EMBRYONIC GROWTH AND INNERVATION OF RAT SKELETAL MUSCLES.

III. NEURAL REGULATION OF JUNCTIONAL AND EXTRA-JUNCTIONAL ACETYLCHOLINE RECEPTOR CLUSTERS

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[Plates 1–8]

CONTENTS

The number and distribution of acetylcholine (ACh) receptors on muscle cells was studied during development of normal, paralysed and aneural embryonic rat diaphragm muscles.

(i) ACh receptors initially are dispersed over the surface of rat embryo myotubes. At day 15½ of gestation junctional receptor clusters ('J-clusters') form in a well ordered band across the midline of the diaphragm muscle; these also form in denervated and paralysed muscles. At about day 18 of gestation additional 'EJ-clusters' develop to either side of the midpoint of treated muscles.

(ii) If a nerve terminal is present, J-clusters increase in length with time. The time course of generation of new endplates calculated from frequency distributions of J-cluster lengths accurately predicts the muscle growth curve established from muscle fibre counts.

(iii) The mean length of J-clusters in paralysed muscles was greater than in controls, due to small new-formed clusters failing to appear. In muscles allowed to recover from paralysis the mean length was less, due to a preponderance of small, new-formed clusters. These observations show that development of new endplates, which is thought to reflect the development of new muscle cells, is halted in paralysed muscles, and recovery from paralysis is associated with the generation of many new endplates.

(iv) J-clusters appeared, but failed to grow, in aneural muscles. In muscles denervated during the later stages of gestation, analysis of the distribution of J-cluster lengths shows that new clusters failed to appear, and existing clusters showed little or no increase in length after the time of removal of the nerve.

(v) EJ-clusters form by aggregation of dispersed receptors, and their mean length increases with time. They do not appear to be stable entities, and are removed within 2 d of recovery from paralysis. In paralysed muscles, with both J-clusters and EJ-clusters present, only J-clusters attract nerve sprouts or become innervated.

(vi) A curve is derived showing development of the total number of synaptic terminals in a muscle. This number increases during days 13–18 of gestation, reaching a peak of about 170 % of the adult value during d18 and d19 of gestation. There are two episodes of terminal elimination, one during days 19–21 of gestation, and another about 2 weeks postnatally. During the first postnatal week the number of terminals remains constant at about 140 % of the adult number, while the average number of inputs per fibre goes down and the number of muscle fibres increases.

(vii) Innervation is essential for muscle development. Motoneurons cannot regulate the number of muscle fibres by requiring a simple one-to-one relation between nerve terminal and muscle fibre, and if their role is regulatory as well as supportive of muscle development then some more complex relationship between nerve terminals and developing myotubes must be postulated.

INTRODUCTION

This is the third in a series of papers concerned with the role of motor nerves in regulating the development of skeletal muscles. Here, the distribution of acetylcholine receptors on the surface of embryonic muscle cells is used as an assay to investigate the mechanisms regulating the time and place of formation of neuromuscular junctions.

Acetylcholine receptors in adult muscles are densely clustered beneath the nerve terminal and are scarce or absent elsewhere (Miledi & Zelená 1966; Fambrough & Hartzell 1972; Kuffler & Yoshikami 1975). Extra-junctional receptors are present in relatively high concentrations on embryonic muscle fibres (Diamond & Miledi 1962; Bevan & Steinbach 1977) and are progressively removed as muscles mature. Junctional receptor clusters can be resolved only one or two days after transmission has begun in rat embryos (Bevan & Steinbach 1977; Braithwaite & Harris 1979*a*) so that it is appealing to assume that their positions on muscle fibres are determined by the nerve (Bennett & Pettigrew 1974; Anderson *et al.* 1977). However, Sanes *et al.* (1978) showed that nerve terminals can be induced to differentiate by contact with endplate basement membrane in the absence of muscle, and in the preceding paper (Harris 1981*b*) it was shown that ChE deposits appear at the normal time and in the normal place in aneural muscles. The possibility that developing embryonic muscles possess a system to direct the place of formation of neuromuscular junctions is further considered here.

Another aspect of endplate development deserving exploration is the functional reason for the presence of redundant innervation in embryonic muscles (Redfern 1970). 'Elimination' of redundant terminals has commonly been assumed to involve a reduction in the total number of terminals in a muscle (Jansen *et al.* 1978) but such studies fail to take into account the concommitant development of new muscle fibres (Ontell & Dunn 1978) or the possibility that an electrophysiologically defined muscle 'fibre' might actually represent several individually innervated filamented cells, perhaps within a common basal lamina sheath (Ontell 1979), that are strongly electrically coupled one to another (Dennis *et al.* 1981).

As this study includes a description of endplate-like structures that develop in the absence of a nerve, it often will be necessary to avoid the use of the word 'junction'. The terms 'J-cluster' and 'EJ-cluster' are used to distinguish ACh receptor aggregates in the central region of muscle fibres (which normally should mark the site of a nerve–muscle junction) from receptor aggregates appearing in other regions of a muscle. I describe special properties of J-clusters that distinguish them from other types of ACh receptor aggregate, and suggest that their time and place of appearance is ordered by some non-neural structure within embryonic muscle tissue that also directs the places at which neurites should differentiate into synaptic terminals. Brief presentations of some of this work have already appeared (Braithwaite & Harris 1979*a*, *b*).

Methods

Dating of pregnant animals, techniques for denervation or chronic paralysis of embryos *in utero*, and the dissection of diaphragm muscles are described in the preceding papers (Harris 1981*a*, *b*).

Labelling acetylcholine receptors

Acetylcholine receptors were visualized by radioautography of [^{125}I]-α-*Naja* toxin (αNT) (Patrick *et al.* 1972). The toxin was purified from *Naja naja* venom (Sigma) and iodinated with the chloramine-T method (see Bray *et al.* (1979) for further detail). Iodine was obtained monthly, and iodinations were done at intervals of 2 weeks.

The tissue to be examined, usually the diaphragm muscle, was dissected and pinned flat in an oxygenated bathing solution containing: NaCl 150 mM; KCl 5 mM; $CaCl_2$ 4 mM; $MgCl_2$ 1 mM; HEPES buffer 4 mM, adjusted to pH 7.2; glucose 11 mM; and foetal calf serum 10 ml/l. After incubation in [^{125}I]αNT (0.33 μg/ml) for 3 h at 20 °C in an oxygenated chamber on a rocking platform tissues were washed overnight in the cold (4 °C) in three changes of 250 ml of con-

tinually stirred bathing medium, and then fixed in freshly made fixative containing: paraformaldehyde 10 g/l; glutaraldehyde 12.5 g/l; HEPES buffer 20 mM, adjusted to pH 7.2; NaCl 120 mM; $CaCl_2$ 5 mM; at 4 °C. Tissues from young embryos were best fixed when kept in fixative for 12 h or more. Tissues were then washed in several changes of buffered saline to remove fixative, and placed in distilled water for frozen sectioning. Sections were cut at 20 μm and dried flat on gelatine-coated slides. Single fibres were prepared by disaggregation of well fixed muscles in a 25 ml Waring blender. Disaggregates of muscles were suspended in distilled water, spun down, resuspended in a small volume of distilled water, spread on coated slides, and dried in a dust-free hood.

Birth-dating endplates

Endplates were birth-dated by injecting 1 or 2 μl of [^{125}I]αNT (0.01 μg/μl) into individual embryos *in utero*, and preparing the tissue for radioautography at later times.

Radioautography

Slides were dipped in Ilford K2 emulsion (50 % (by volume), in distilled water) and exposed at 4 °C in sealed containers with silica gel as dessicant. Tissues labelled *in vitro* were developed with fresh D-19 developer (Kodak) at 50 % normal strength for 2 min after exposure for 2–5 d. Tissues labelled *in vivo* were exposed for up to 4 weeks. Slides were then dehydrated and permanently mounted.

Analysis of radioautographs

Cluster sizes were measured from photographs taken through a × 16 planachromat objective and enlarged to a final magnification of × 570 or × 915. Measurements of grain densities were made from photographs taken with a × 40 planapochromat oil immersion objective, n.a. 1.0, at a final magnification of × 715. Depending upon age and previous experimental treatment, from 3–12 sections (20 μm) could be made through an embryonic hemidiaphragm. These were mounted on a single slide, and two slides were obtained from each embryo, bearing left and right hemidiaphragm muscles respectively. After processing, the unstained sections were located by means of a stereo microscope with dark-field illumination, and the position of each section was ringed. Each section was then examined with the compound microscope, the central band of J-clusters was located, and a photograph was taken at a 'randomly' located position: i.e. care was taken to use the first portion of the endplate zone to be seen, and not to select particular regions. Photographs were measured with a dial micrometer to a resolution of 10 μm (i.e. < 0.2 μm final resolution). At least 200 endplates were measured from each embryo, equal numbers being taken from left and right hemidiaphragms. Care was taken to include all clusters from each print so that problems of subjective selection of particular size classes could be minimized. Embryos of d15 did not provide a sufficient number of clusters, and measurements from litter mates were pooled.

A slightly different procedure was used for neonates and young rats. A strip 1 cm wide running from left to right costal borders and including the nerve entry zones of left and right hemidiaphragms was labelled and fixed. A series of ten sections from each hemidiaphragm was processed and measured as above. Reproducibility of the technique is exemplified by measurements from left and right hemidiaphragms of individual animals not being significantly different one from the other, and measurements from litter-mate pairs not being significantly different.

Results

Junctional receptor clusters

Measurements

Aggregations of ACh receptors near the midpoints of muscle fibres (J-clusters) were made visible by making radioautographs of [^{125}I]αNT-labelled receptors. Most observations were made on frozen sections of diaphragm muscles. There were two advantages for the present work over use of teased or dispersed single fibres (Bevan & Steinbach 1977): the distribution of J-clusters within whole muscles could be described; and a large volume of material could be analysed (figure 1, for example, is derived from measurements of nearly 5000 individual endplate clusters).

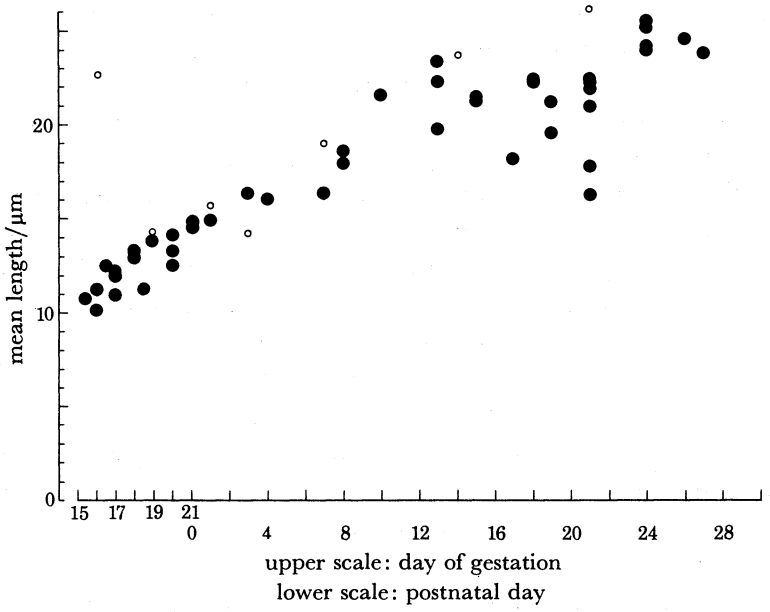

FIGURE 1. Mean lengths of J-clusters in normal muscles from d15½ until postnatal day 27: ●, each point is the mean of at least 200 measurements from both hemidiaphragms of a single embryo (except d15½, where data from two embryos are pooled); o, data replotted from Bevan & Steinbach (1977). The s.e. of each mean is less than the size of the symbols.

Values for mean lengths of receptor clusters measured from photographs (figure 1) were similar to those obtained by Bevan & Steinbach (1977) (o) from unmounted radioautographs of single fibres, with two exceptions: at d16 and at postnatal day 21. These differences may reflect a different pattern of growth in the sternomastoid muscle (J. H. Steinbach, personal communication). The correspondence of the other values justifies use of the simpler frozen section technique. Similar values were obtained from control experiments from dispersed single fibres (not stained for AChE as in the work of Bevan & Steinbach, and permanently mounted).

First appearance of J-*clusters*

Aggregates of ACh receptors were first seen in muscles taken from embryos during the afternoon of d15. They were not seen in muscles from d14 embryos, or in any muscle from a litter taken at 9 a.m. on d15, although dispersed receptors were present at these times. Most,

but not all, muscles from embryos taken in the afternoon or evening of d15 had a central band of receptor clusters which, if present, crossed the full width of each hemidiaphragm. The density of receptors within clusters initially was low, but by the morning of d16 clusters were all distinct and obvious.

J-clusters from muscles taken at d15, d16 and d18 are compared in figure 2, plate 2. Muscles for this experiment were labelled and processed together to allow comparison of grain densities. The dimensions of J-clusters at the three ages were not very different, but the density of receptors within the d15 clusters was less than at later times. Quantitative estimates of the density of receptor packing were not made: qualitatively, these results agree with those of Bevan & Steinbach (1977).

Growth of J-clusters

Lengths of d15 J-clusters were difficult to measure due to problems in defining their borders, but from d16 onwards measurements with a good degree of reproducibility could be made. A summary of measurements from muscles ranging from d15 gestational to 26 d postnatal is shown in figure 1. Mean values for cluster lengths steadily increased from d16 onwards, beginning at about 10 μm and reaching 20 μm after about 2 postnatal weeks. Mean values were unchanged during the period 2–3 postnatal weeks and then increased again.

The scatter of points in the postnatal data is thought to reflect real differences between animals rather than limitations in the measuring technique. Animals came from different litters, with at most two litter mates being used at one time. The mass and size of young rats varied both with age and with the size of the litter and age and size of the mother. It could be argued that smaller animals may have had smaller endplates than larger animals of the same age, and some fortuitous selection of animals might account for the lack of change between postnatal days 13 and 21. An extreme example to counter this argument is given by four rats from two litters at postnatal day 21 with a mean mass of 31.85 g and mean J-cluster length of 21.7 μm, compared with two rats from a litter of unusually small rats at postnatal day 15 with mean mass 21.8 g and mean J-cluster length 21.4 μm.

Changes in value of the mean length of clusters with time should reflect two processes: the addition of new endplates to the muscle; and the growth of endplates on older fibres. Frequency distributions of J-cluster lengths in muscles of different ages are illustrated in figure 3. Of the clusters in d15 and d16 muscles, 70 % are < 12 μm long (figure 3*a*). Older embryonic muscles have an increasing proportion of larger clusters, although all have clusters in the < 12 μm range. From birth up to postnatal day 8 (figure 3*b*) clusters lie within the range 8–24 μm, with an increasing proportion of longer lengths within this range with time; the range remains constant at 16–32 μm between postnatal days 10 and 21; after that time a small proportion of clusters of adult length (*ca.* 40 μm) has appeared.

If it is assumed that small clusters (e.g. < 12 μm) reflect the presence of recently developed muscle fibres, then these distributions can be used to calculate the time course of formation of new endplates. As the majority of J-clusters in d15 and d16 muscles were < 12 μm long, this value is arbitrarily chosen as a definition of small clusters. The percentage of clusters < 12 μm long in muscles of different ages is plotted in figure 4. During d17, when the rate of addition of new muscle units is at a maximum (Harris 1981*a*), 60 % of clusters were in this size range; at birth 35 %; and by postnatal day 10 the proportion had declined to 2 %. As only 200 clusters were measured from each muscle the data at later times are of limited accuracy.

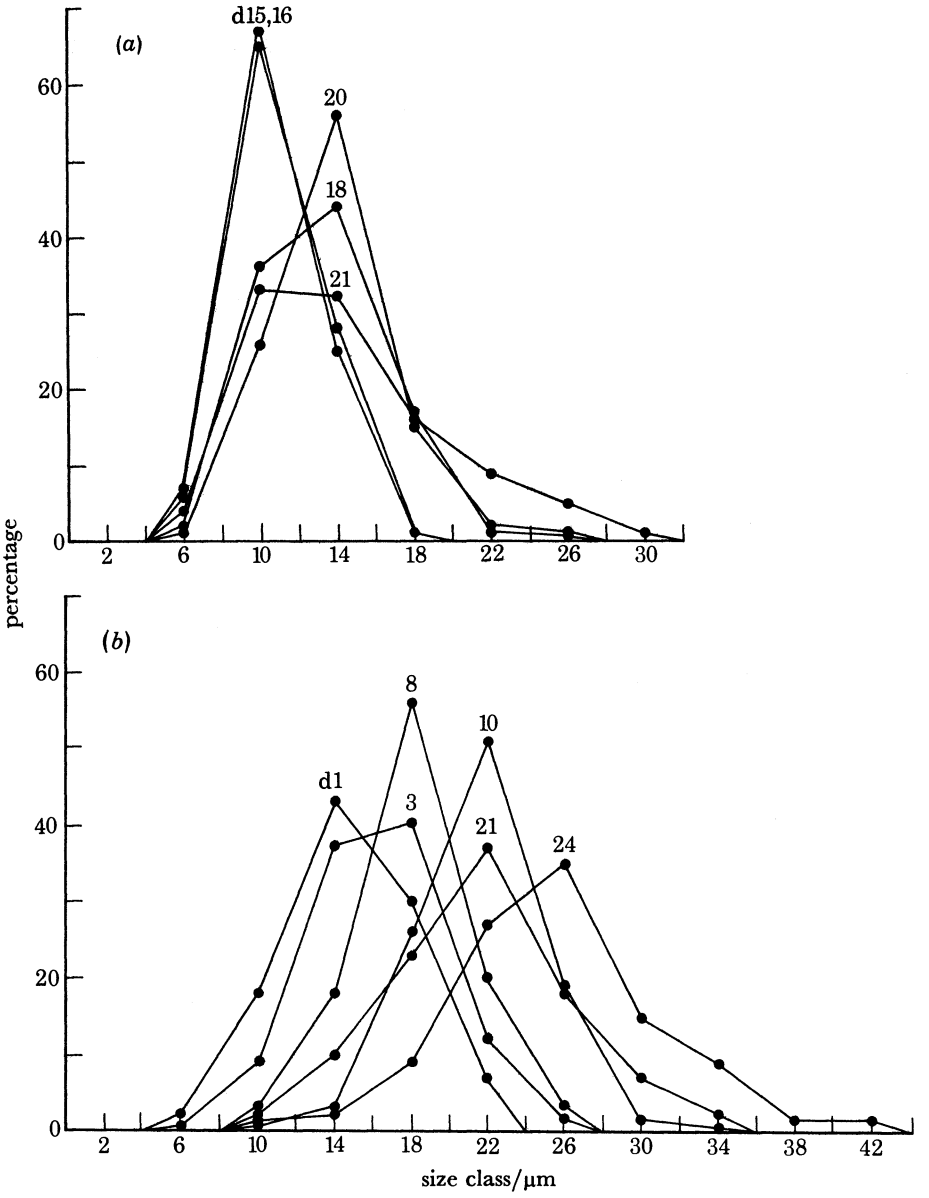

FIGURE 3. Examples of frequency distributions of J-cluster lengths. (*a*) Diaphragm muscles from embryos. (*b*) Diaphragm muscles from young rats. The number above each set of connected points represents the age of the animal. Points are plotted in the midpoint of each range; for example a point at 10 μm represents the proportion of clusters in the range 8–12 μm.

Values of the number of muscle units present at different times during development (Harris 1981 *a*) were used to construct curves showing the percentage of units in a muscle at any given time that were formed during the last 1, 2, or 3 d, respectively (figure 4*b*). A good match with the curve for the proportion of clusters < 12 μm long can be made by assuming that clusters of this length were formed within a period extending no more than 2 d previously. This correlation supports the suggestion that small clusters have formed more recently than larger ones.

The validity of this analysis can be tested further by using values of the percentage of small

J-clusters present each day to construct growth curves predicting the number of muscle fibres present at each embryonic and early postnatal day. A family of such curves is illustrated in figure 5.

The curves are calculated as follows. Suppose x_{14} fibres are present on d14, x_{15} on d15, and so on. On d15, 74% of J-clusters are < 12 μm long, and 26% > 12 μm long. If the latter

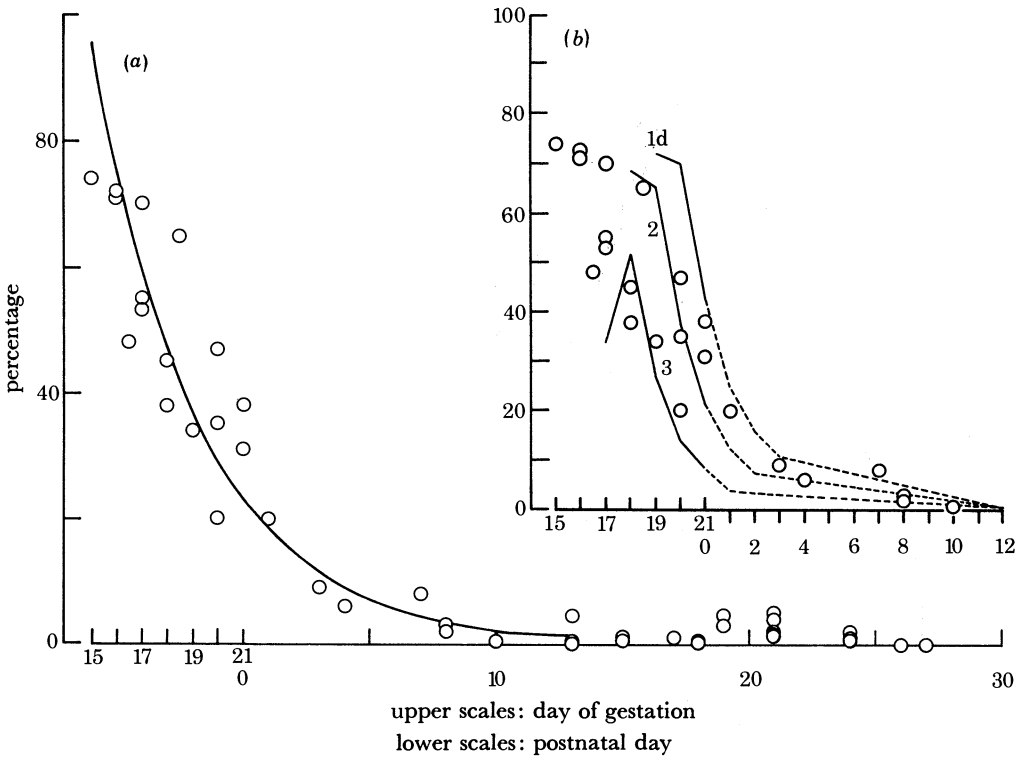

FIGURE 4. (*a*) Proportion of J-clusters < 12 μm long in diaphragm muscles from embryos and young rats. The regression line is fitted to the logarithm of each percentage value ($r = 0.95$). Each point represents values from both hemidiaphragms from a single animal; sample size > 200 J-clusters for each datum. (*b*) Same data as in (*a*), compared with estimates of the number of muscle units formed 1, 2 or 3 d previously (Harris 1981*a*).

represent endplates on fibres formed more than 1 d previously, so that they were all present on d14, then $x_{14} = \frac{26}{100}x_{15}$, i.e. $x_{15} = x_{14}/0.26$. The number on d16 is $x_{15}/0.285$ (71.5% of d16 J-clusters were < 12 μm long), and so on. Given that x_{33} (postnatal day 12) is 11918, the values for the earlier days can be calculated. These calculations were made for intervals of 1, 2 or 3 d, as illustrated (figure 5), with measured values of daily means of the percentage of J-clusters whenever possible. When daily data were not available they were obtained by extrapolation with a logarithmic curve fitting program. Another series of calculations (not shown) employed the logarithmic regression line ($R = 0.95$) shown in figure 4*a*. This gave smoother theoretical lines at the longer time intervals (> 3 d), but was not otherwise much different from the curves for which the measured data were used.

The best fit between observed and theoretical data in figure 5 is obtained if clusters less than 12 μm long were formed less than 2 d previously, but if some are more than 1 d old. This result justifies the use of an analysis of J-cluster lengths in a single muscle to provide a history of growth for one individual.

Location of J-clusters within the muscle

Receptor clusters formed a band along the middle of the muscle, in the same general area occupied by the intramuscular nerve. Seven muscles, including four left and three right hemidiaphragms, from embryos examined at various times in the afternoon and evening of d15 were subjected to a detailed analysis. All 44 sections obtained from the muscles were photographed. A grid was laid on the photograph and four uniformly spaced points were marked. The maxi-

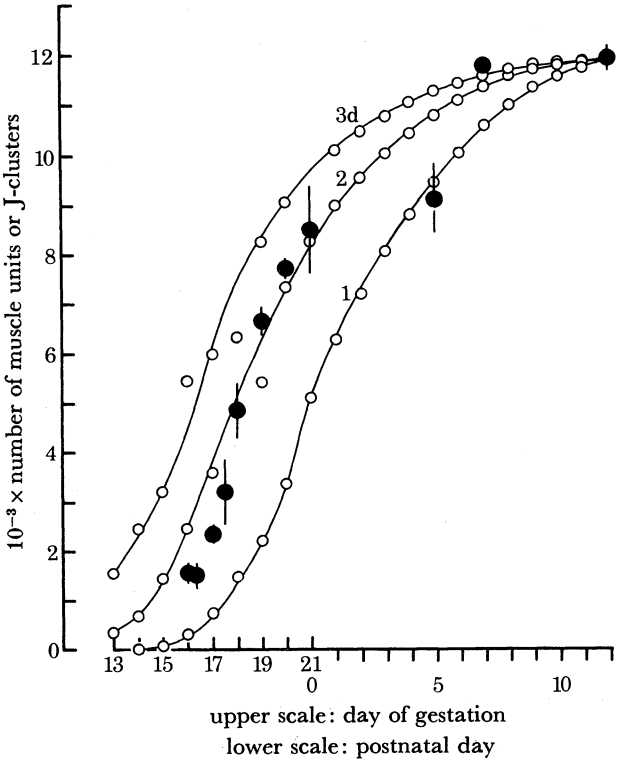

FIGURE 5. Curves predicting the number of J-clusters present at different times, based on the hypothesis that clusters < 12 μm long are < 1, < 2, or < 3 d old, respectively (○). These curves are compared with data points (●, ± s.d.) representing the growth in number of muscle units, from Harris (1981*a*). Details of the calculations are given in the text.

mum longitudinal distance between the clusters nearest each point was then measured. Clusters formed bands which were never more than 100 μm across (mean 49 ± 16 μm) and were continuous from edge to edge of the muscle. No muscles were seen with clusters in only one region or at only one level of sectioning. The precise relation between nerve terminals and receptor clusters at early times is not known. The nerve, as revealed with silver or AChE staining (see Harris 1981*b*, figure 1) covered an area of muscle about three times as wide as the J-cluster band. It was not possible, with light microscopy, to distinguish synaptic terminals at this age; axons ended in fine sprouts and growth cones.

At later times clusters were more dispersed, but they still tended to be arranged in orderly rows. Left and right hemidiaphragm muscles were different, the right muscle developing several rows of clusters in contrast to the one or two in the left muscle. Occasional muscles had receptor clusters in other regions, usually near the tendon ends of fibres in the widest part of the muscle, although silver or ChE staining never revealed nerve fibres in this region.

Birth-dating J-clusters

[^{125}I]-labelled αNT was injected into embryos of d16–d19 and radioautographs made of their muscles at d17–d21. Injection of the toxin caused paralysis that was prolonged when compared to the effects of injecting unlabelled toxin. For example, following injection of 1 μg of unlabelled αNT some embryos were still paralysed when examined 1 d later, but all were mobile within 2 d. Injection of 0.06 μg of [^{125}I]αNT left some embryos fully paralysed even 4 d later. Tritium-

TABLE 1. MEAN SIZE OF RADIOAUTOGRAPHIC GRAIN DEPOSITS OVER J-CLUSTERS AFTER LABELLING *IN VIVO*, COMPARED WITH CONTROL J-CLUSTERS LABELLED ACUTELY

(For each observation n = 200.)

labelled *in vivo*		
time when labelled	time when examined	mean size of cluster, ± s.d./μm
d16	d18	10.38 ± 2.29
d17	d21	10.32 ± 1.83
d17	d21	11.32 ± 2.20
controls (acutely labelled)		
time		mean size of cluster/μm
d16		10.11 ± 3.18
		11.12 ± 1.72
d17		12.18 ± 2.69
		10.85 ± 2.67
		12.00 ± 3.47
d18		13.10 ± 2.85
		12.85 ± 3.79
d21		14.77 ± 4.76
		14.47 ± 5.28

labelled thymidine had a similar effect, which was not seen after injections of unlabelled thymidine or of saline or distilled water. This effect of radiation is important, as paralysis of muscle retards the development of new fibres (Harris 1981*a*). Thus tissues that have developed in labelled embryos cannot directly be compared with control tissues.

Muscles labelled with [^{125}I]αNT *in vivo* required 2–4 week exposures to provide satisfactory images. Labelled J-clusters were present in a band across the whole width of the muscles and had mean lengths similar to those expected at the time of injection, rather than the time of examination (table 1).

Location of ACh *receptors*

Acetylcholine receptors labelled *in vivo* and examined at later times were dispersed along the whole length of extra-junctional parts of the muscle cells. Injection of unlabelled toxin in a 100-fold greater dose than the labelled toxin (1 μg cf. 0.01 μg) did not paralyse embryos for much more than 24 h, indicating that there was no maintained pool of unbound labelled toxin. Thus labelled toxin should bind only to receptors present close to the time of injection. Labelled muscle fibres were present throughout the muscles, showing that new fibres formed since the time of labelling were randomly positioned in the muscles. Labelled J-clusters also could be found across the whole width of each muscle. Staining for AChE revealed unlabelled endplates that had developed since the time of labelling (see figure 10). These findings confirm the pattern of growth seen with [^{3}H]thymidine radioautography (Harris 1981*a*).

Paralysed muscles

Development of receptor clusters

Embryos were paralysed by insertion of slow-release capsules as described previously (Harris 1981 *a*), and their diaphragms were dissected for examination of ACh receptor distribution. The earliest time at which TTX capillaries were inserted was the morning of d15, before receptor clusters are seen in normal muscles. Muscles dissected from control embryos at that time and mounted in an organ chamber contracted in response to nerve stimulation.

J-clusters formed in paralysed muscles despite the absence of nerve and muscle action potentials. Muscles examined at d18 were smaller than controls and contained areas with no myotubes between the epimysia (figure 6, plate 2; Harris 1981 *a*, fig. 1). Radioautography revealed bands of J-clusters running from edge to edge of each muscle. These bands were characteristically narrower in left hemidiaphragms than in right hemidiaphragms (figure 6). No EJ-clusters were seen in muscles paralysed during d15–d17 or d15–d18. Embryos left for longer periods, with the same (3–4 mm length) capillaries, did not remain paralysed. Two litters examined at d20 possessed varying degrees of spontaneous motility, and all gave reflex responses to stimulation of the nose. Their diaphragm muscles were more comparable to controls than the d18 muscles. They were fully formed with no areas lacking multi-nucleate myotubes, but muscle fibres still were shorter than in control litter mates and there were areas of distortion in the direction of muscle fibre growth within some muscles. Slight differences from controls were noted when the spread of ChE deposits and of J-clusters was examined in muscles from 15 embryos that were paralysed at d15 or d16, and examined at d20 or d21. Seven embryos had recently recovered from the paralysis; the rest had not. Muscles from five of these animals had a greater than normal spread in distribution of J-clusters and ChE deposits in sections from within the muscles (see figure 9), despite the shorter overall length of the muscles. As a criterion for 'normal', the point of greatest width of the central J-cluster band in d20 and d21 control muscles (including muscles from litters outside this series) was measured. While the mean width of the central band was about 400 μm in right hemidiaphragms and 250 μm in left hemidiaphragms, widths of up to 1110 μm (right hemidiaphragm) and 890 μm (left hemidiaphragm) were recorded at single points, usually near the nerve entry. Widths of the central band of J-clusters in sections from the interior of the TTX-treated muscles ranged as high as 2220 μm (right hemidiaphragm) and 1510 μm (left hemidiaphragm). Narrower bands, as in d15–d18 paralysed muscles, appeared in superficial sections. Careful phase-contrast examination of individual fibres, to the extent that the frozen section technique allows, revealed no multiple junctional structures on single fibres.

J-clusters and muscle growth

Mean lengths and size distributions of J-clusters from TTX-treated muscles are shown in figures 7 and 8, and compared with those from control muscles. Muscles remaining paralysed until the time of assay had J-clusters with mean lengths equal to or greater than those in controls. In contrast to this, muscles that were paralysed and then allowed to recover had shorter than normal mean J-cluster lengths.

These findings can be accounted for by assuming an effect of nerve and muscle activity on muscle development. If the generation of new J-clusters was inhibited by TTX paralysis while existing endplates continued to grow, then the mean J-cluster length should become greater than in control muscles due to the absence of newly formed, and therefore smaller, clusters.

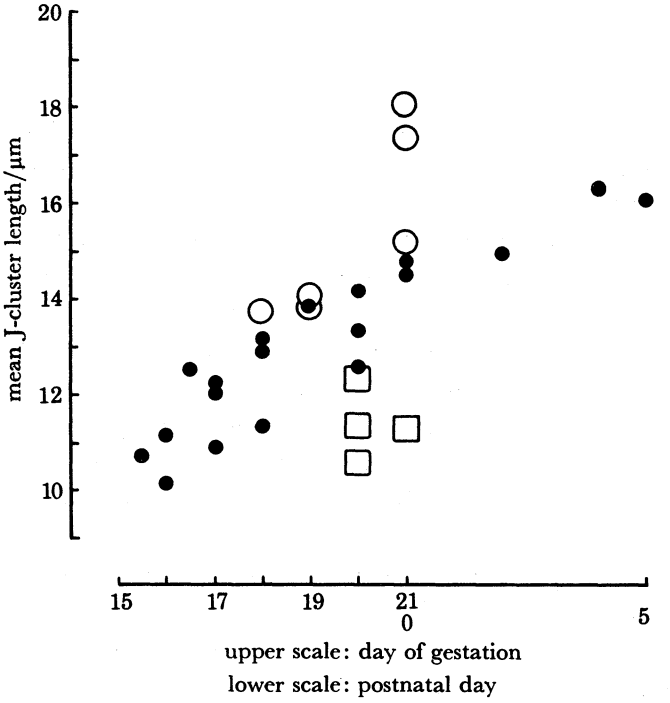

FIGURE 7. Effects of paralysis with TTX on the mean length of J-clusters in rat embryo diaphragm muscles: ●, control data replotted from figure 1; ○, muscles paralysed from d16 to d21, d16 to d19 and d15 to d18, respectively; □, muscles paralysed at d15 and recovered before examination at d20, and paralysed at d16 and recovered before d21, respectively.

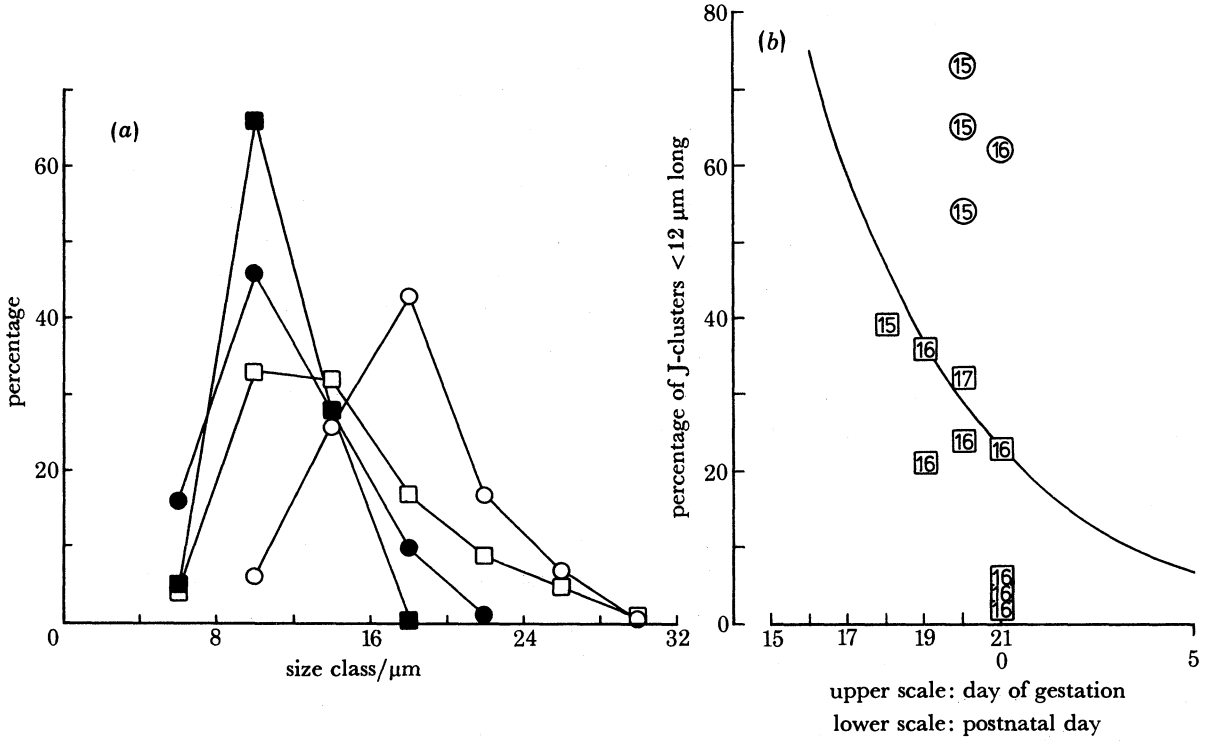

FIGURE 8. (*a*) Examples of frequency distributions of J-cluster length in paralysed and control rat embryo diaphragm muscles: ○, J-clusters in muscle paralysed during d16–d21; ●, paralysed at d16 and recovered before examination at d21; □, d21 control muscle; ■, d16 control muscle. Examples are of experimental muscles with mean values well away from control values, to illustrate the basis for the divergence. (*b*) Proportion of J-clusters < 12 μm long in paralysed muscles and muscles recovered from paralysis, to indicate generation of new endplates: ——, proportion of small clusters in control muscles (replotted from regression line in figure 4); ○, muscles recovered from paralysis; □, continually paralysed muscles. The numbers inside the symbols represent the day of gestation when paralysis was initiated.

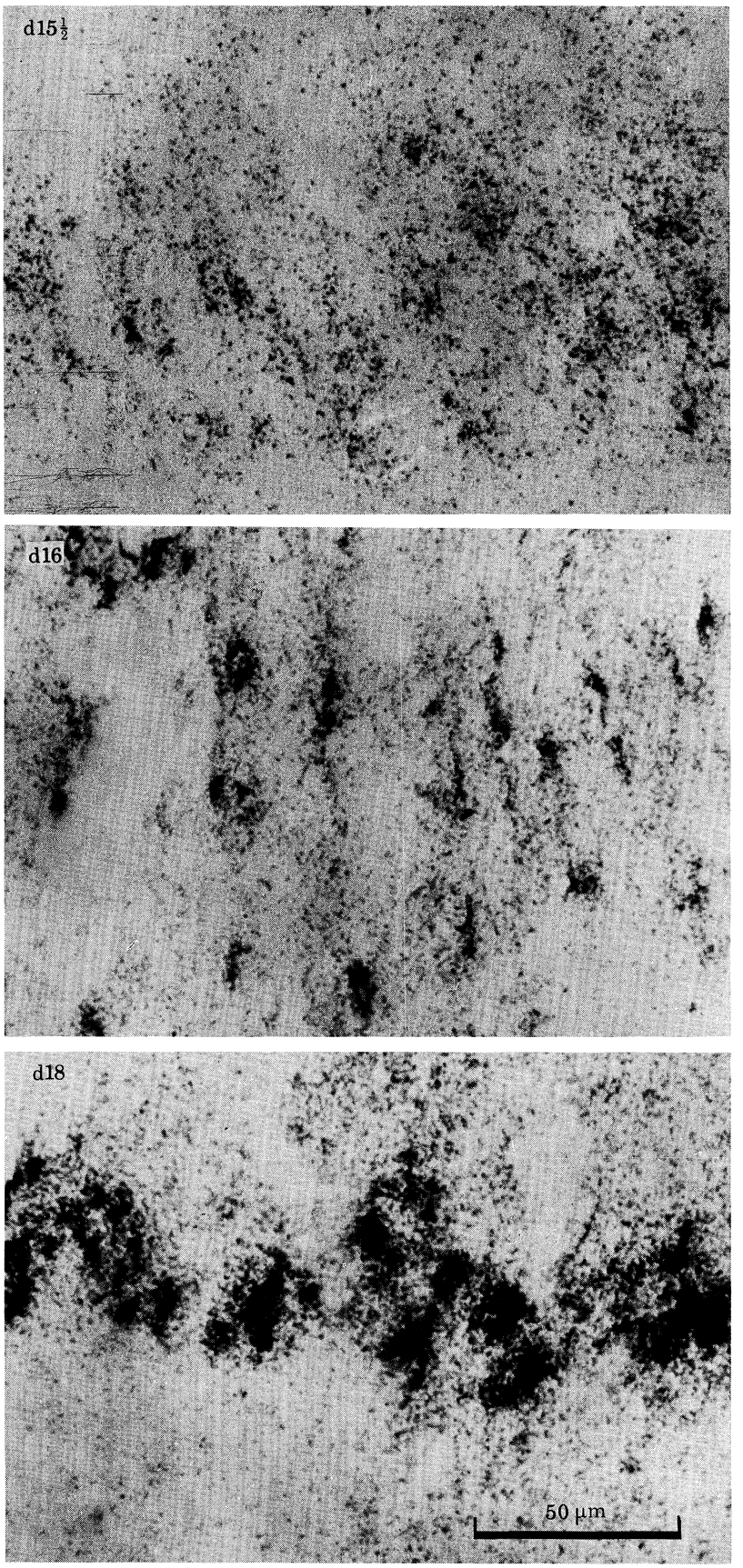

FIGURE 2. Illustrations of J-clusters in muscles of d15½, d16 and d18. The muscles were labelled and processed in parallel to allow direct comparison of grain densities.

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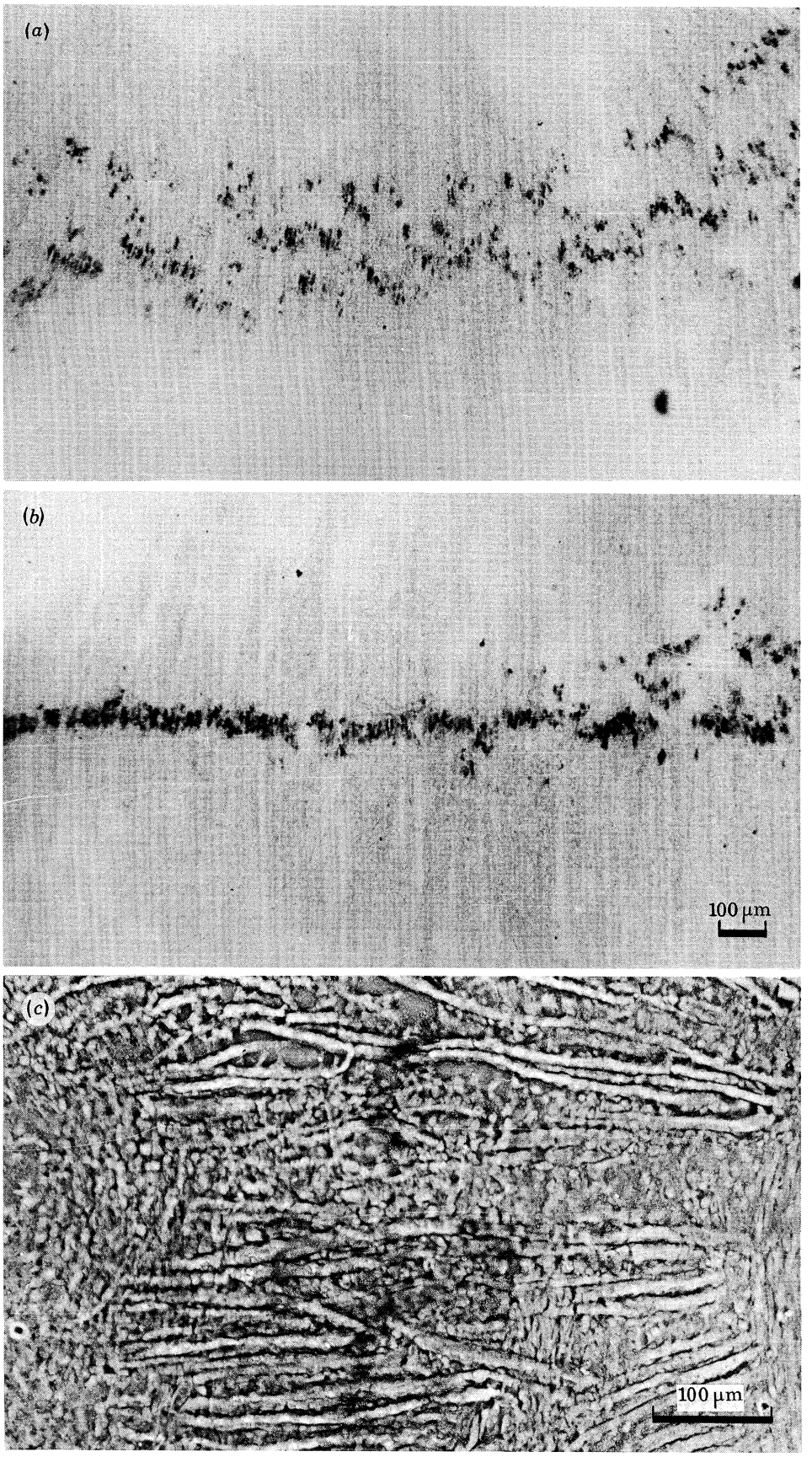


FIGURE 6. J-clusters and myotubes in muscles from embryos paralysed from d15 to d18. (*a*), (*b*) Radioautographs of J-clusters and dispersed extra-junctional ACh receptors in (*a*) right and (*b*) left hemidiaphragm. (*c*) Phase contrast photograph of unstained radioautograph.

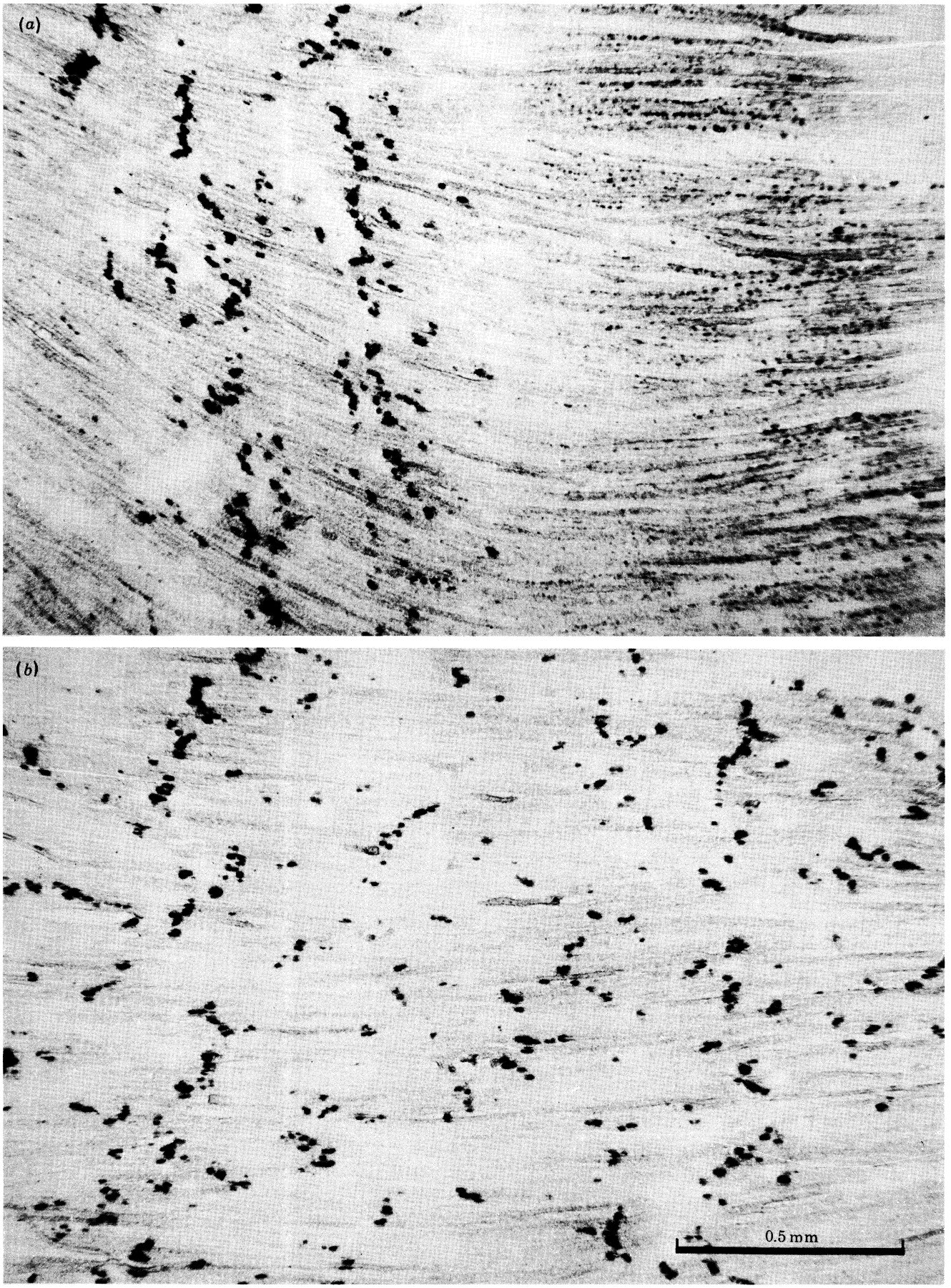


FIGURE 9. J-clusters and EJ-clusters in a muscle from a TTX-treated embryo paralysed from d16 to d21. Both sections are from the same muscle (right hemidiaphragm). (*a*) Superficial section, showing J-clusters with normal distribution, and EJ-clusters near fibre ends. (*b*) Deep section, showing unusually wide band of J-clusters (this is the widest distribution seen in any muscle).

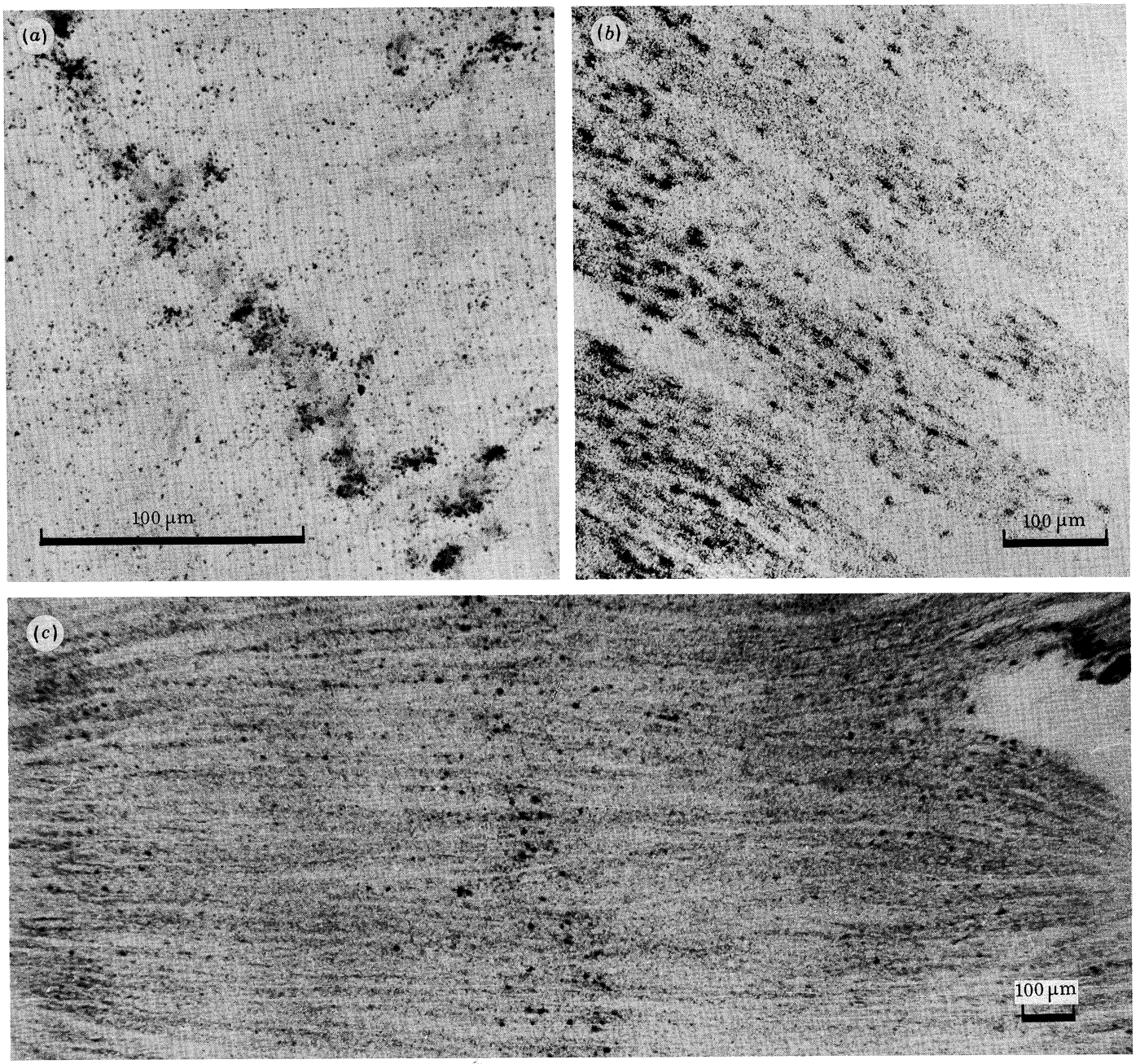


FIGURE 10. Effects of paralysis with αNT or curare. (*a*) Paralysis with [^{125}I]αNT from d16 to d18. Radioautograph stained for ChE. Note ChE deposits between labelled J-clusters, indicating that new endplates have formed since the time of labelling. (*b*) Paralysis from d17 to d19; formation of EJ-clusters. There is a band of J-clusters along the left border of the photograph, and EJ-clusters spread through the rest of the area. (*c*) Muscle paralysed with dTC from d17 to d20. Note formation of EJ-clusters.

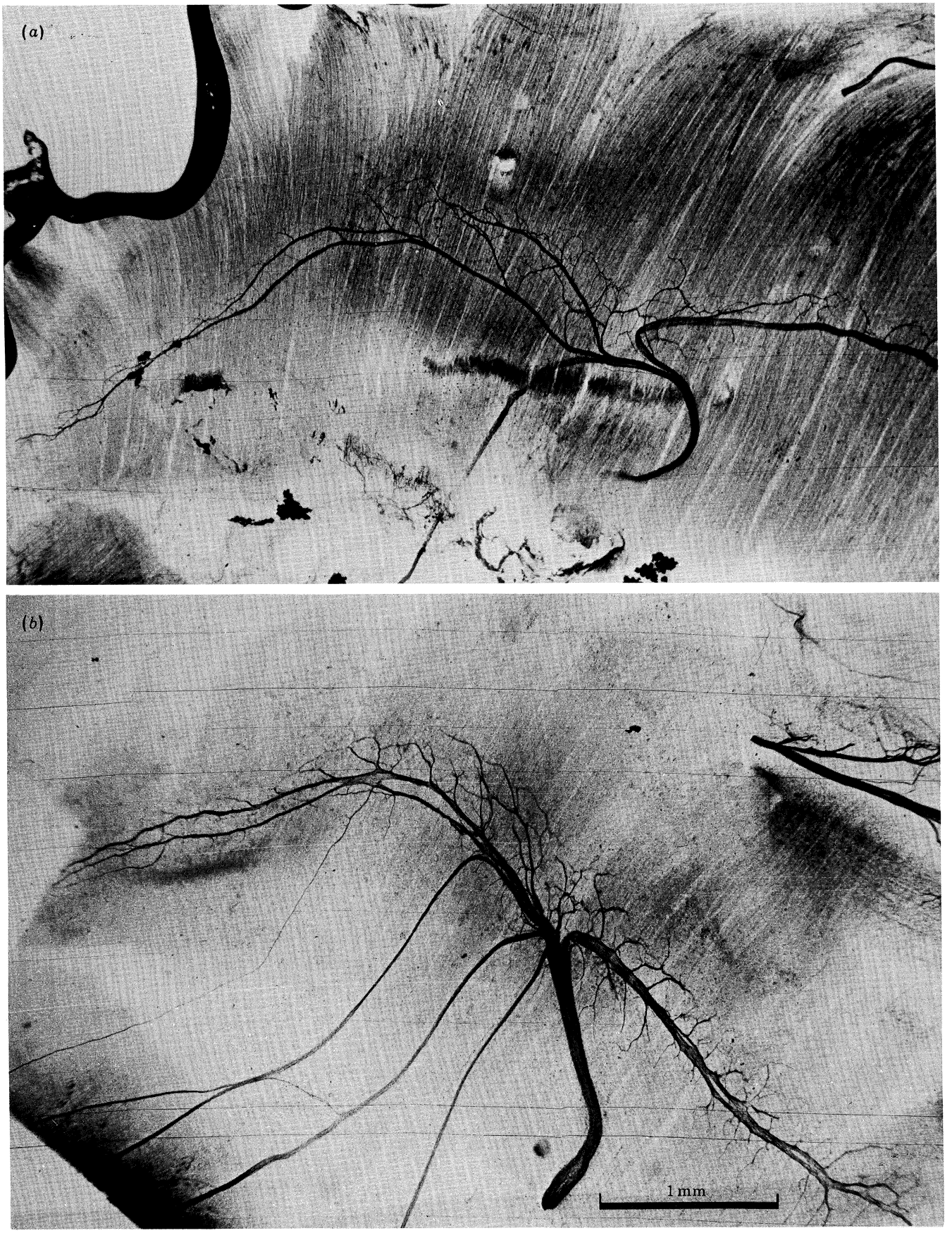


FIGURE 11*a*, *b*. For description see p. 299.

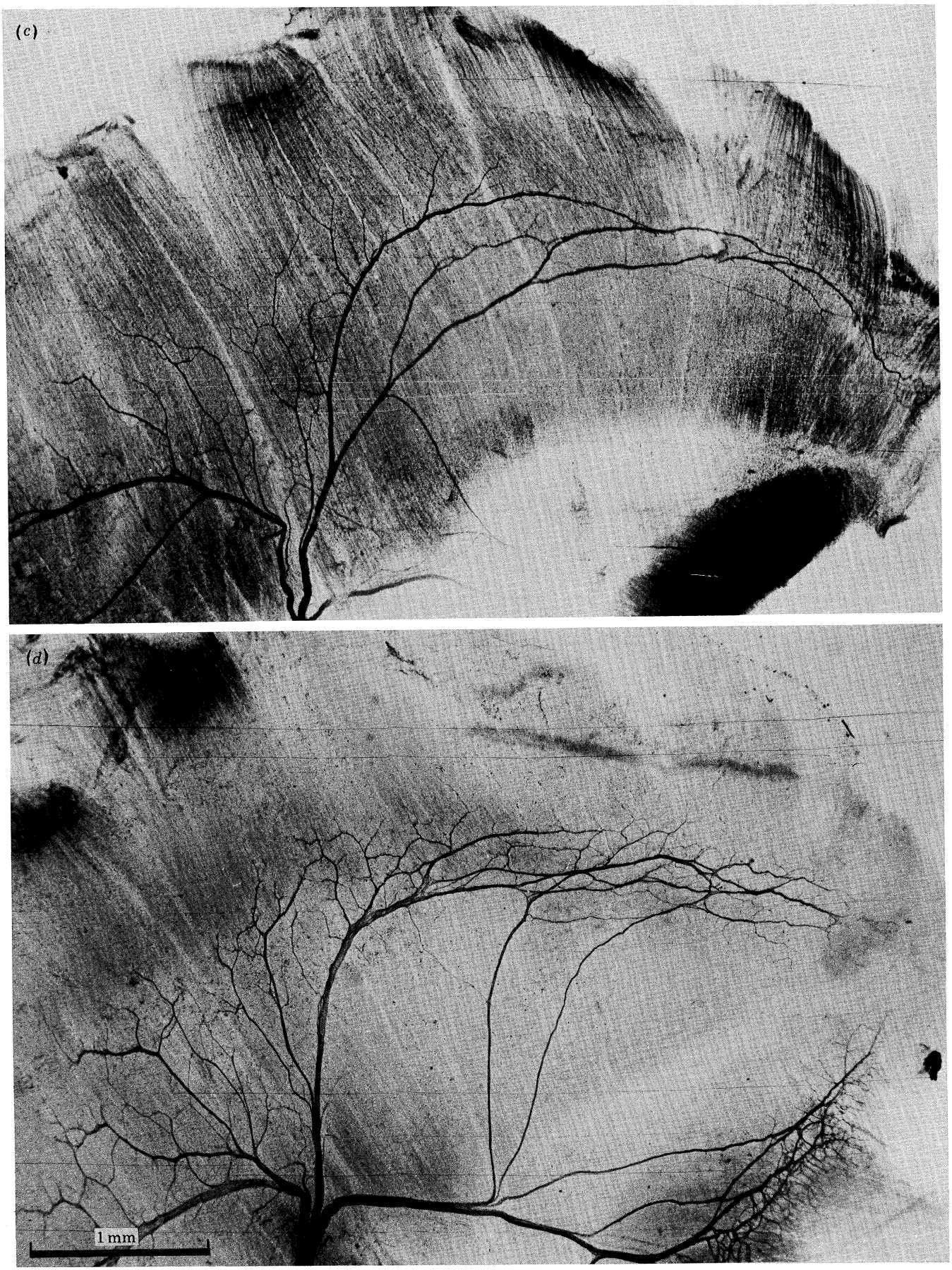
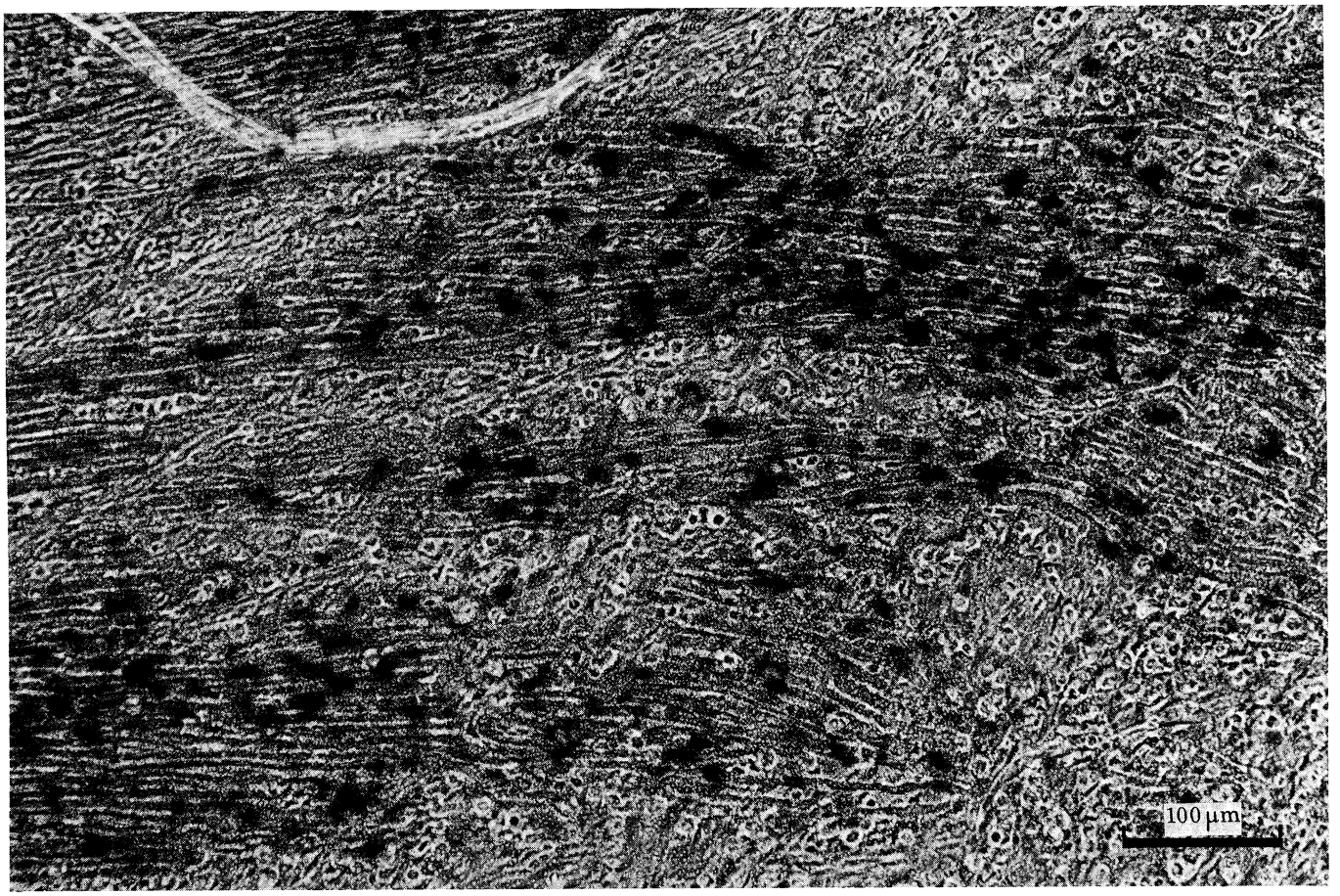


FIGURE 11*c*, *d*. For description see p. 299.



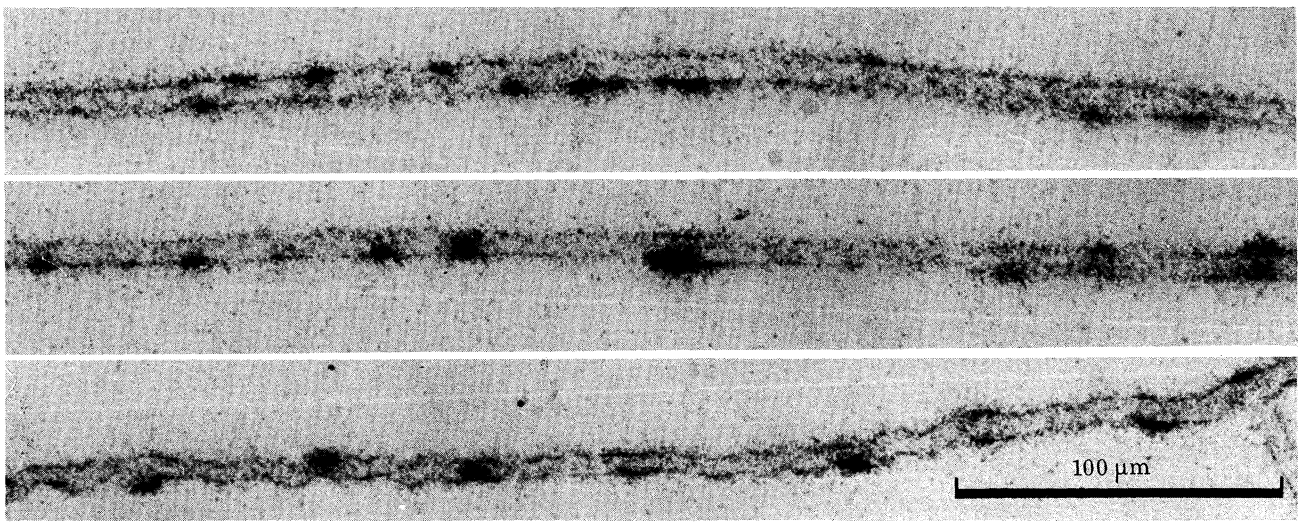


FIGURE 12. Multiple receptor clusters and areas lacking multi-nucleate myotubes in a diaphragm muscle from a d20 embryo treated with β-BTX at d15. Phase-contrast micrograph of an unstained radioautograph.

FIGURE 16. Multiple receptor clusters along the length of single β-BTX-treated muscle fibres. Embryo injected at d17 and examined on d21. Unstained radioautographs of mechanically disaggregated single fibres.

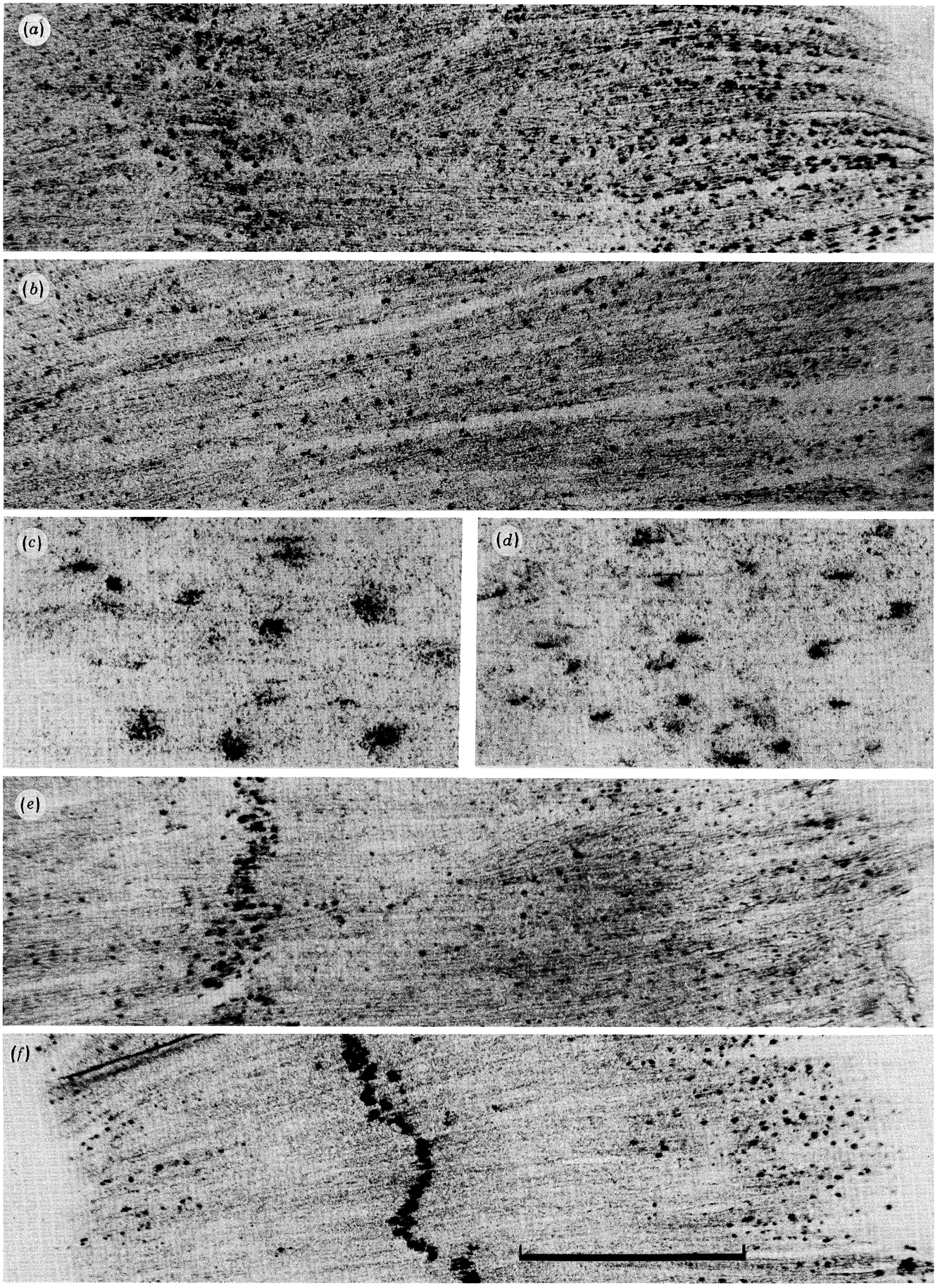


FIGURE 13. For description see opposite.

Release from paralysis and resumption of development of new muscle fibres would result in the presence of many small clusters, so that their mean length should be smaller than in controls.

The proportion of J-clusters < 12 μm long in paralysed and in recovered TTX-treated muscles is shown in figure 8*b*. The proportion in muscles kept paralysed was the same or smaller than in controls, whereas the proportion in temporarily paralysed muscles was approximately twice that expected. These results, together with observations on the size of paralysed muscles provide evidence that electrical activation of muscle is essential for the development of new J-clusters in muscles older than d16. This is no doubt due to the failure of generation of new secondary myotubes in paralysed muscles (Harris 1981 *a*), and reflects a correlation between the formation of endplates and the formation of new myotubes.

EJ-*clusters in paralysed muscles*

EJ-clusters are defined as aggregates of αNT binding sites that could appear at any point along a muscle fibre. Evidence is presented below to show that such clusters are truly extrajunctional in that they do not attract or receive innervation.

EJ-clusters had formed on paralysed muscles examined at d19 or later. Multiple EJ-clusters formed along single muscle fibres (Braithwaite & Harris 1979*a*, fig. 2). Muscles from embryos paralysed on d16 and examined on d19 had EJ-clusters extending all the way along muscle fibres from the band of J-clusters in their centres to the tendonous junctions at the ends of the fibres. This was not true in muscles examined a day or two later. On d20 or d21 there was a clear area between J-clusters and EJ-clusters (figure 9, plate 3). Furthermore, EJ-clusters tended to be smaller the nearer they were to the J-clusters (figure 9).

Not all muscles paralysed on d17 and examined on d19 had EJ-clusters. When EJ-clusters were seen they were located near the tendon ends of the longest fibres in the muscles, those in the middle of the muscles near the nerve entry. A day later, in muscles paralysed during d17–d20, J-clusters were present from edge to edge of the muscles, but there still was a clear area between J-clusters and EJ-clusters. The same was true of muscles paralysed for 3 d from d18–d21.

Litters were prepared with short and long TTX capillaries inserted at d16, so that all embryos were paralysed through d19 but by d21 some had recovered motility. Muscles from the

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Description of plates 5 and 6

Figure 11. Distribution of nerves within paralysed and control rat embryo diaphragm muscles. Experimental muscles were paralysed during d16–d21. Left hemidiaphragms: (*a*) control; (*b*) TTX. (Scale bar in (*b*) also applies to (*a*).) Right hemidiaphragms: (*c*) control; (*d*) TTX. (Scale bar in (*d*) also applies to (*c*).) Bodian silver stains.

Description of plate 8

Figure 13. Unstained radioautographs showing J-clusters and EJ-clusters in β-BTX-treated muscles. (*a*), (*b*) β-BTX injected on d12, muscles examined on d20. Receptor clusters have similar dimensions, but in the muscle in (*a*) a relatively clear area can be distinguished between the central band of clusters and peripherally located clusters; no such clear area is apparent in the muscle illustrated in (*b*). (*c*), (*d*) Enlargements of clusters in a muscle injected at d15 and examined at d20. (*c*) J-clusters in the central band. (*d*) EJ-clusters located peripherally. In muscles treated at this time and later, J-clusters and EJ-clusters can be distinguished by their mean lengths as well as by their location. (*e*) J-clusters and EJ-clusters in a muscle from an embryo injected at d17 and examined at d21. Clusters can be distinguished both by size and by location. (*f*) Clusters in a muscle treated at d18 and examined on d19. EJ-clusters have formed within 24 h of injecting an embryo of this age. Scale bar, 0.5 mm ((*a*), (*b*), (*e*), (*f*)) or 100 μm ((*c*), (*d*)).

embryos that had recovered had no EJ-clusters at all. A few muscles paralysed from d15, and examined on d20 after recovery from paralysis some time after d18, retained some EJ-clusters, which must have formed after d18.

Effects of other forms of paralysis

Curare was a less effective paralysing agent than TTX due to its relatively lower potency. Open-ended capillaries packed with solid *d*-tubocurarine (*d*TC) released the drug at the rate of 0.25 mg/d. No embryo was fully paralysed at the end of 3 d of treatment, although a number were immobile and gave very slight reflex responses to stimulation of the nose or head. Muscles from these embryos had EJ-clusters in variable numbers (figure 10, plate 4), while EJ-clusters were absent from the muscles of treated embryos with even a moderate amount of spontaneous motility.

Direct injection of [^{125}I]αNT into embryos *in utero*, as described above, produced a paralysis greater than that attributable solely to the effect of the toxin. Muscles examined 1 or 2 d later had some EJ-clusters, usually at a low density, but there were occasional areas with a high density (figure 10). This finding demonstrates that clusters could form by aggregation of dispersed receptors present on the surface of the muscle at the time of injection. A converse experiment was to inject [^{125}I]αNT into d18 and d19 embryos previously treated with β-BTX so that EJ-clusters were already present (see below). When examined 2 or 3 d later, J-clusters were distinct and obvious, but EJ-clusters sometimes could not be seen, and if present were no more frequent than in control muscles labelled *in vivo*. These findings indicate that ACh receptors were not stable within EJ-clusters, but were continually added and removed. Receptors could be added by diffusion in the plane of the membrane; the mechanism of removal cannot be inferred from these experiments. Whether or not EJ-clusters maintain a constant spatial location is not known.

Receptor clusters and endplate formation

It has been postulated that ACh receptor clusters may direct the formation of endplates on embryonic muscles (Sytkowski *et al.* 1973; Fischbach & Cohen 1973) or on denervated adult muscles (Ko *et al.* 1977). Paralysis of muscles or motor nerves in adult animals provokes sprouting and extension of nerve terminals (Duchen & Strich 1968; Brown & Ironton 1978). If embryonic nerves are induced to sprout and to form terminals by the presence of non-innervated ACh receptor clusters, then muscles in TTX-paralysed embryos should have an aberrant pattern of innervation.

Embryos were paralysed for the period d16–d21. Their diaphragm muscles were then stained with the Bodian technique and the pattern of innervation was compared with normal control muscles. Twelve experimental muscles were compared with the same number of control muscles: the results are illustrated in figure 11, plates 5 and 6. No difference in the pattern of innervation could be seen between paralysed and control muscles. Nerve fibres were present only in the centre regions of the muscles, and did not extend towards the EJ-clusters present in the periphery. A careful search with a ×40 oil immersion objective revealed no fine neurites leaving the intramuscular nerve, or sprouting from endplates, to extend distally along muscle fibres. The adequacy of the staining is justified by the resolution of fine terminal sprouts in endplates and the resolution of autonomic nerve fibres surrounding blood vessels within the muscles (see Harris 1981*a*, fig. 2).

Endplates may be recognized by the associated deposit of ChE (Koelle & Friedenwald 1949). TTX-treated muscles were stained for this enzyme (see Harris (1981*b*) for technical details) and compared with normal and aneural muscles. The distribution and intensity of staining of ChE deposits in normal and paralysed muscles were nearly always identical. As mentioned above, in 5 out of 15 muscles treated with TTX on d15 or d16 and examined on d20 or d21, J-clusters and junctional ChE deposits in sections from within the tissues formed a band about twice as wide as controls (figure 9). The spread never covered an area wider than the distribution of nerves seen in normal muscles, and was not correlated with whether or not a muscle had recovered from paralysis.

Aneural muscles

J-clusters in aneural muscles

Muscles were denervated by injection of β-BTX as described previously (Harris 1981*a*). Receptor clusters appeared in aneural muscles at the same time and with the same pattern of distribution as in normal muscles. These 'J-clusters' proved to have properties distinguishing them from other ACh receptor aggregates, indicating that in normal muscles they would define the site of an endplate.

One series of experiments was to compared muscles from litter mates of d15 or d16, with or without injection of β-BTX at d12 or d14. Nine embryos from two litters injected at 9 a.m. on d14 were examined at 4.30 p.m. or 7.30 p.m. on d15, and compared with six litter-mate controls. Twelve embryos from four litters injected at 9 a.m. on d14 were examined, at 10 a.m., 10 a.m., 11 a.m. and 7.30 p.m. respectively, on d16 and compared with 13 litter-mate controls. Five embryos in one litter were injected on d12 and examined at 9 a.m. on d16, and compared with two litter-mate controls. Examination on d16 had the advantage that embryos could be tested to ensure the complete absence of reflex responses, as well as checked for the presence or absence of nerve-induced contraction in the isolated muscles (this was the only check possible in embryos injected on d12). The numbers of experimental animals given here refer to embryos in which both hemidiaphragms were successfully used for radioautography; thus there were totals of 52 experimental muscles and 42 litter-mate controls. Eighteen aneural hemidiaphragms were examined on d15. Of these, eight had distinct bands of receptor clusters along their midlines extending from edge to edge of the muscle, six had bands of greater receptor density along their midlines but only occasional clearly recognizable clusters, and four had receptors uniformly dispersed along their lengths. The twelve control hemidiaphragms included six with bands of J-clusters along their midlines, two with a band of greater density of dispersed receptors, and four with uniformly dispersed receptors. All 64 hemidiaphragms examined on d16 were similar, whether or not β-BTX had been injected. All had a narrow band of J-clusters across the centre of the muscle and nowhere else.

Aneural muscles examined on d17 could be distinguished from normal muscles by the greater width of the central band of clusters. As an example, all the sections obtained from one experimental and one litter-mate control diaphragm at this time were photographed and measured. The mean widths of J-cluster bands in the left hemidiaphragms, β-BTX-treated cf. control, were 280 and 160 μm respectively. Mean widths of J-cluster bands in the right hemidiaphragms were 521 and 208 μm, respectively. EJ-clusters were not obviously present in aneural muscles until d18. There was no clear area between J-clusters and EJ-clusters at this time, so that the width of the J-cluster band could not be determined, but EJ-clusters did not

always extend to the tendon junctions. By d19 aneural muscles had EJ-clusters scattered from tendon to tendon (figure 12, plate 7). This is comparable with muscles paralysed with TTX from d15, in which EJ-clusters were not seen at d18 but were present at later times.

A second series of experiments involved examination of muscles from d21 embryos, following injection of β-BTX at various times from d12 onwards. In muscles injected on d16 or later it always was possible to distinguish a central band of clusters corresponding to the J-clusters in normal muscles. Such bands were sometimes, but not always, apparent in muscles from embryos injected earlier (figure 13, plate 8).

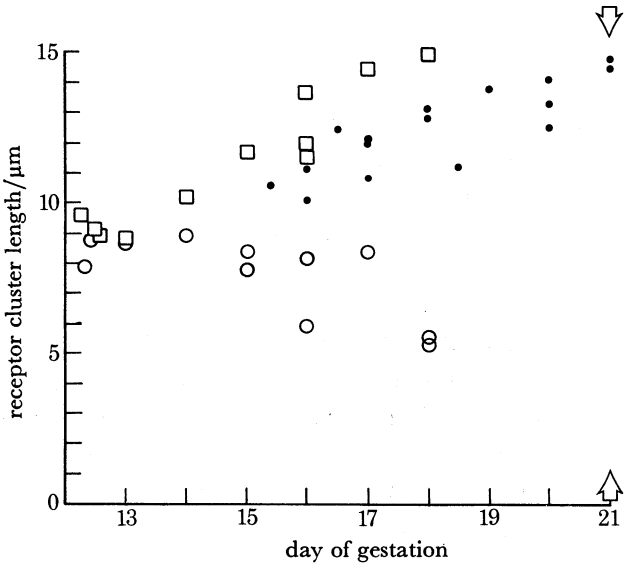

FIGURE 14. Mean lengths of ACh receptor clusters in β-BTX-treated muscles examined at d21. The time of examination of all the toxin-treated muscles is marked by the arrows. Symbols: ●, mean lengths of J-clusters in control muscles examined from d15–d21 (replotted from figure 1): □, mean lengths of J-clusters in muscles from embryos injected with β-BTX at the times plotted, and examined at d21; ○, mean lengths of EJ-clusters in β-BTX-treated muscles, examined at d21.

The mean lengths of clusters from this central band, in muscles from embryos injected with β-BTX at times from d12 to d18 and examined at d21, are shown in figure 14. Control data from figure 1 are replotted here to allow comparison with normal endplate clusters. The mean length of the J-clusters is that expected in control muscles about 2 d older than at the time of injection of β-BTX. Comparison of frequency distributions of cluster lengths (figure 15) is more informative. The β-BTX-treated muscles can be divided into two groups. Those treated during the period d12–d16 and examined at d21 have distributions similar to control muscles examined at d16–d17, with the majority of clusters < 12 μm and few or none > 20 μm. Those treated on d17 and d18 have mean lengths comparable to d20–d21 controls; this coincidence is due to treated muscles having fewer clusters in the < 12 μm class, as well as fewer larger clusters. This is in contrast to TTX-paralysed muscles (figure 8), which also have few clusters < 12 μm, but a greater number in the > 20 μm class.

The simplest model to fit these data is one where J-clusters stop growing shortly after the nerve terminal has been destroyed, and new J-clusters do not develop in toxin-treated muscles of d17 or older. New muscle fibres must have appeared following injection of β-BTX at early

times, as otherwise muscles from embryos injected at d12, for example, should not have developed at all. If, however, normal numbers of new J-clusters had continued to appear from d17 onwards it would be difficult to account for the frequency distributions seen, as they should have been dominated to a greater extent by new, small clusters. The inhibition of development of secondary myotubes in β-BTX treated muscles (Harris 1981*a*) is consistent with this explanation.

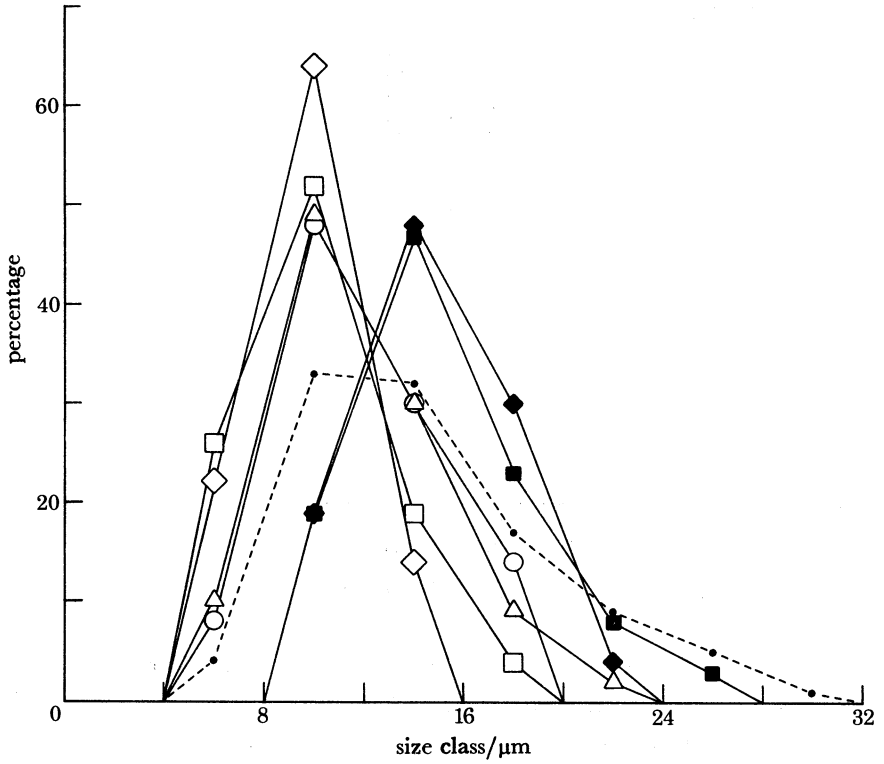

FIGURE 15. Examples of frequency distributions of J-cluster lengths in muscles treated with β-BTX at various times and examined at d21 (——); d21 control muscle (– – – –). Open symbols: muscles from embryos injected at d12–d16 (◇, d12; □, d14; ○, d15; △, d16). Closed symbols: embryos injected at d17 or d18 (◆, d17; ■, d18). Note the paucity of small clusters in the d17–d18 treated muscles, as compared to muscles treated earlier, or to the control.

Development of EJ-*clusters*

Muscles treated with β-BTX on d16 or earlier and examined at d21 had receptor clusters scattered over the whole length of the muscle (figures 12, 13). Treated muscles examined at d17 had a wider than normal central band of receptor clusters, but no clusters near their tendon ends. By d19 clusters were scattered along the whole length of muscle fibres. EJ-clusters first appeared near the tendon ends of muscle fibres, leaving a clear area between EJ-clusters and the central band of J-clusters (figure 13). Fibres in the widest part of the diaphragm muscles, at the level of the nerve entry, were the first to acquire EJ-clusters, and these appeared within a day if toxin was injected at d18. Two days after injection EJ-clusters were present across the whole width of the muscle, although the clear area was maintained. By d21 EJ-clusters were scattered throughout the clear area as well, although it was still marked by a relatively lower

density of clusters (figure 13). Examination of single fibres showed that multiple EJ-clusters were scattered along individual muscle cells (figure 12 and figure 16, plate 7). Not all muscle fibres possessed clusters, despite having high levels of dispersed receptors, and clusters were not necessarily uniformly spread along the length of individual fibres.

The mean length of EJ-clusters increased with time. When they first appeared they were smaller than early J-clusters, and it is not clear whether they had a minimum size. One day

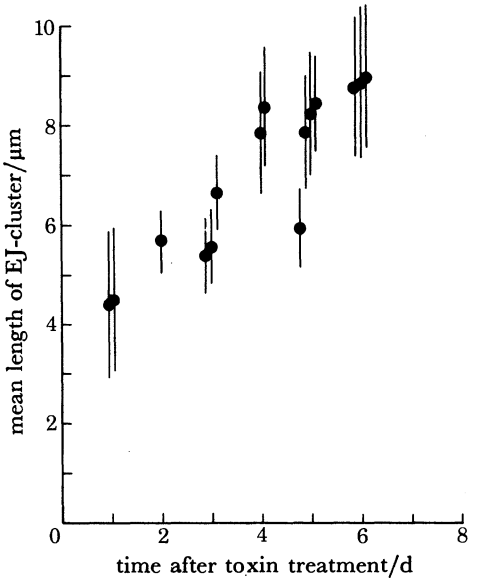

FIGURE 17. Mean lengths (± s.d.) of EJ-clusters in β-BTX-treated muscles, 1–6 d after injection of toxin.

after injection of β-BTX their mean length (probably over-estimated, owing to the limits of detection of small clusters) was 4.5 μm, increasing to 9 μm by 6 d (figures 14, 17). The density of receptors within clusters in any one muscle appeared to be constant and independent of cluster size (a fully quantitative analysis was made difficult by the lack of any way of locating clusters in the briefly exposed radioautography required for accurate grain counts).

DISCUSSION

The first in this series of papers (Harris 1981*a*) studied embryonic muscle growth in terms of histologically defined muscle units. An early stage of muscle growth continued through d16, with the formation of about 2000 primary myotubes in the left hemidiaphragm muscle. The appearance of secondary myotubes on d17, generated on the cellular framework provided by the primary myotubes, marked the time of most rapid increase in number of muscle units, with 20% of the adult number being generated in 1 d. About 70% of the adult number of units was present at birth, and it is assumed that the adult number, or close to it, of about 12000 fibres in the left hemidiaphragm, was reached by postnatal day 12. It was shown that both innervation and muscle electrical or contractile activity were essential for the development of secondary myotubes, but primary myotubes could develop autonomously. The second paper (Harris 1981*b*) added to this the observation that deposits of cholinesterase developed on primary myotubes at the appropriate position and time for endplate formation even in the

absence of innervation, indicating that in the normal course of events the cellular framework provided by primary myotubes includes instructions for locating endplates. This paper adds the further observation that clusters of ACh receptor molecules also formed, at the time and place appropriate for endplate formation, in aneural muscles. A dependence on neural regulation for further growth of these 'J-clusters', and for maintenance of their position in a narrow band across the midline of the muscles, was demonstrated. Measurements of the size distribution of J-clusters were correlated with muscle unit growth curves to show that appearance of new J-clusters is linked with the generation of new secondary myotubes. These quantitative data on skeletal muscle growth are correlated with data on development of synaptic function (Dennis *et al.* 1981) and calculations are presented below giving a measure of the time course of development of the total number of synaptic terminals in the muscles.

Receptor clusters

Development of J-*clusters*

J-clusters were first seen at d15½. Their time of appearance was synchronized, and their positions within the muscle were highly ordered and restricted to a band about one-third of the width of the rather diffuse early innervation. Clusters had a minimum size, and receptors within clusters were less dense at early times than later in development (quantified by Bevan & Steinbach (1977)). The initial formation of clusters was autonomous, but their growth required the presence, but not the activity, of innervation. If labelled αNT was injected into embryos at d17 and clusters were examined at d21 it was found that the d17 dimensions had been maintained, indicating that individual receptors were not mobile within a cluster. The use of *Naja* toxin, which binds reversibly to ACh receptors, precluded accurate measures of turnover.

Individual J-clusters had an initial length of about 10 μm. From d16 onwards the mean length of J-clusters steadily increased, reaching a plateau of about 20 μm after two postnatal weeks. The continued increase in the mean reflected a progressive increase in the population of larger J-clusters as pre-existing endplates grew larger and new small clusters were added at a diminishing rate. The existence of the temporary plateau at 20 μm mean length suggests that individual J-clusters grew to this length and then remained stable until the end of the third postnatal week, when growth was resumed. The time required for clusters to grow from 10 to 20 μm length can be roughly calculated by determining the first day at which the absolute number of J-clusters > 20 μm long was equal to the number of units in a d16 muscle. This occurred at postnatal day 1 (figure 3; Harris 1981*a*, tbl. 1), indicating that 6 d was the minimum time required. The smallest size class of J-clusters was no longer present by postnatal day 15 (figure 4*a*), indicating that by this time no further addition of new fibres was taking place. It seems likely that the plateau in the growth curve (2–3 weeks postnatally) marks the time at which the muscle achieved its mature complement of muscle fibres. For example, Rosenthal & Taraskevich (1977) found that withdrawal of multiple innervation within the rat diaphragm was complete by postnatal day 14, and that this withdrawal left bare postsynaptic receptor clusters. The plateau may mark a period during which these 'bare' areas become reoccupied. The resumption of J-cluster growth 3 weeks postnatally shows an interesting coincidence in timing with the ability to regenerate endplates following neonatal denervation (Dennis & Harris 1980).

Development of EJ-*clusters*

EJ-clusters were clearly distinguishable in β-BTX- and TTX-treated muscles at d19; they were not distinguishable in treated muscles examined on d17, although in β-BTX-treated muscles the band of J-clusters was wider than normal at that time. EJ-clusters were present 1 day after injection of β-BTX at d18. Disaggregates of d21 muscles, injected with β-BTX at d17, had a proportion of fibres without EJ clusters, although all fibres had similar densities of dispersed ACh receptors. The timing indicates that EJ-clusters appeared only after secondary myotubes should have begun to form (Kelly & Zacks 1969*a*; Harris 1981*a*). As EJ-clusters were seen on muscles injected with β-BTX at early times, so that secondary myotubes were not formed, they must be presumed also to form on primary myotubes at d18 and later.

Muscles injected *in vivo* with [^{125}I]αNT, which as well as labelling ACh receptors caused paralysis, had areas of EJ-clusters 1 or 2 d later, indicating that they had formed by aggregation of dispersed receptors. The density of receptors within clusters appeared to be independent of their size. The mean length of EJ-clusters progressively increased with time, and clusters did not appear to have a minimum dimension. A plot of the square of cluster lengths (proportional to area) against time, was fitted by a regression line that extrapolated close to zero area at zero time (not shown). All these data indicate that EJ-clusters formed by aggregation of dispersed ACh receptors that were mobile in the cell membrane, as seen in cultured muscle cells by Poo *et al.* (1978). Further evidence for this mobility was given by the uniform distribution of label along muscle fibres that had doubled in length since labelling *in vivo* 4 d previously, and the dissolution of EJ-clusters following *in vivo* labelling of aneural muscles. Recovery from paralysis caused total removal of all EJ-clusters within a 2 d period. EJ-clusters have been described on denervated adult muscle fibres by Ko *et al.* (1977).

Regulation of the pattern of muscle innervation

J-clusters and ChE deposits appeared in normal, aneural and paralysed muscles at the same times and with the same patterns of distribution. No differences between control and experimental muscles were noted in any facet of development until d17.

J-clusters when first seen extended across the whole width of a muscle. Examination of several litters of animals during d15 revealed that clusters either were present or were not; there was no evidence for progressive development from the centre outwards. In confirmation of Bennett & Pettigrew (1974), nerves at this time were seen to be spread over a band several hundreds of micrometres wide, about three times wider than the band of J-clusters. These authors, however, proposed that the diaphragm muscle grows by addition of new fibres at its dorsal and ventral edges, where nerve sprouts could be seen extending along the whole length of myofibres. They suggested that nerve sprouts define the sites of endplate formation at random points along these fibres. The present study does not support their hypothesis. The diaphragm muscle does not grow in the way that they propose; instead, new fibres develop throughout the muscle. A critical part of their evidence was a 300 μm scatter in the position of endplates in more mature muscles, corresponding to the length of the myotubes when the junctions first formed. The scatter in position of J-clusters in young muscles is one-third of this amount. Their theory does not account for the ordered appearance of J-clusters and ChE deposits in aneural muscles. The evidence presented here better supports the view that determination of the pattern of innervation is intrinsic to developing muscle, which directs the

nerve where to form junctions. The relationship between axon terminals and J-clusters in d15 muscles is not known (I confirmed the observation by Bennett & Pettigrew (1974) that nerve sprouts could extend along much of the length of an individual muscle fibre at this time). Dennis *et al.* (1981) describe electrophysiological muscle units in d15 muscles that were not innervated. Cholinesterase staining in d16 muscles (the stain reveals nerve fibres, glial sheaths, and endplate AChE) showed clearly that axon branches terminated at sites of junctional AChE deposits (Harris 1981*b*). The similarity between the pattern of distribution of ChE deposits and of J-clusters, and the known coincidence of their positions in mature muscles, strongly suggest that they occur together in d16 muscles. Thus J-clusters are contacted by nerve terminals at least as early as d16, less than 24 h after first formation of clusters.

Paralysed muscles developed large numbers of EJ-clusters scattered along the length of muscle fibres, with particularly high densities toward the ends of the fibres. These were first seen on d19, 2 d after the major period of generation of secondary myotubes and (presumably) of innervation of new J-clusters. Silver staining demonstrated a normal pattern of intramuscular innervation, and revealed no innervation in the vicinity of EJ-clusters. Thus there was no evidence that ACh receptor aggregates, *per se*, can induce embryonic motor axons to sprout and grow towards them. (Evidence was presented that receptors are not immobilized in EJ-clusters, suggesting that these may not be stable, but even so, if high densities of ACh receptors could induce embryonic axons to sprout and grow to them from a distance, this should have occurred.) It is not known whether nerve sprouts were still growing in embryonic muscles during the period that EJ-clusters were present. It has recently been shown, on the basis of partial denervation experiments, that sprouting from intact motor axons is not induced by the presence of denervated J-clusters in neonates (Betz *et al.* 1980; Dennis & Harris 1980). The experiment does not, therefore, fully answer the question as to whether a growing embryonic axon, meeting any aggregate of ACh receptors, might be induced to form a synaptic terminal, as there may have been no growth of neurites in the vicinity of EJ-clusters.

Of fifteen muscles treated with TTX from d15 or d16 until d20 or d21 five had a wider than normal spread of J-clusters and ChE deposits in sections from their interiors. Muscles from embryos treated with β-BTX on d12 or d14 showed broadening of the central band of J-clusters by d17, and ChE deposits were similarly spread in aneural muscles examined on d20 or d21; thus there is no evidence for the spread in paralysed muscles being a secondary consequence of abnormal nerve sprouting. Paralysis at least until d19 was a common denominator in all these muscles, and the abnormal distribution is probably related to distortions in growth of the tissue. Restriction of the distribution of endplates in normal muscles would then be the consequence of a normal pattern of muscle growth (as suggested by Bennett & Pettigrew (1974)) and not directed by innervation.

Sanes *et al.* (1978) showed that frog muscle basal lamina became reinnervated at the site of old endplates in the absence of muscle cells, Sanes & Hall (1979) have made antibodies specific to the subsynaptic part of the muscle basal lamina, and Burden *et al.* (1979) have shown that old synaptic sites on basal lamina can induce the formation of J-clusters in regenerating muscle cells. Bader (1980) studied reinnervation in experimentally damaged regenerating rat muscles. He found that new endplates formed only if the portion of damaged muscle included a region that had previously contained endplates. As muscle fibres had broken down before regenerating, the significant component of the old endplate zone may be presumed to be the basal lamina, as in the experiments of Sanes *et al.* in frogs. It seems likely that a marker on the extracellular

connective tissue skeleton of developing muscle directs muscle cells where to form J-clusters, serves as an anchorage site for junctional AChE, and directs exploring axons within the muscle where to form their contacts. Distribution of the marker may become less orderly at the time of formation of secondary myotubes, when there are substantial changes in relation between muscle cells and basement membranes.

This suggestion is not without precedent. For example, postsynaptic structures develop in cerebellum in the absence of afferent innervation (Hirano & Dembitzer 1973; Hirokawa 1979). Also, multiply innervated nerve cells have been shown to possess a limited number of sites that can receive synapses (Gottlieb & Cowan 1972).

Innervation in relation to the pattern of muscle growth

During the course of muscle growth endplates maintain their pattern as a narrow band extending along the midline of the muscle. Two endplates that are adjacent to one another in a d15 muscle will be separated by three or four new muscle fibres in a d21 muscle; two endplates that are 1 mm apart in a d15 muscle will be 8 mm apart in a d21 muscle. Furthermore, a muscle cell nucleus that was close to an endplate in a d15 muscle could be 2.5 mm away from it by d21. The present data do not exclude the possibility of a constant relation between a particular nucleus and an endplate (Fambrough & Devreotes 1978), or nerve terminal and endplate, but they do not require it. There may be a dynamic process of terminal withdrawal and reformation as new endplates are added to the muscle. Physiological studies (Dennis *et al.* 1981) show that the quantal content of transmitter release from single terminals remains remarkably constant from d16 until postnatal day 7. In developing muscle, J-clusters and junctional AChE deposits form on the new muscle units that continue to appear for a short time after removal of the nerve. This may reflect a normal process where the postsynaptic components of an endplate develop independently of innervation, and become innervated by a terminal that has withdrawn from a neighbouring pre-existing endplate and grown out towards the new one. Similar suggestions have recently been made by Betz *et al.* (1979). Another system where a process of formation, withdrawal and reformation of synaptic contacts has been described is during the development of projections from retina to optic tectum in *Xenopus* (Gaze *et al.* 1974). The events here proposed to occur during development of innervation in skeletal muscles should be of general relevance to the understanding of synaptic development in other systems.

Muscle growth

Neural regulation

The developmental stages covered in these experiments were marked by continued rapid growth of the muscles. There are two parts to growth of the diaphragm muscle: fibres become elongated, with new nuclei being added at points all along their length; and new fibres are formed throughout the muscle (Kelly & Zacks 1969*a*; Ontell & Dunn 1978; Harris 1981*a*). The continued changes in the age distribution of muscle fibres, and in their absolute number, must be taken into account in any interpretation of the results of experiments involving developing muscles.

It was shown that one single assumption, that J-clusters < 12 μm long mark the presence of muscle units not more than 2 d old, makes it possible accurately to predict the rate of addition of new units to developing muscles. For example, both predicted and empirical growth curves show the most rapid increase in unit numbers occurring during d17 (figure 5), the time at

which secondary myotubes were first seen histologically (Harris 1981*a*). The proportion of J-clusters less than 12 μm long in experimental muscles was then measured to indicate the effects of innervation and of electrical activity on the development of endplates in normal muscles.

In the preceding paper (Harris 1981*a*) it was shown that both denervation and paralysis inhibit the production of new muscle units, assayed histologically. Paralysed muscles possess a lower proportion of small (< 12 μm) J-clusters than do normal muscles (figures 7, 8). This is evidence that, following paralysis, new J-clusters fail to appear. Conversely, muscles that had been paralysed and then recovered had a higher than normal proportion of small clusters. This is evidence that development of new J-clusters was temporarily suppressed and then a large number appeared synchronously when muscle electrical activity was restored.

The precise time relation between appearance of a new, histologically defined, muscle unit and the generation of a new J-cluster cannot be determined at present, but is certainly less than 2 d. In some muscles kept paralysed from d16 to d20 the proportion of small J-clusters was close to zero. This shows that the partial suppression of new unit development was due to secondary myotube generation being halted for some period, rather than its being slowed throughout the period of analysis. The initiation of secondary myotube formation near the midpoint of primary myotubes may be explained by its dependence on innervation. Nerve terminals are available only in this region of the muscle; initiation of secondary myotube formation elsewhere could not be supported.

Number of nerve terminals cf. *number of muscle fibres*

Kelly & Zacks (1969*a*) proposed that 'secondary' myotubes develop on a framework of 'primary' myotubes. Their nomenclature was endorsed by Ontell & Dunn (1978), who extended their observations by making serial reconstructions of electron micrographs of neonatal rat extensor digitorum longus muscle, as schematized in figure 18. They describe primary myotubes as extending from tendon to tendon and being enclosed in basal lamina. Secondary myotubes begin to form near the midpoint of primary myotubes, beneath the basal lamina. A primary myotube with secondary myotubes within one basal lamina is named a 'muscle unit' and appears as one cell in light microscopy. Secondary myotubes split off longitudinally and formation of the new muscle fibre is defined by the acquisition of its own basal lamina.

Units defined by electrophysiology appear to include a primary myotube, at least at early times. Dennis *et al.* (1981) injected Lucifer Yellow into units in embryonic rat intercostal muscles over a range of gestational ages, and found that labelled cells always extended from tendon to tendon. Dye sometimes diffused into neighbouring cells but to a lesser extent than expected from measurements of electrical coupling. It is possible that an electrophysiological unit corresponds to a histological unit, i.e. it may consist of more than one cell contained within a single basal lamina.

Embryonic muscle innervation was studied by Kelly & Zacks (1969*b*), who observed bundles of small axons that from d16 onwards made contacts with myotubes. They could not say whether two axons seen in cross section were branches of a single parent axon. Ontell (1977) observed that both primary and secondary myotubes within one muscle unit could have endplates, and (Ontell 1979) suggested that the apparent polyneuronal innervation of electrophysiologically or light microscopically defined units (Redfern 1970) might actually represent the coupling together of a number of singly innervated myotubes within one muscle unit.

This hypothesis can be tested in several ways. First, polyneuronal innervation should not

be seen before secondary myotubes begin to form. Dennis *et al.* (1981) found multiply innervated electrophysiological units from d15 onwards, which does not support the hypothesis. However, electrical coupling between neighbouring myotubes was powerful at that time and the criterion of a rapid rate of rise of the endplate potential may not have been adequate to discriminate between inputs to two closely coupled cells.

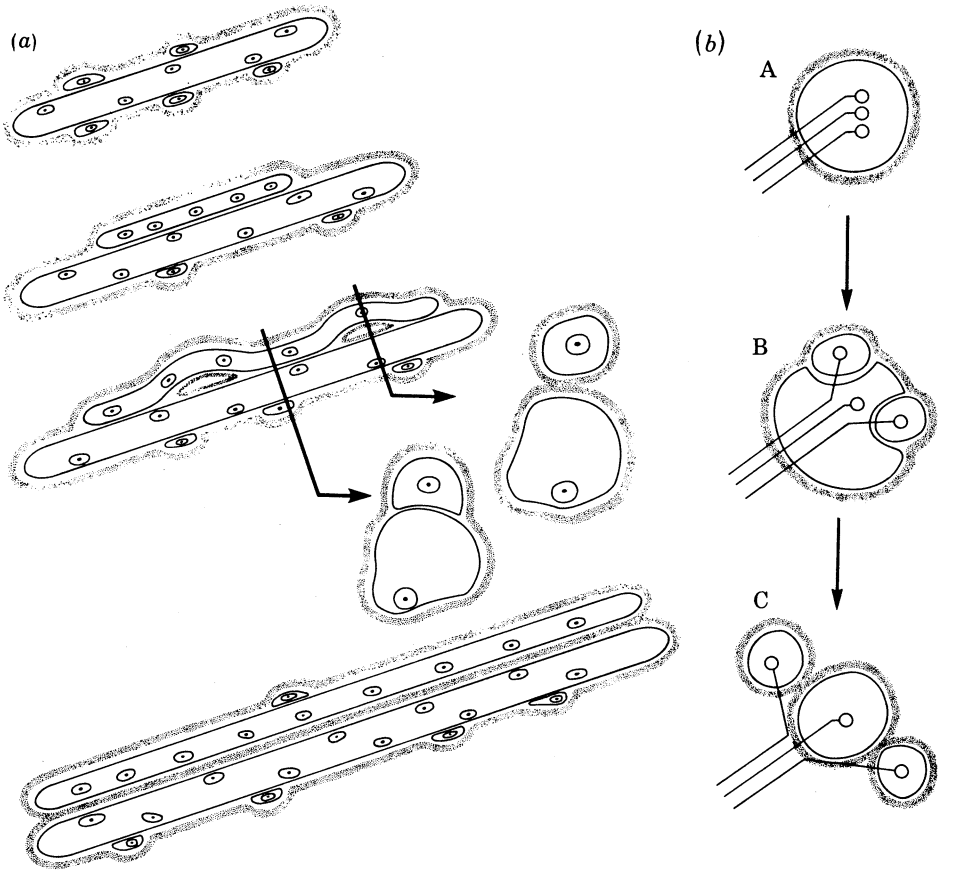

FIGURE 18. (*a*) Schematic illustration of the development of primary and secondary myotubes (after Ontell & Dunn 1978). Primary myotubes extend from tendon to tendon. Secondary myotubes form near their mid-points, within the same basal lamina, and grow longitudinally. Formation of a new muscle cell is defined by the secondary myotube acquiring its own basal lamina, and separating from the cluster. During the process of separation light microscope sections at different levels may resolve the same unit as including either one or two cells. (*b*) Hypothetical relations between nerve terminals, muscle cells and muscle units. If state B was assayed as one unit electrophysiologically and three units histologically, then multiplying the number of units by the average number of synaptic inputs would over-estimate the total number of terminals in the muscle.

A second, more complex prediction is as follows. Assume (i) that light microscope and electrophysiological muscle units correspond, and (ii) that there are no surplus nerve terminals within the muscle that later degenerate. Then, if at any time the number of histological units is multiplied by the average number of synaptic inputs per electrophysiological unit, the product will give the total number of synaptic terminals in the muscle and this should at no time be greater than the number of endplates in an adult muscle.

The results of this calculation are presented in figure 19. Data for the mean number of

synaptic inputs per unit are from intercostal muscles (Dennis *et al.* 1981); data for muscle unit numbers are from the diaphragm (Harris 1981*a*). The number of terminals calculated in this way reaches a peak of > 170 % of the adult number at d19–d20 gestation, and then declines. Calculations that assumed the relationship between electrophysiological and histological units to be 1 or 2 d displaced in time still generated a surplus of terminals. This result does not support the postulate being tested. Nor does it support Ontell's (1979) suggestion that there may never be more than one synaptic input to a single cell. It does, however, rest on the unproved assumption that histological and electrophysiological units are the same. This point is illustrated in figure 18*b*. Suppose that the adult number of nerve terminals is formed before the adult

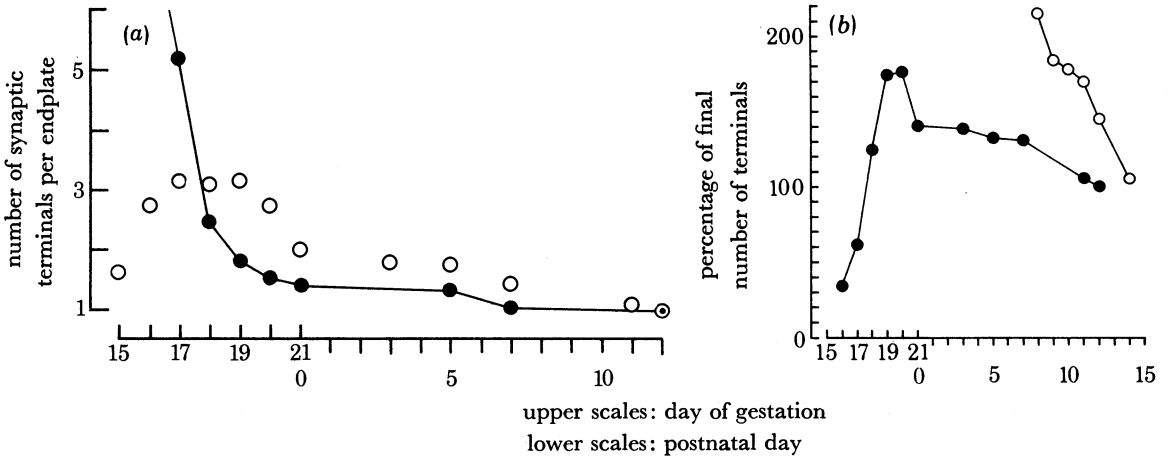

FIGURE 19. Calculation of the total number of synaptic terminals in a muscle during development. (*a*) Symbols: ○, mean number of synaptic inputs per muscle unit (electrophysiological data from Dennis *et al.* (1981); internal intercostal muscles); ●, predicted number of inputs per unit, assuming that the adult number of terminals was present in the muscle at all times. (*b*) Total number of terminals (as a percentage of the adult number) calculated as the product of average number of inputs per unit (electrophysiological) and the number of units (histological, from Harris (1981*a*)): ●, data for electrophysiological units from Dennis *et al.* (1981); ○, data from Rosenthal & Taraskevich (1977).

number of muscle fibres, as in A→B→C, and suppose that state B is reached at d19. Then if histological studies described three units, and electrophysiology one unit with three inputs, the calculation would give the erroneous conclusion of there being nine terminals in the muscle, as compared with three at A and C. This may well explain the peak at d19–d20, but is less able to explain the conclusion that 140 % of the adult number of terminals is maintained during the first postnatal week, or the finding (Rosenthal & Taraskevich 1977) that rat diaphragm muscle fibres still receive an average of two synaptic inputs each at the end of the first postnatal week. The data of Rosenthal & Taraskevich (also plotted in figure 19) predict an even greater surplus of terminals than do the results of the study by Dennis *et al.* (1981).

Taken at face value, the results of the calculations presented in figure 19 suggest that there are two episodes of synapse elimination during development of the rat diaphragm. The first, from d19–d21, may be correlated with elimination of 'mistakes' in innervation, which occurs at that time only, and not at any later period (Dennis & Harris 1979; Dennis *et al.* 1981). The second episode occurs at some time during the second postnatal week, and is correlated with achievement of the adult state of one synaptic input per muscle fibre at that time, which is

accompanied by exposure of 'bare' ACh receptors on the muscle surface (Rosenthal & Taraskevich 1977), and a hiatus in the growth of endplate ACh receptor clusters (figure 1). Betz *et al.* (1979) measured twitch tensions in neonatal rat lumbrical muscles and concluded that the number of muscle fibres innervated by a motoneuron at birth was about 140 % of the adult value, and that this number remained constant for the first ten postnatal days and then decreased to the adult level. Their figures, derived from an independent set of experiments and from a different muscle in the rat, are very similar to those calculated here.

In conclusion, innervation is required to support development of the proper number of skeletal muscle fibres. Whether innervation regulates this number is still undetermined. The following facts can be regarded as established. (i) There is a genetically determined limit to the number of muscle fibres in a muscle (Hooper 1978). (ii) Motoneurons cannot generate new synaptic terminals following damage in the early postnatal period (Betz *et al.* 1980; Dennis & Harris 1980); therefore there is an upper limit to the number of nerve terminals that can be present during this period. (iii) A new secondary myotube cannot be generated unless there is a nerve terminal available for it (Betz *et al.* 1980; Harris 1981 *a*). (iv) There may be a 40 % excess in the number of nerve terminals compared with the number of muscle fibres in a muscle at the time that development of new muscle fibres stops.

Betz *et al.* (1979) make the attractive suggestion that generation of a new muscle fibre requires contact with *two* synaptic terminals; hence motor nerves *are* responsible for regulating the number of fibres in a muscle. It then is necessary to know how the number of motoneurons is regulated, and how the number of synaptic terminals that may form is controlled.

Trophic actions of innervation

Comparison of the effects of β-BTX *and* TTX

Although many or all of the effects of denervating an adult muscle may be reversed by an appropriate pattern of direct electrical stimulation (Lømo & Westgaard 1975), nerves do have some actions that are independent of electrical activity (Pestronk *et al.* 1976; Lavoie *et al.* 1977; Gilliatt *et al.* 1978). A possible explanation for the coexistence of trophic and activity-related mechanisms could be to allow the size, strength and endurance of mature muscles to reflect the extent to which they have been needed and used. It could then be argued that trophic actions should be more important during embryonic development, before there is opportunity for interaction with the external world. The present study lends little immediate support to this idea, as there were few obvious differences between aneural and paralysed muscles.

Development of muscle through the primary myotube stage was little affected by denervation or paralysis. The normal number of myotubes was generated, ChE deposits and J-clusters appeared at the normal time and in their normal positions, and the nerve-specific 16*S* form of AChE was synthesized by muscle cells. It is likely that myotubes were shorter than normal, but that both classes of experimental muscle were equally affected. The only differences were that junctional ChE deposits were more dense and better organized in paralysed than in aneural muscles, and that J-clusters increased in size with time, i.e. that there was a more normal development of the endplate in the physical presence of the nerve. Normal muscles exhibit myogenic contractions while in the primary myotube stage, and electrical coupling between myofibres is so powerful that the muscle acts as one functional unit.

Both denervation and paralysis halted the development of new secondary myotubes. Both

classes of treated muscle retained their electrical coupling, as indicated by the unitary response of isolated muscles to direct stimulation, and aneural muscles continued to exhibit myogenic contractions (unfortunately it was not possible to quantify spontaneous muscle activity in the embryo). No conclusion can be drawn as to whether an adequate amount of electrical activity alone might support normal generation of secondary myotubes. A dependence on the physical presence of innervation is required in any scheme where innervation regulates the numbers of muscle fibres. The strongest hint of this is paradoxical; muscles denervated on d16, d17 or d18 continued to produce new myotubes for a longer time than if they were paralysed (Harris 1981*a*; table 3). It is reasonable to assume that myogenic activity was not reduced by denervation, but it supported the generation of new myotubes for only 1½ d, indicating that a nerve-induced signal remained in the muscle for that time. Neurogenic activation of muscle was very effective in promoting growth, as the slightest signs of recovery from paralysis marked the presence of nearly normal numbers of myofibres. Inactive nerve terminals regulated the growth of J-clusters, partially regulated EJ-clusters (they were removed from regions close to innervated junctions), enhanced the development of junctional ChE deposits and suppressed the appearance of EJ ChE deposits, and partially regulated the density of dispersed extra-junctional ACh receptors (data not given: sets of muscles treated and processed in parallel gave relative densities of 1:2:4 in control, TTX- and β-BTX-treated muscles respectively).

It is concluded that normal development of skeletal muscle depends absolutely on electrical activity, and this must be neurogenic once the secondary myotube stage of development is reached. Additional trophic actions can be defined, but their relative importance is much the same as in the adult.

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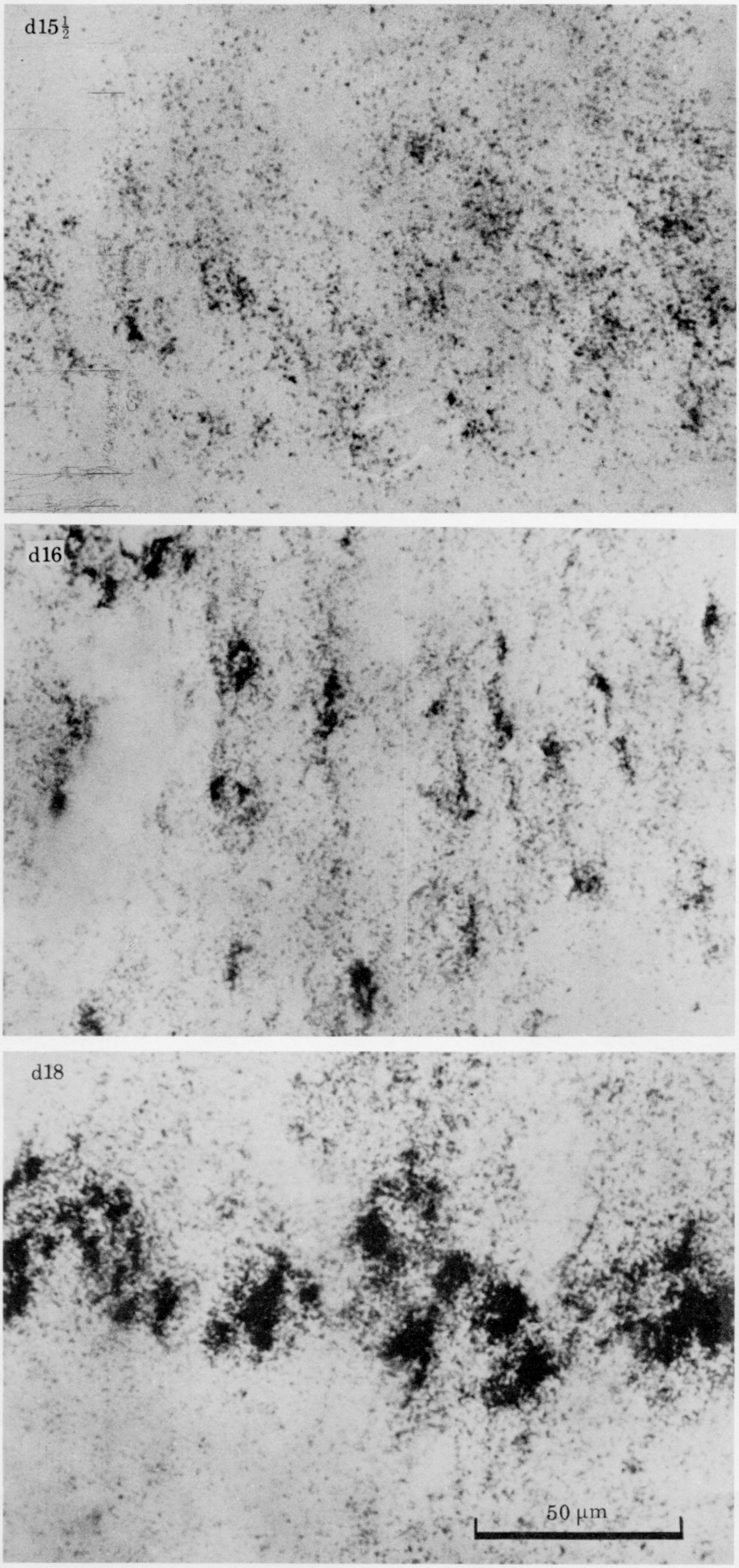


FIGURE 2. Illustrations of J-clusters in muscles of d15½, d16 and d18. The muscles were labelled and processed in parallel to allow direct comparison of grain densities.

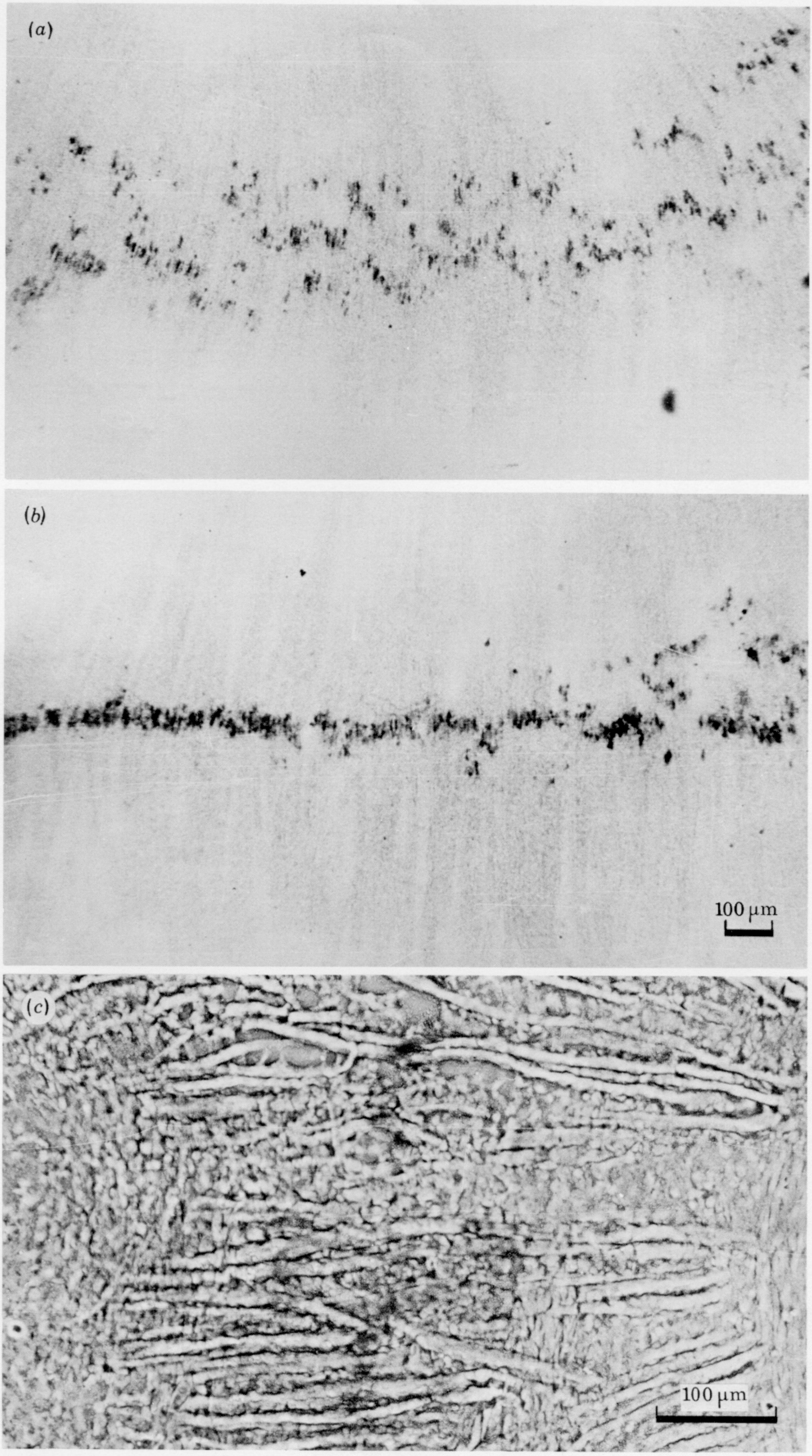


FIGURE 6. J-clusters and myotubes in muscles from embryos paralysed from d15 to d18. (*a*), (*b*) Radioautographs of J-clusters and dispersed extra-junctional ACh receptors in (*a*) right and (*b*) left hemidiaphragm. (*c*) Phase contrast photograph of unstained radioautograph.

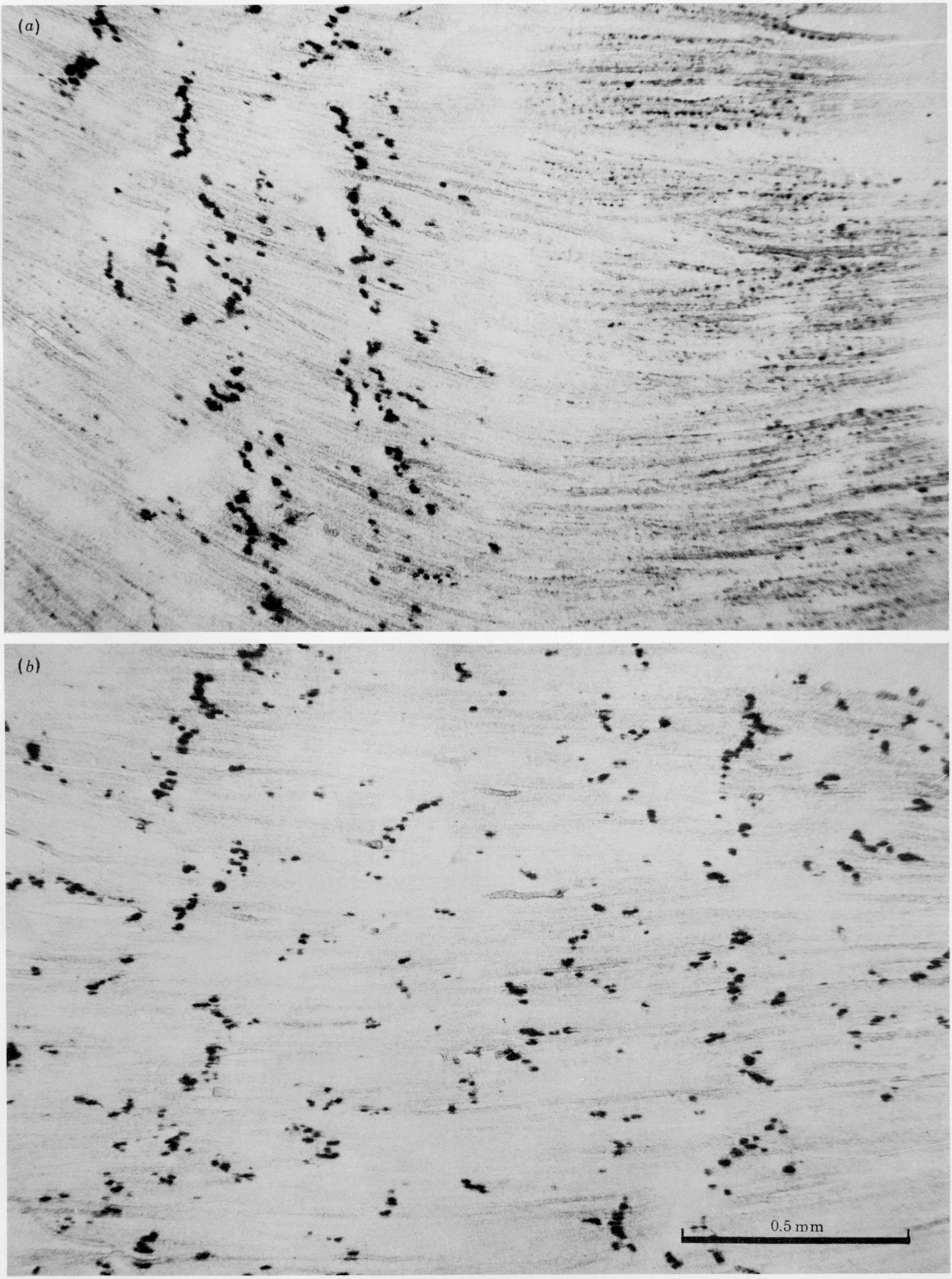
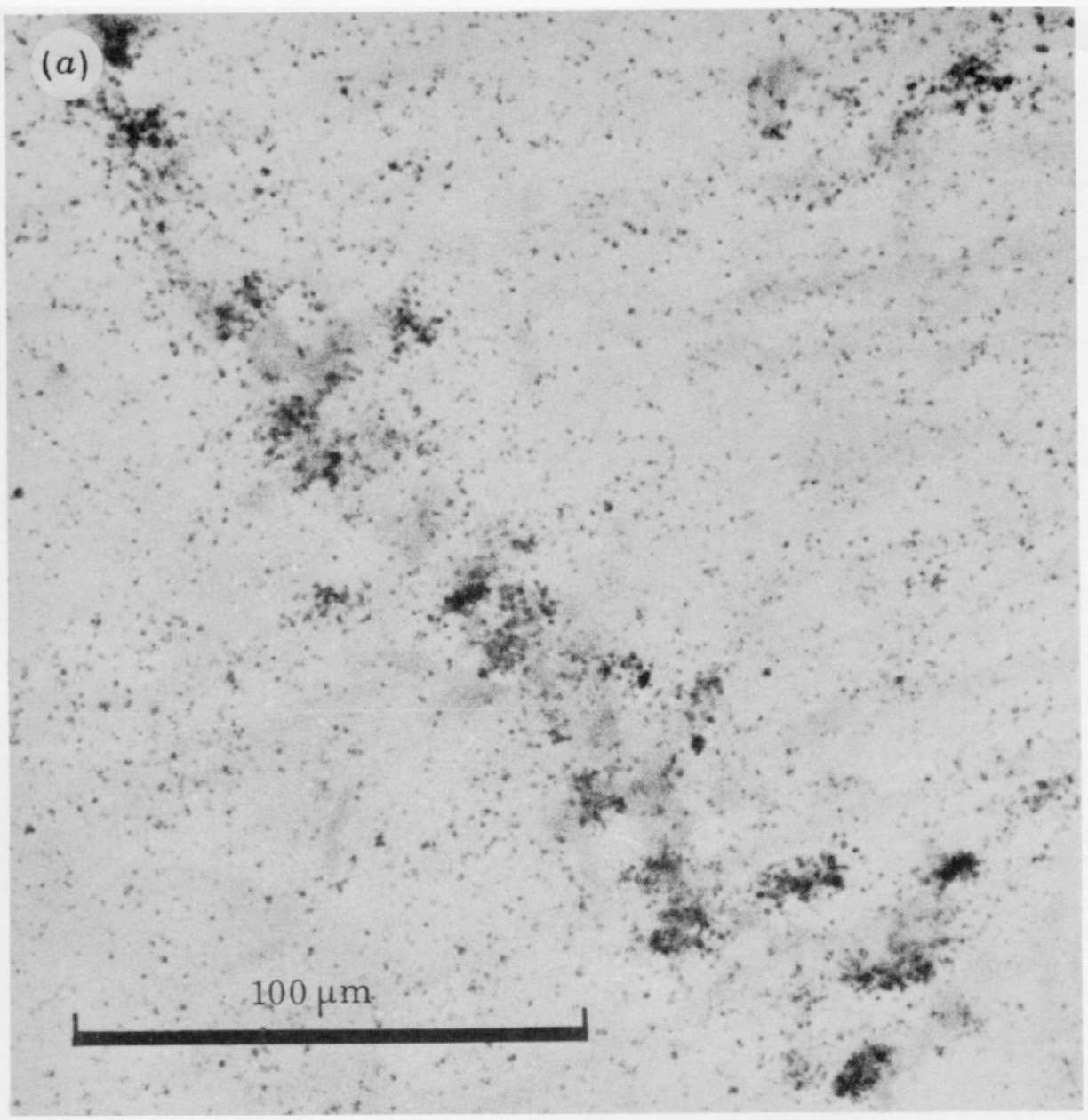


FIGURE 9. J-clusters and EJ-clusters in a muscle from a TTX-treated embryo paralysed from d16 to d21. Both sections are from the same muscle (right hemidiaphragm). (*a*) Superficial section, showing J-clusters with normal distribution, and EJ-clusters near fibre ends. (*b*) Deep section, showing unusually wide band of J-clusters (this is the widest distribution seen in any muscle).



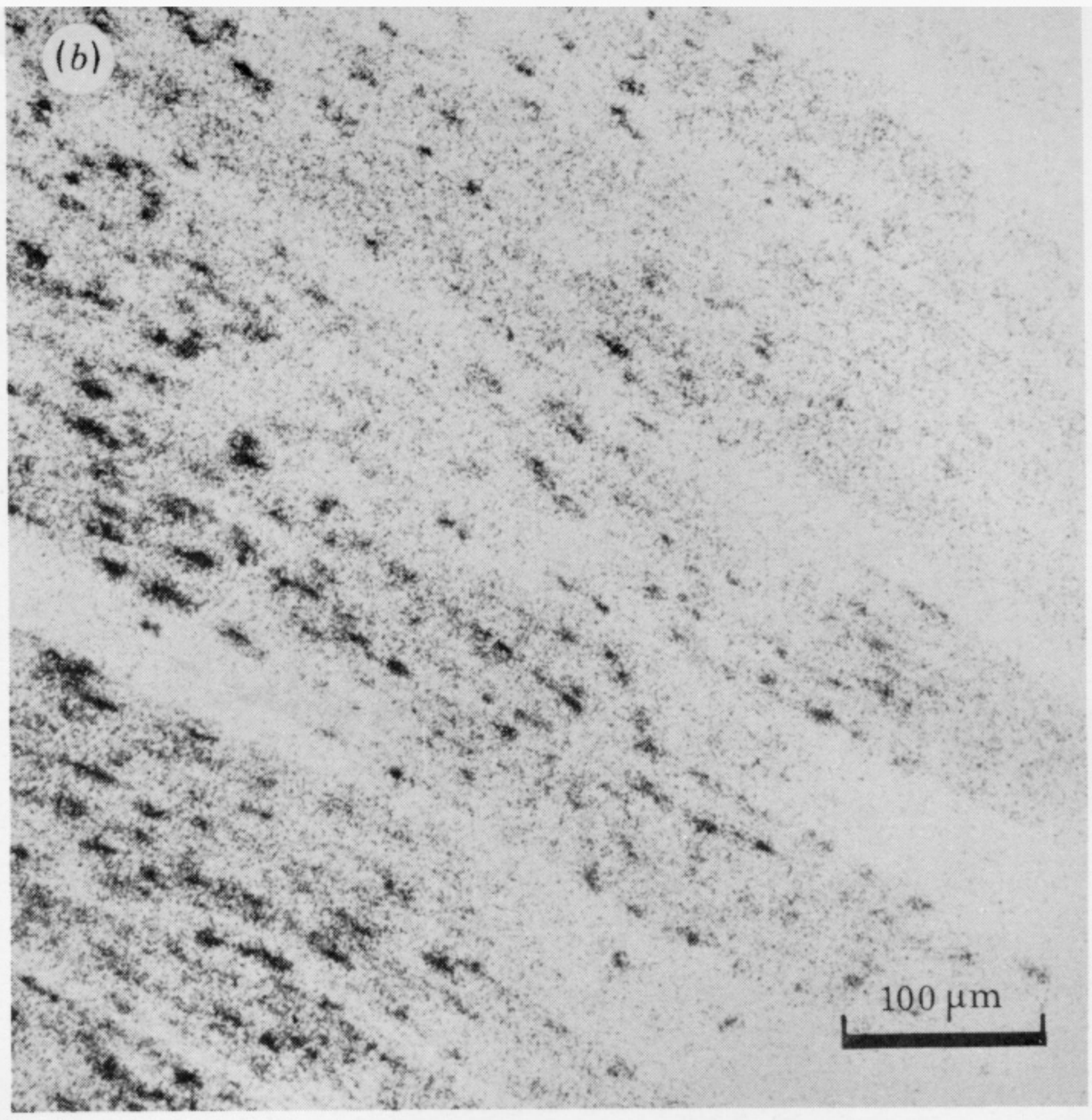


FIGURE 10. Effects of paralysis with αNT or curare. (*a*) Paralysis with [^{125}I]αNT from d16 to d18. Radioautograph stained for ChE. Note ChE deposits between labelled J-clusters, indicating that new endplates have formed since the time of labelling. (*b*) Paralysis from d17 to d19; formation of EJ-clusters. There is a band of J-clusters along the left border of the photograph, and EJ-clusters spread through the rest of the area. (*c*) Muscle paralysed with dTC from d17 to d20. Note formation of EJ-clusters.

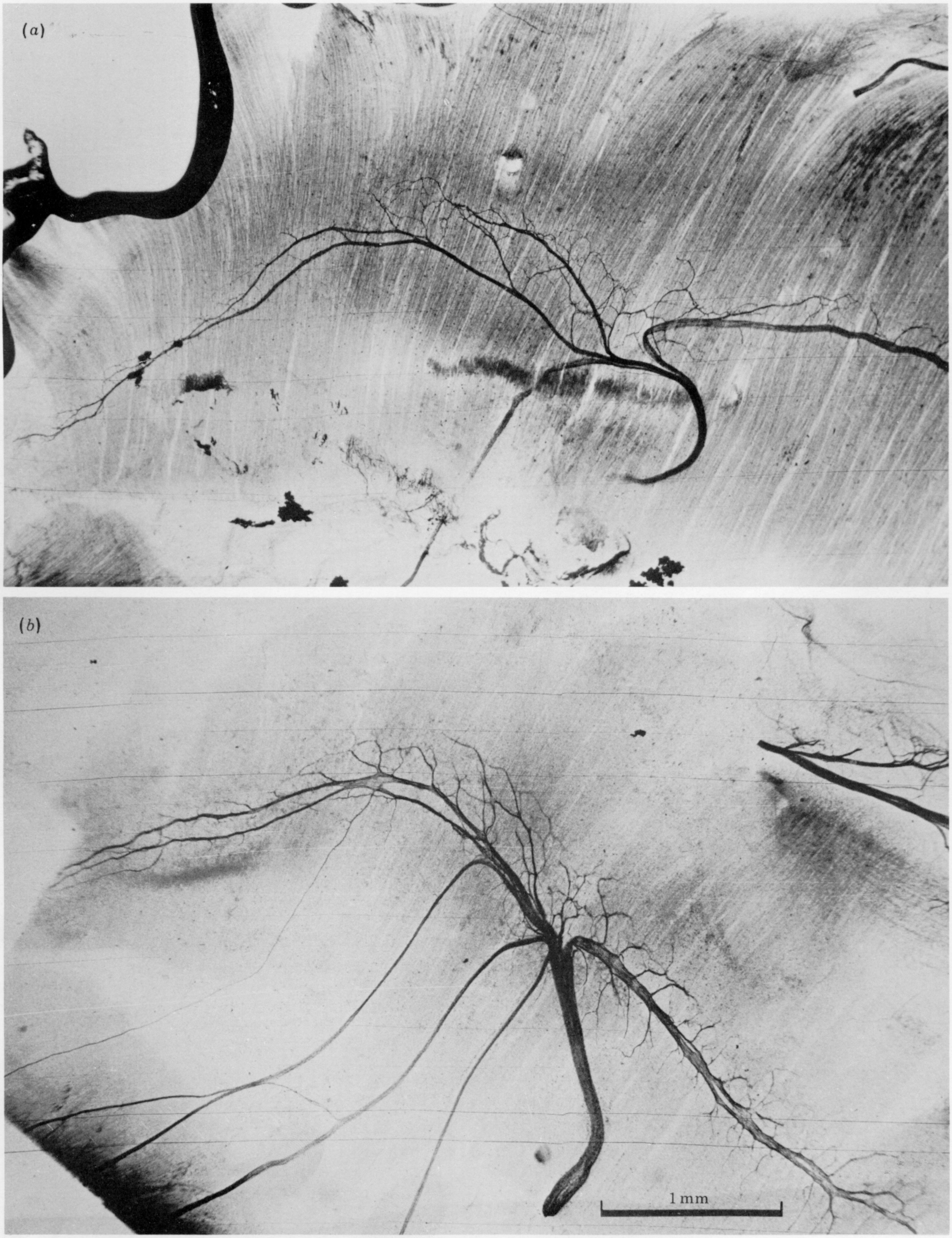


FIGURE 11*a*, *b*. For description see p. 299.

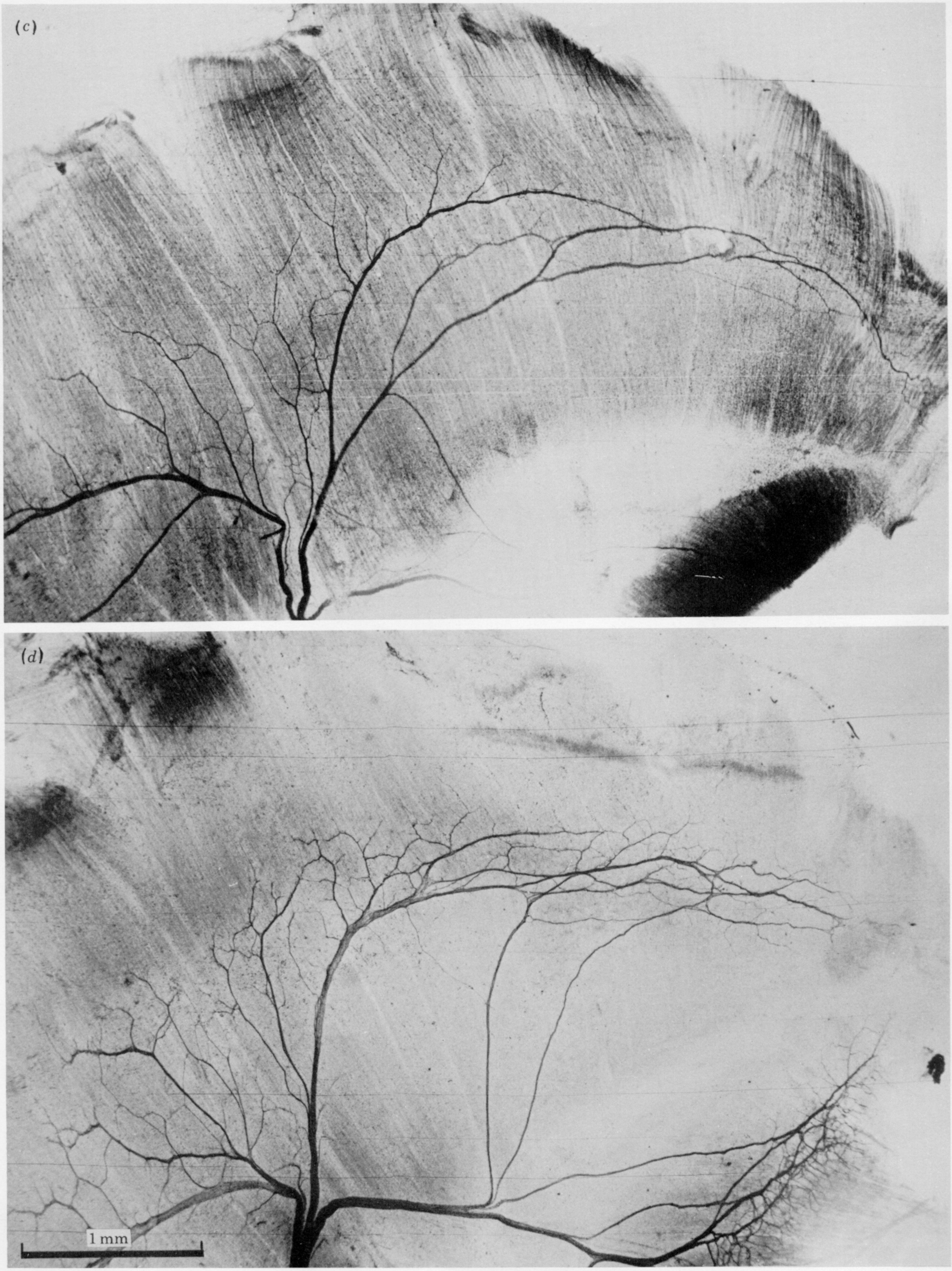
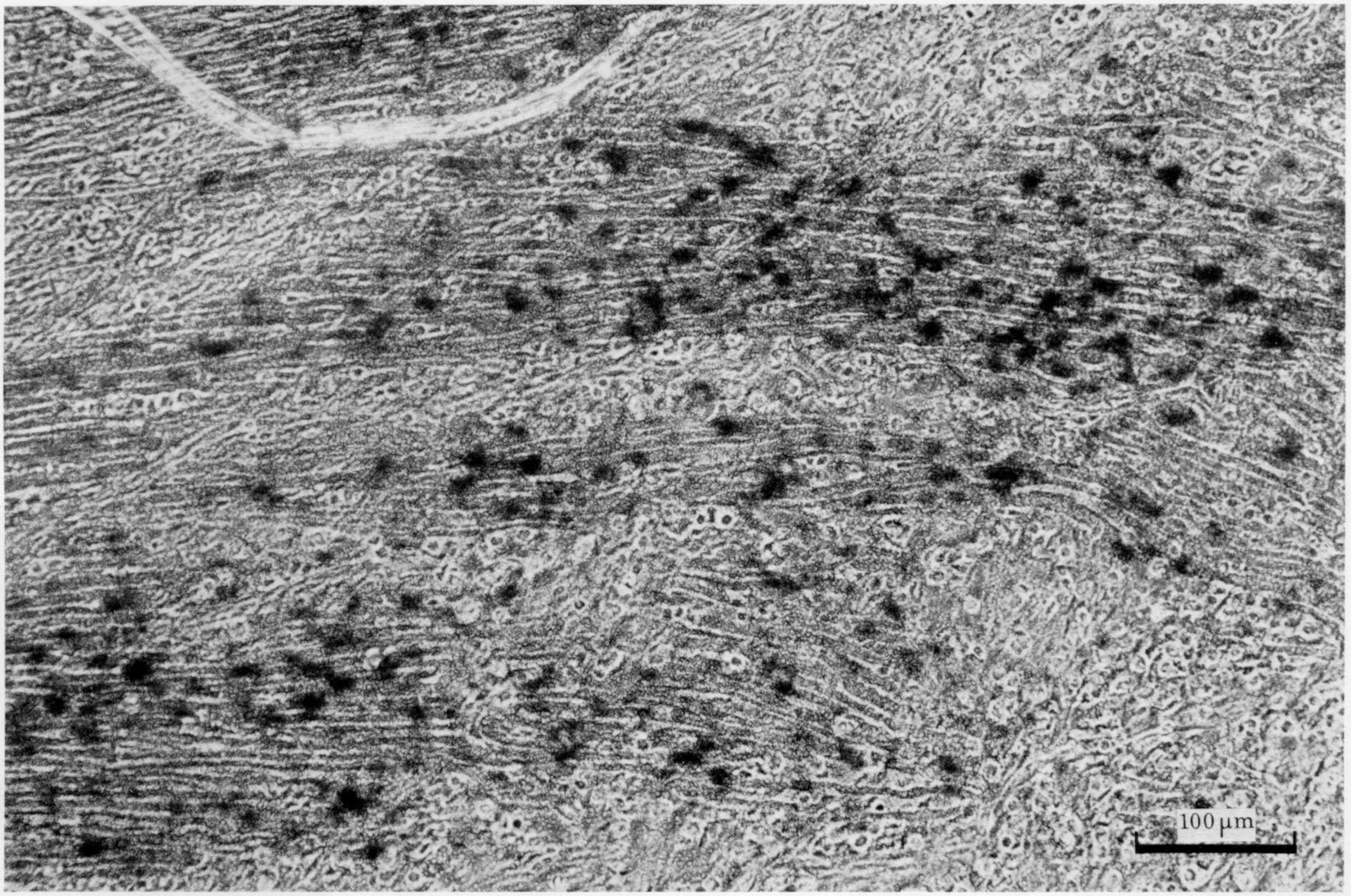


FIGURE 11*c*, *d*. For description see p. 299.



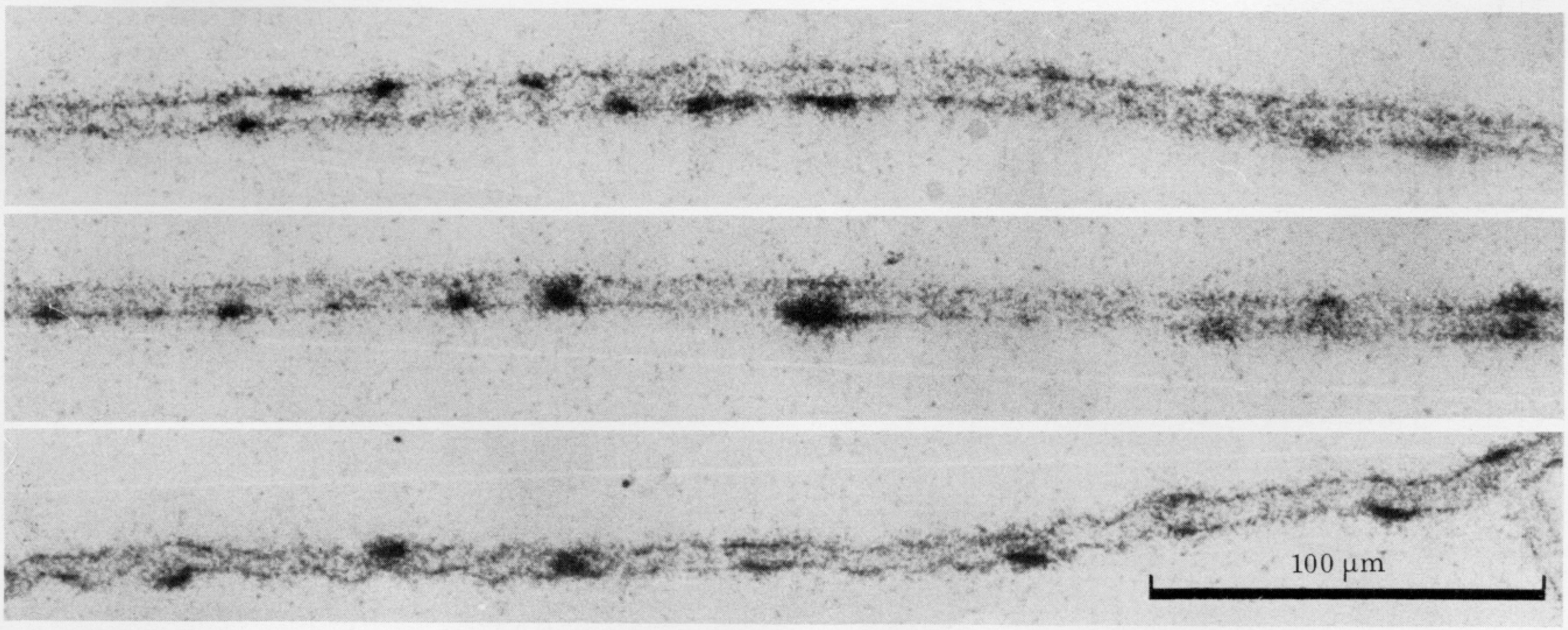
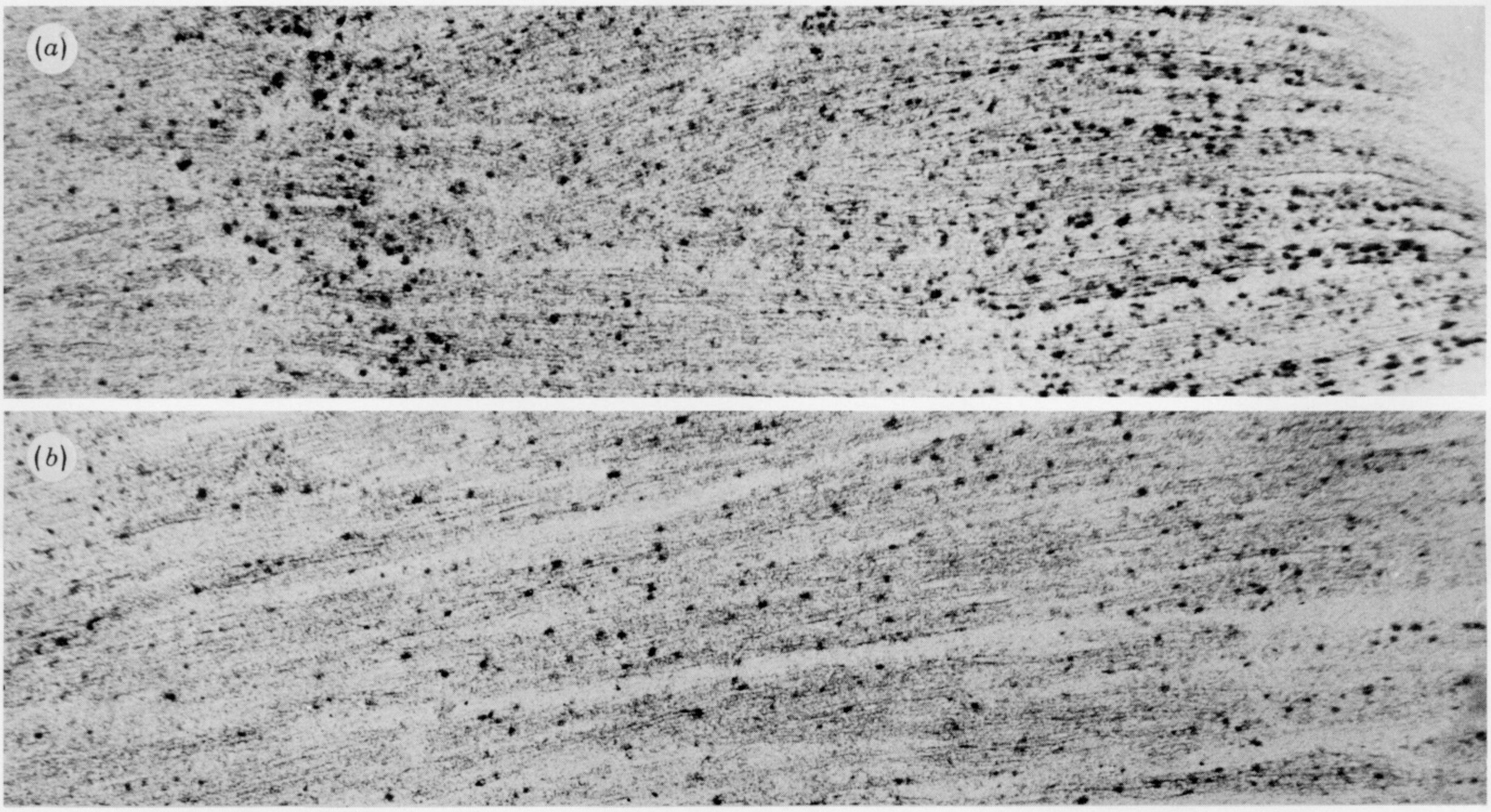
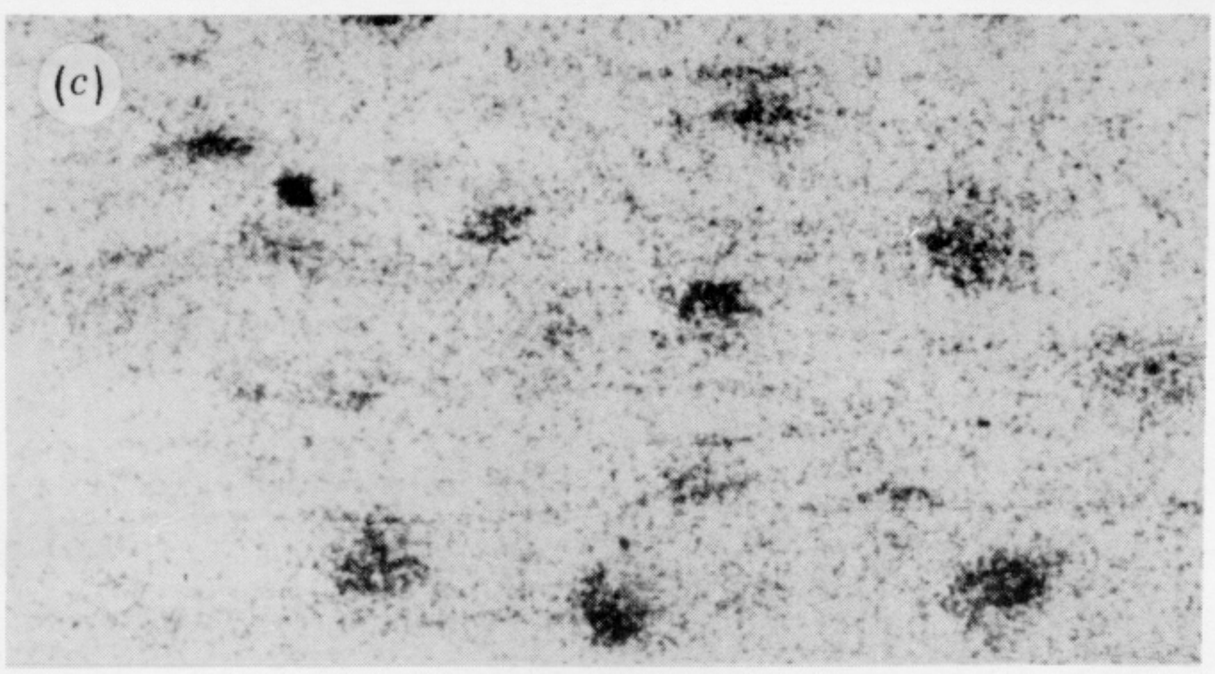
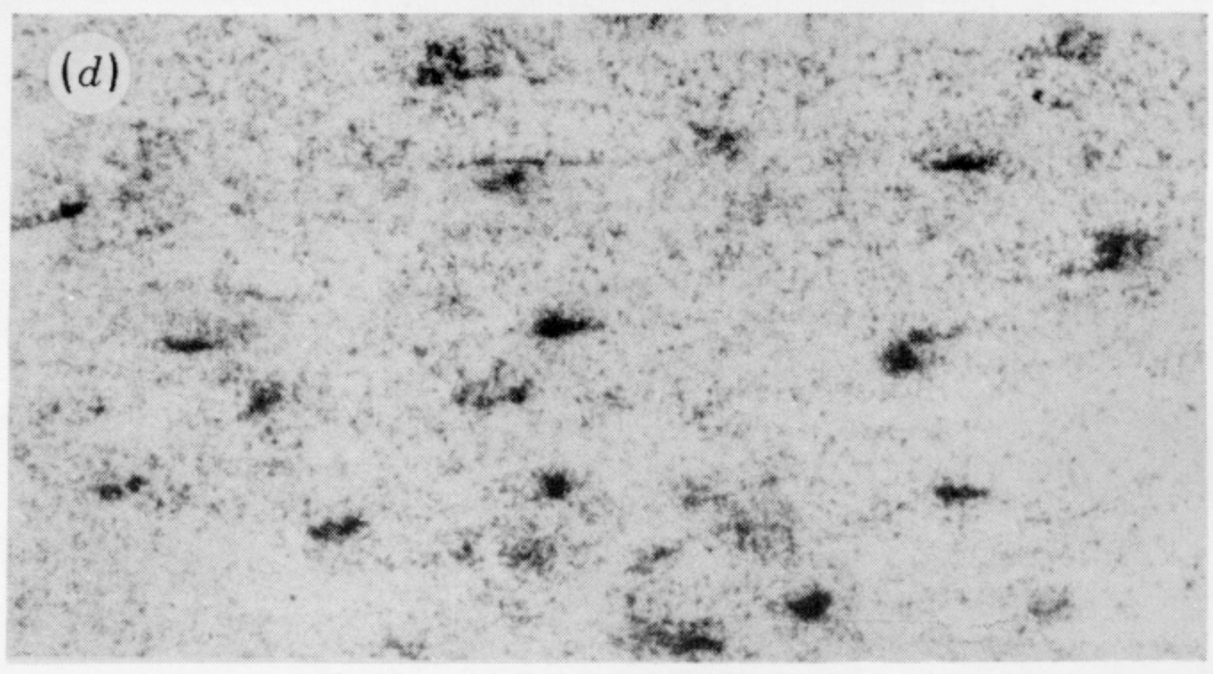


FIGURE 12. Multiple receptor clusters and areas lacking multi-nucleate myotubes in a diaphragm muscle from a d20 embryo treated with β-BTX at d15. Phase-contrast micrograph of an unstained radioautograph.

FIGURE 16. Multiple receptor clusters along the length of single β-BTX-treated muscle fibres. Embryo injected at d17 and examined on d21. Unstained radioautographs of mechanically disaggregated single fibres.







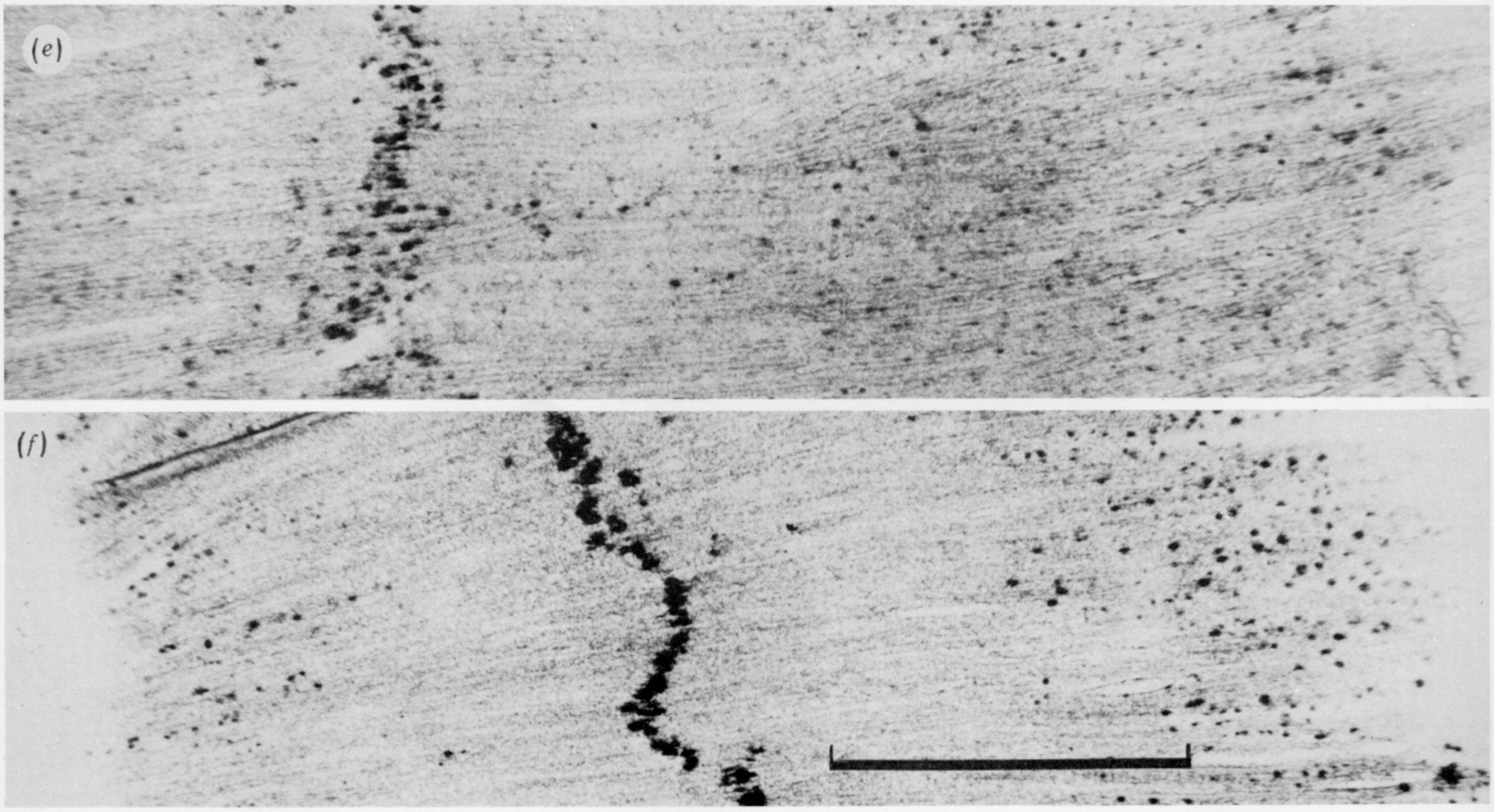


FIGURE 13. For description see opposite.