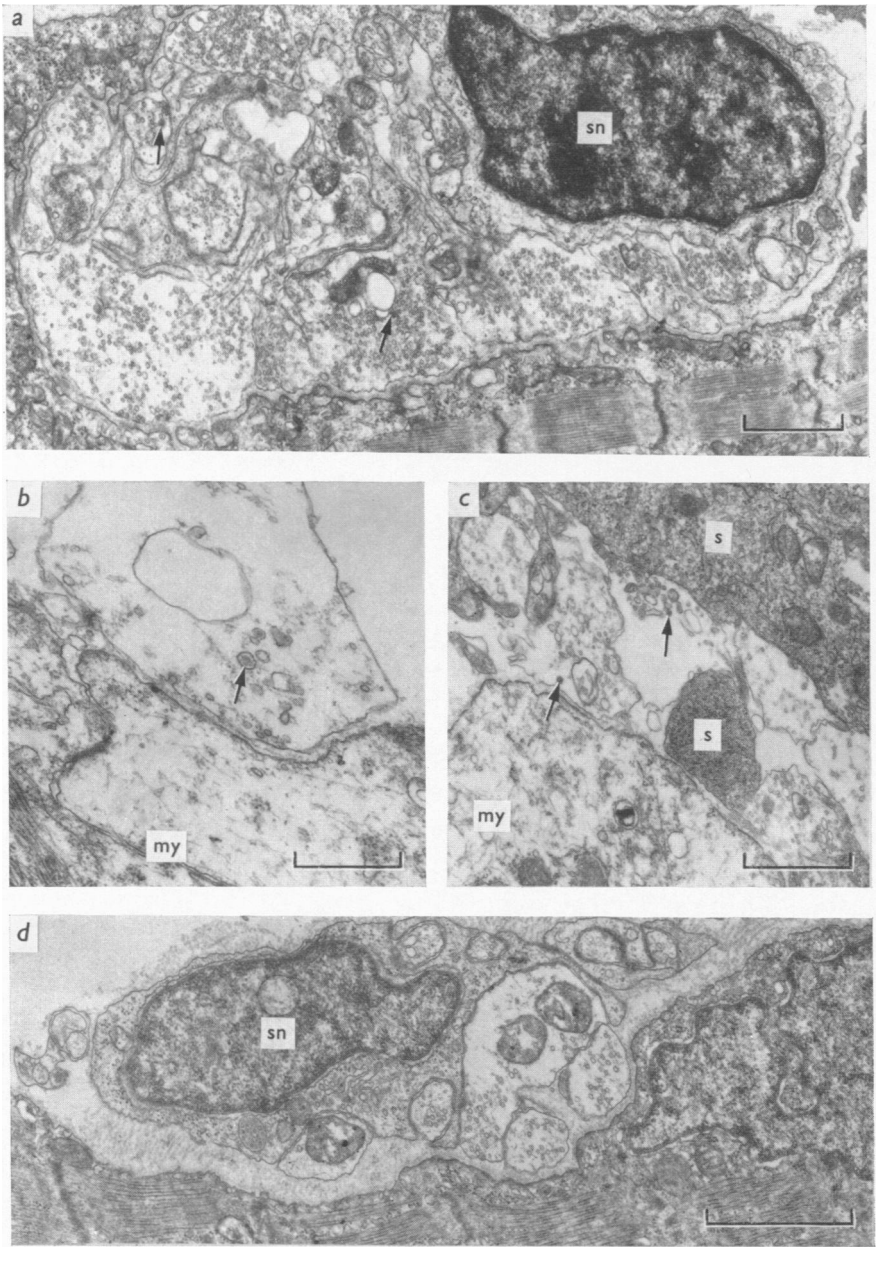
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silver impregnated axons. Calibration 20 $\mu$m. *e*, at 16 days incubation the last group of ChE deposits across individual fasicles is maintained at 170–200 $\mu$m from its adjacent and more centrally located group of ChE deposits. The end of the ALD muscle is at the top of the plate. Calibration 100 $\mu$m. *f*, ChE deposits on ALD myofibres at 16 days gestation when the first indication of the 'en grappe' type of terminal becomes apparent. Calibration 25 $\mu$m.

## Plate 4

Ultrastructure of nerve terminals in the developing rat hemidiaphragm and chick ALD muscle. *a*, fine structure of an end-plate in the rat hemidiaphragm at 2 days post-natal. A large number of axon profiles with varying densities of agranular vesicles and a few large dense-cored vesicles (arrows) lie in synaptic contact with the muscle fibre in the same primary synaptic cleft. The axon profiles are covered by a Schwann cell (sn, Schwann cell nucleus) and small secondary synaptic folds can be seen indenting the cytoplasm of the muscle fibre. Calibration 1000 nm. *b*, nerve terminal in the ALD muscle at 9 days incubation. The terminal is in synaptic contact with two myotubes (my) which appear to be fusing and contains a few agranular vesicles, a dense-cored vesicle (arrow) and a coated vesicle budding off the synaptic membrane. Calibration 500 nm. *c*, a number of axon profiles in the region of synaptic contact with a myotube in the ALD muscle at 13 days incubation. The profiles contain agranular vesicles and dense-cored vesicles (arrows) and are surrounded by Schwann cell cytoplasm (s). Calibration 1000 nm. *d*, fine structure of an end-plate in the chick ALD muscle at 6 days post-hatched. At least three large axon profiles containing agranular vesicles are in synaptic contact with the muscle fibre at this point and a number of intervaricose structures can be seen in the surrounding Schwann cell cytoplasm (sn, Schwann cell nucleus). No secondary synaptic folds can be seen at these end-plates. Calibration 1000 nm.