EMBRYONIC GROWTH AND INNERVATION OF RAT SKELETAL MUSCLES

I. NEURAL REGULATION OF MUSCLE FIBRE NUMBERS

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Individual rat embryos were chronically paralysed during development *in utero* by insertion of small slow-release capsules containing tetrodotoxin (TTX). In others, motoneurons were destroyed by individual injections of β-bungarotoxin (β-BTX). These embryos were compared with normal controls so that the effects of innervation and of use on embryonic development of skeletal muscles could be defined. The use of Sirius Red to stain muscle basal lamina made it possible to count muscle units (one or more myotubes contained within a single basal lamina sheath) with light microscopy.

(i) In left hemidiaphragm muscles from control embryos about 20% of the adult number of muscle units was present on day 17 of gestation, the time at which formation of secondary myotubes was first observed, and about 70% at birth. New units continued to form during the first postnatal week.

(ii) Skeletal muscles still formed following destruction of muscle innervation at day 14 of gestation, although at day 21 of gestation (birth) they were smaller and thinner than controls. The number of units in these muscles remained constant from day 18 through day 21 of gestation, and was similar to that in day 17 controls. No secondary myotubes were seen histologically.

(iii) Following destruction of motoneurons at day 16, 17½ or 18 of gestation, new muscle units continued to form for about 1½ d, indicating that the temporary presence of innervation affected later development of muscle tissue. The denervated muscles contracted spontaneously during this time.

(iv) TTX-induced paralysis from day 16 onwards was at least as effective as denervation in inhibiting the generation of new muscle units. There was a dose–response relation in the ability of TTX to reduce the generation of new muscle units beyond the amount necessary to paralyse nerves, and it is suggested that the higher concentrations also blocked myogenic contractions. High doses of TTX stopped generation of new units within half a day.

(v) Even the slightest degree of recovery from paralysis was accompanied by restoration of the proper number of muscle units. This shows that there is no 'critical period' for muscle fibre development within the last five prenatal days in the rat.

(vi) It is concluded that development of primary myotubes is autonomous, but that development of secondary myotubes, which form the majority of muscle cells in the adult, is dependent on innervation. Even when nerves are present, new muscle fibres cannot be generated in the absence of muscle contraction.

Introduction

This is the first in a series of three papers presenting the results of studies on the embryonic development of skeletal muscle and its innervation in the rat (Harris 1981*a*, *b*; and see also Dennis & Harris, 1979, 1980; Braithwaite & Harris 1979*a*, *b*; Dennis *et al.* 1981). The basic plan of the experiments was to compare muscles from rat embryos in which motoneurons were destroyed at an early stage in development ('aneural' muscles) and muscles from embryos chronically paralysed with tetrodotoxin (TTX) ('paralysed' muscles) with muscles from normal embryos. This comparison should make it possible to define the relative importance of innervation *per se*, that is the trophic effects of innervation, and of nerve or muscle electrical activity, on muscle development. This paper is principally concerned with the regulation of the number of fibres developing in skeletal muscles, while the presence and distribution of cholinesterase (ChE) deposits, and of acetylcholine (ACh) receptors, provide the principal assays for the experiments described in the following two papers.

That the number of fibres in skeletal muscles is genetically controlled has been shown by Luff & Goldspink (1970) and Hooper (1978), but the way in which this control is exerted is not

known. Development of skeletal muscles has been extensively studied: the following brief description is based on the work of Kelly & Zacks (1969*a*, *b*), Ontell (1977, 1979) and Ontell & Dunn (1978). Myotubes are formed by fusion of mononucleate myoblasts. 'Primary' myotubes extend from tendon to tendon of the developing muscle. On day 18 of gestation† 'secondary' myotubes were first seen in rat intercostal muscles. These form near the midpoint of primary myotubes, and initially are contained within the same basal lamina. There is a period of development when more than one filamented cell, as well as undifferentiated cells, may be contained within a single sheath of basal lamina, forming a multicellular 'muscle unit' which appears as a single cell in light microscopy (Ontell & Dunn 1978). Formation of a new muscle fibre from within a muscle unit is defined by its acquisition of a separate basal lamina. This process may give the appearance of longitudinal splitting of the muscle unit when seen with light microscopy. Muscle units in the present work were defined by staining basal lamina with Sirius Red, and viewing sections with light microscopy.

Innervation is an absolute prerequisite for proper development of skeletal muscles (Engel & Karpati 1968; McArdle & Sansone 1977; Betz *et al.* 1980). The determination of muscle fibre types, and consequently the speed of muscle contraction, is regulated by their innervation (Buller *et al.* 1960; Close 1969), but it is not known whether innervation is regulatory or simply permissive as far as generation of the proper number of muscle fibres is concerned.

Methods

Dated pregnant animals

All experiments were done on white Wistar rats. Mating was dated by the presence of a copulation plug, and pregnancies were timed from 9 a.m. on the morning that the copulation plug was found. Animals were checked at 5 p.m. each day to eliminate daytime matings (these were infrequent). The rat colony was maintained on a fixed light–dark cycle.

Destruction of motoneurones

Motoneurons were destroyed by injecting 1 μg of β-bungarotoxin (β-BTX) into individual embryos. Purified toxin (Boeringer) was dissolved in sterile water (1 μg/μl) and portions were kept frozen at −20 °C until required. The mother was anaesthetized with ether and the uterus was exposed via a laparotomy. Embryos were made visible by trans-illumination through the uterine wall with a fibre-optic light. By using the liver, head and limb buds as landmarks it was possible to place injections either intra-abdominally or intra-thoracically in embryos of d14 or older; the site of injection in younger embryos was less well controlled. A standard dose of 1 μg of toxin was injected by means of a microlitre syringe with hypodermic needle of 0.15 mm external diameter.

Paralysis of embryos

Embryos were paralysed *in utero* by chronic application of TTX (Sankyo) by the capillary method of Bray *et al.* (1979). One end of a 0.5 mm internal diameter glass capillary was

† Throughout this and the succeeding two papers 'day 18 of gestation' will be abbreviated to 'd18'.

constricted in a De Fonbrunne microforge to form a pore 20 μm in diameter and 100 μm in length. The capillary was then cut to the desired length, filled by capillarity through the open end with 0.03 M TTX in citrate buffer, and the open end plugged with modelling clay (Plasticine). These capillaries provided about 3 d of paralysis per 2 mm filled length; e.g. a capillary 4 mm long, with 1 mm occupied by the plasticine plug and 3 mm filled with TTX solution, would maintain paralysis for at least 4 d. Capillaries were held in forceps with a longitudinal groove at the tip and inserted into embryos through a hole in the uterine wall made with a hypodermic needle. Capillaries were fully internalized in either the thoracic or the abdominal cavity of the embryo, and did not physically interfere with normal development. A maximum of four embryos were treated in any one litter so as not to overload the mother's ability to excrete the TTX. More details of these procedures are given in Braithwaite & Harris (1979*b*) and Mills & Bray (1979).

Preparation of tissues

Pregnant rats were killed with a blow to the head so that embryos were not anaesthetized, and the embryos were removed. The effectiveness of paralysis or denervation was tested immediately by applying stimuli of 100 V × 0.5 ms at 10 Hz via bipolar platinum stimulating electrodes to the nose and head, and looking for reflex body flexion. This procedure reliably evoked responses from normal embryos of d17 or older; responses from younger embryos were less obvious and easily fatigued. The embryos were then placed on ice to anaesthetize them. The diaphragm muscle was dissected out and pinned under oxygenated bathing medium 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 1 % (by mass).

Muscle fibre numbers

Before fixation the left hemidiaphragm muscle was carefully distorted as it was pinned out, to make it possible to cut transverse sections along the midline of the whole muscle. The tissue was fixed with formaldehyde 40 g/l, sucrose 150 g/l, lightly stained with haemotoxylin, dehydrated and embedded in paraffin, and the midline of the muscle was marked on the paraffin block. Sections were cut at 2–5 μm, stained with Sirius Red F3Ba (Sweat *et al.* 1964), and permanently mounted. A section traversing the muscle across its midline, as defined by inclusion of the point of entrance of the nerve (except for aneural muscles, where the midline marked on the block served as reference), was selected for photography. Photographs of neonatal muscles were taken through a × 40 planachromat (numerical aperture (n.a.) 0.65), and of embryonic muscles through a × 63 oil immersion planapochromat (n.a. 1.4), with use of bright-field illumination and a deep green filter. Prints were made at final magnifications of × 714, × 892 or × 1125, and large photomontages were constructed; those of neonatal muscles were about 10 m long. Muscle units were counted with the aid of an electric counter that marked each unit as it was recorded.

Birth-dating muscle nuclei

Muscle fibre nuclei were birth-dated with tritiated thymidine (Amersham) by injecting 1–5 μCi (specific activity 5 or 28 Ci/mmol) into individual embryos. Label was sometimes distributed in a gradient across the diaphragm, especially at the higher specific activity, a

technical artefact which limited the usefulness of this technique. Diaphragm muscles were fixed in a freshly made solution 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. After overnight fixation tissues were thoroughly washed in several changes of buffered saline to remove fixative and prevent fading of the latent image during radioautography, and frozen sections were cut at 14 or 20 µm. For radioautography, slides were dipped in Ilford K2 emulsion and exposed at 4 °C in sealed containers, with silica gel as dessicant. After exposure for 2–6 weeks, the emulsion was developed in fresh D-19 developer (Kodak) for 2 min, and tissues were lightly stained with Harris's haemotoxylin to make nuclei visible. Slides were then dehydrated and permanently mounted.

Silver staining

The Bodian technique proved the most reliable and successful of many silver stains tried, giving good staining of fine neurites and growth cones, with little background. Tissues were fixed in Bodian fixative for 24 h, and then incubated at 37 °C for 3–5 d in two or three changes of protargol, 5 g/l, (Rocques or Schmid) in the presence of a short length of clean copper wire. Bleaching was not required. After development tissues were toned in gold chloride, 10 g/l and mounted as whole mounts. Good results could also be obtained from tissues fixed in formalin–sucrose or paraformaldehyde–glutaraldehyde, provided that the copper wire was omitted.

Spinal cord histology

Embryos were fixed by intra-cardiac perfusion with ice-cold paraformaldehyde–glutaraldehyde. The thoracic region of the spinal cord was dissected and immersed in fixative. The tissue was embedded in Epon and 1 µm sections were cut with a glass knife. Sections were stained with toluidine blue and examined with light microscopy.

Results

Paralysis with tetrodotoxin

TTX capillaries were inserted into embryos of d15–d18, and diaphragm muscles examined at various later times. Control experiments with new-born rats showed that insertion of a TTX capillary produced total paralysis in less than 5 min. Embryos of d15 were not paralysed for more than 3 d, due to limitations in the size of capillary that they could accept, so that when longer periods of paralysis were required a second capillary was inserted later. Larger embryos, from d16 onwards, could be paralysed up until the time of birth (d21) with a single capillary.

Embryos paralysed for more than 2 d were lighter than controls by up to 20 %. Width of the diaphragm muscles (i.e. spinal cord to sternum) was not affected by paralysis, but muscle length was reduced (figure 1*a*). Areas of muscles from TTX-treated, β-BTX-treated and control diaphragms are compared in figure 1*b*; from d18 onwards there is a retardation in the growth of the experimental muscles. Not only were muscle fibres shorter than controls, but embryos paralysed from d15 had some areas within their diaphragm muscle with no myotubes present between the abdominal and thoracic epimysia.

Muscles isolated from paralysed embryos and placed in an organ bath initially showed no spontaneous contractions. This is in contrast to aneural muscles (see below). After a few minutes washing they began to fibrillate vigorously. Fibrillation was accompanied by spontaneous

contractions of the whole muscle, and was maintained for several hours *in vitro*. Isolated muscles did not at first respond to electrical stimulation, but after a brief wash responses could be obtained to both direct and indirect stimulation. Direct stimulation, even of d21 muscles, produced a contraction that slowly propagated across the whole width of the muscle. This response, which reflects the presence of physiologically effective electrical coupling between muscle fibres, is normally lost at d18 or d19 (Dennis & Harris 1979; Dennis *et al* 1981).

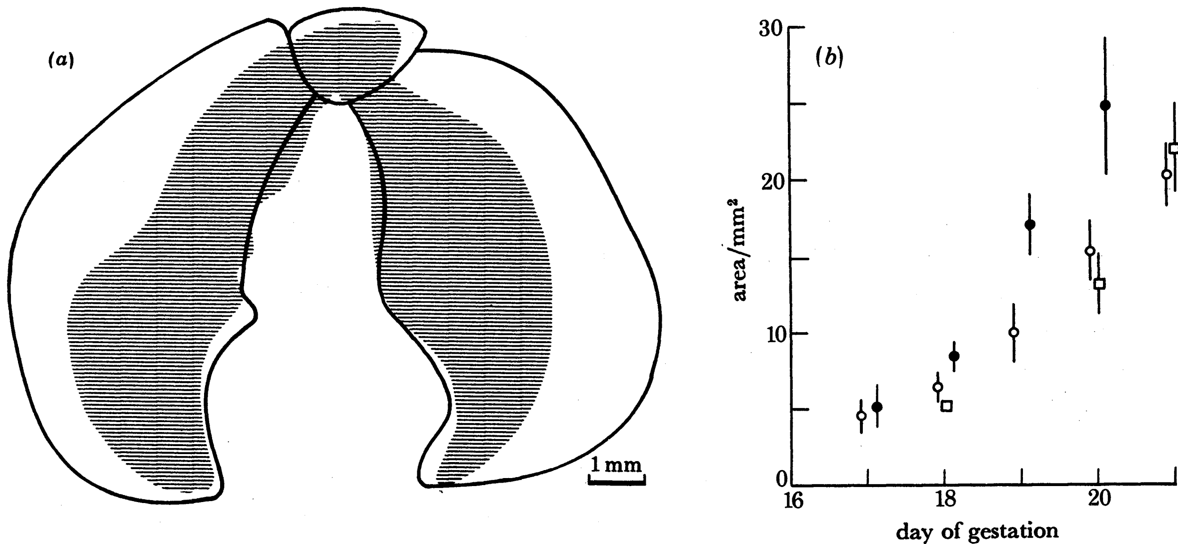

FIGURE 1. (*a*) Tracings of d16–d20 TTX-treated muscle and control. The paralysed muscle had 47% of the area of the control. (*b*) Area of diaphragm muscles in toxin-treated and control embryos of different ages. Treatment for > 3 d in each case; *n* = 4–10 for each point; error bars, ± s.d.; ●, controls; ○, β-BTX treatment; □ TTX treatment.

Development of aneural muscles

Destruction of motoneurons

Abe *et al.* (1976) showed that β-BTX destroys nerve terminals in adult muscles, and Hirokawa (1978) found that it destroys the cell bodies of certain selected classes of chick embryo neurons, including motoneurons. In a control experiment, injection of the standard dose of 1 μg of β-BTX into four 2 d old rats (mean mass 7.7 g) killed all four in less than an hour. Injection of this same dose into individual rat embryos of d14 or older appeared to cause total loss of motoneurons, as in chick embryos (Hirokawa 1978). Evidence for this observation is that embryos examined at later times were completely paralysed. They did not move spontaneously or give reflex responses to stimulation of the nose or head. Isolated muscles maintained in an organ bath contracted spontaneously. Muscle nerves could not be seen, and electrical stimulation applied to places where they would normally lie, for example the suspensory ligaments of the phrenic nerve, did not evoke contraction. Direct stimulation of the muscle was effective, but there were no points of lower threshold marking the presence of an intramuscular nerve. The contraction slowly propagated through the whole muscle, showing that electrical coupling between muscle fibres was maintained, as in the TTX-treated muscles. No synchronous contraction of muscle fibres, which would reflect the presence of innervation, was ever seen.

A group of embryos was injected with 1 μg β-BTX on d15 and their spinal cords examined on d18. Control embryos from d17, d18 and d19 were used for comparison. The ventral quadrant of the control spinal cords had a well developed marginal zone, and prominent ventral horn nuclei. Motoneuron cell bodies were easily recognized. The marginal zone in β-BTX-treated spinal cords was minimal, and sometimes absent. No groupings of cells into nuclei could be recognized, and no motoneurons could be distinguished. The cells present had very little cytoplasm surrounding their nuclei and there were lacunae between them. Without some independent test it is not possible to say that these cells did not include some extremely atrophied motoneurons.

Ventral roots could not be seen under a dissecting microscope, and ventral root fibres could not be distinguished with electron microscopy (it is not possible to be certain that appropriate bits of tissue were examined). Dorsal root ganglia could not be found. A litter of embryos injected on d14 with 0.002 μg of β-BTX each (i.e. $\frac{1}{500}$ of the usual dose) was examined at d20. The embryos moved spontaneously, and showed reflex responses to stimulation of the head. Some isolated internal intercostal muscles contracted normally, others gave no response to stimulation of their nerves, or responded along a part of their length. No spontaneous miniature endplate potentials could be recorded from muscles unresponsive to nerve stimulation, even in the presence of hypertonic bathing solution (Dennis & Harris 1980). Ventral roots in some treated embryos were obviously thinner than controls, and were examined with the electron microscope. They were deficient in collagen when compared with controls, and neuroglial cells had little cytoplasm. Larger diameter axons were still present, in reduced numbers, while Remak bundles tended to be larger than normal, reflecting the relative atrophy of the glial cells. There was no indication that the smaller size of the nerve root reflected the presence of a normal number of axons of less than normal diameter.

Aneural muscles stained with the Bodian technique possessed autonomic nerve fibres surrounding their blood vessels (figure 2, plate 1), but showed no signs of motor innervation (see figure 3, plate 2). Normal muscles gave a very reliable response to this stain, even at early stages of innervation (see illustrations in Harris (1981 *b*)). Cholinesterase staining with the Karnovsky technique (Harris 1981 *a*) produced a strong response from the glial cells surrounding young nerves (figure 2), which declined with age, becoming less prominent by the time of birth. This response was lost within a day of β-BTX treatment, being, for example, absent in d15 diaphragms from embryos injected with toxin at d14. That both silver and ChE staining reliably demonstrated motor nerve fibres in control muscles, and autonomic fibres in both control and experimental muscles, is evidence that the techniques were adequate to have demonstrated motor innervation in β-BTX-treated muscles if it had been present.

Selective destruction of phrenic motoneurons

Embryos injected with β-BTX at d12 and examined at later times were not paralysed. They moved spontaneously, showed spontaneous gasping movements, and possessed a reflex response to stimulation of the nose or head. Their body masses were up to 20 % lower than litter-mate controls and they were not viable if born naturally. Death appeared to be due to respiratory insufficiency.

Diaphragm muscles from these embryos were aneural. Physiological tests applied to isolated muscles, as described in the preceding section, gave no indications of innervation. Nerves

could not be seen with silver or ChE staining (figure 3, and see Harris (1981 *a*) for illustrations of ChE staining). The possibility of a temporary innervation being withdrawn (Dennis & Harris 1979; Pettigrew *et al.* 1979) was not upheld by the results of studying embryos at d15, d16, d17, d18, d20 or d21, following injection of β-BTX at d12, as no muscle in this series was found to be innervated. In contrast, physiological and histological examination of intercostal muscles from these animals revealed the presence of functional innervation (figure 3). All intercostal muscles contained nerve trunks and contracted in response to nerve stimulation. Treated embryos assumed grotesque postures in response even to gentle stimulation and their responses were unusually prolonged. Patterns of spontaneous movement also were abnormal. However, the incidence of 'mistakes' in innervation of internal intercostal muscles examined at d18 was no greater than normal (Dennis & Harris 1979; Dennis *et al.* 1981), indicating that the abnormal movements might reflect central nervous system deficiencies. It was also of interest to note that aberrant innervation of the aneural diaphragm was rare, despite the close proximity to its borders of motor nerves. Figure 3 illustrates the single example seen of a small axon bundle entering the left hemidiaphragm from the oesophageal plexus; two other examples were noted of fibres of unknown origin entering left or right hemidiaphragms at their dorsal poles, and penetrating a short distance into the muscle. It is not known if this aberrant innervation was functional.

The basis for selective action of the toxin is not clear. Rat embryos at d12 have no recognizable muscle cells in the region of the presumptive diaphragm, which at that time consists solely of ligament separating liver and lung primordia. The embryos were seen in outline by trans-illumination of the uterus at the time of injection, but penetration of the hypodermic needle could not be made visible. Most injected embryos survived. I cannot be certain that toxin actually was injected inside d12 embryos and not merely into the amniotic fluid so that it attained a selective access, rather than had a selective action.

Development of embryos without motoneurons

Aneural embryos were not grossly different from normal embryos so far as overall development is concerned. The relative loss of mass was comparable to that of TTX-treated embryos. They were tightly enclosed in the amnion, indicating that embryonic movements may have a role in promoting growth of the tissues enclosing the embryo, and in consequence of this assumed a tightly flexed posture and often had rather elongated heads. Some were oedematous, with subdermal oedema giving rise to a 'saggy baggy elephant' look. Oedema did not occur in TTX-treated embryos. Their bones remained cartilagenous and poorly calcified. Muscle growth was affected in much the same way as in TTX-treated embryos (figure 1). Muscle width, which reflects the growth of the rib cage, was normal, but muscles were shorter and much thinner than normal and sometimes contained regions without myotubes, or regions in which the direction of fibre growth was abnormal, for example forming spirals. Fatty degeneration or replacement of muscle with connective tissue as seen in aneural and paralysed chick embryos (Eastlick 1943, Drachman 1967; Pockett 1977; Shellswell 1977) was not seen in rat embryos.

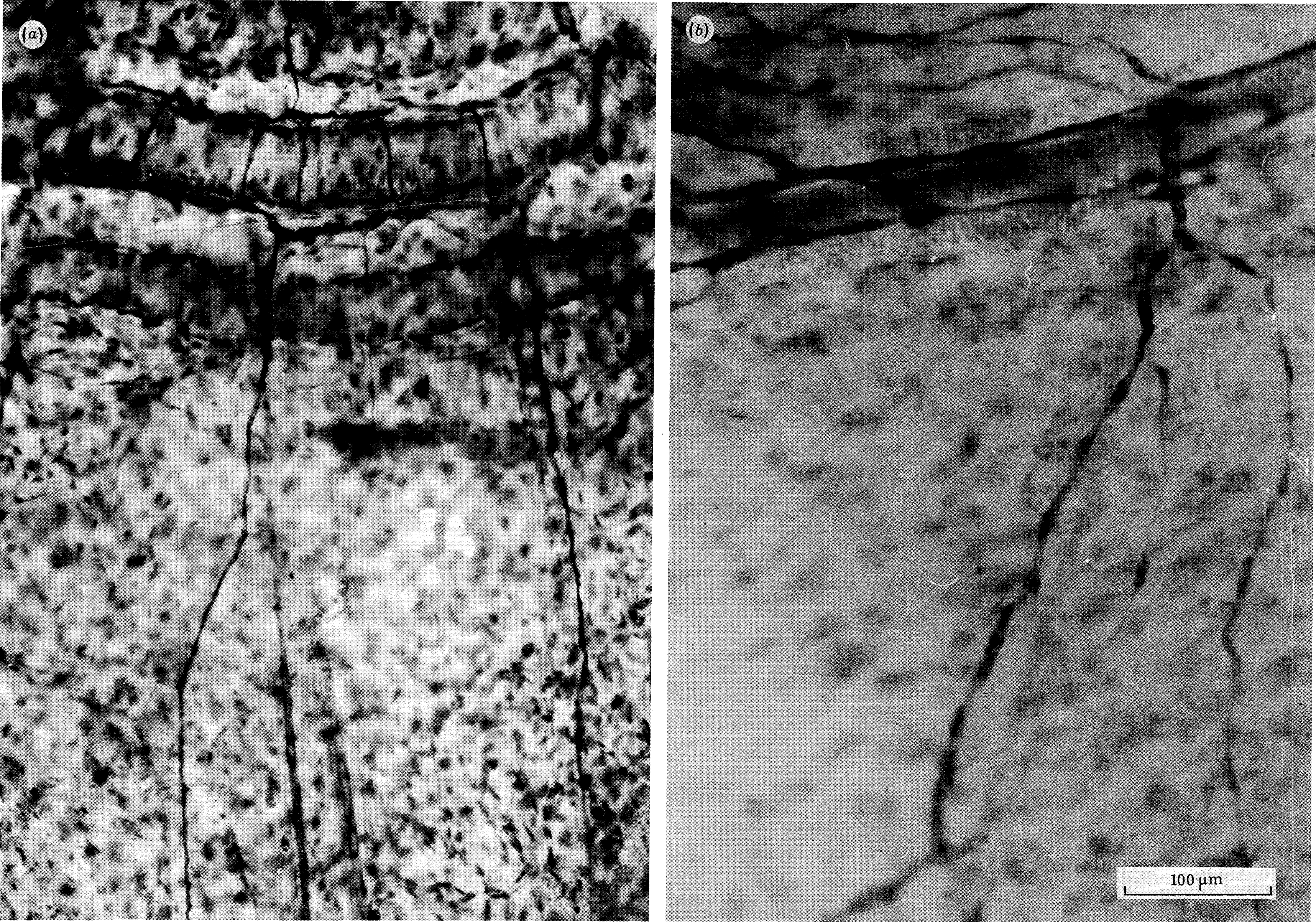

FIGURE 2. Autonomic nerve fibres surrounding blood vessels near the costal borders of β-BTX-treated muscles (embryos injected at d12 examined at d21). (*a* ilver stain (Bodian); (*b*) AChE stain.

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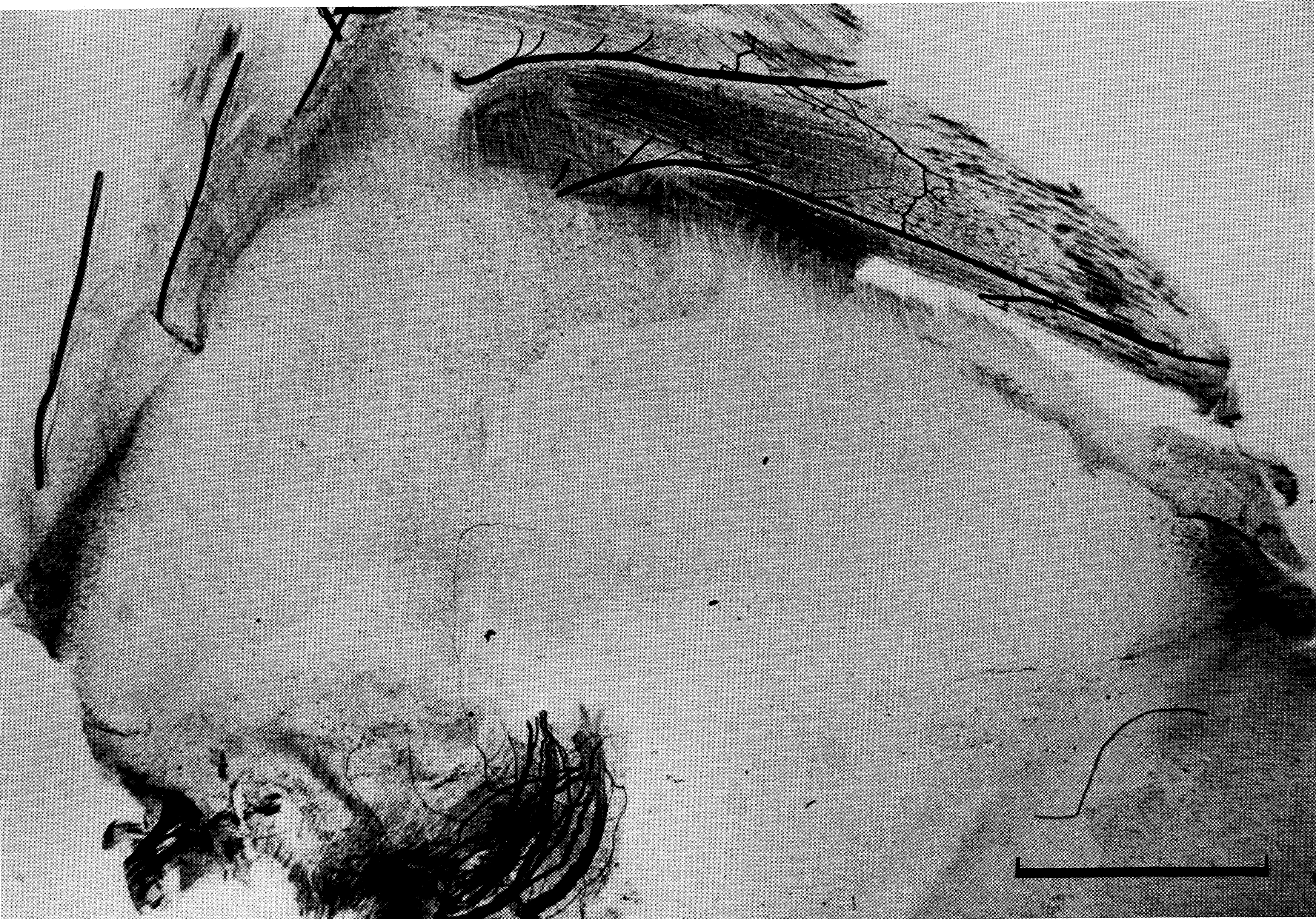

FIGURE 3. Selective denervation of diaphragm muscles by injecting β-BTX at d12. Left hemidiaphragm muscle from d18 embryo treated at d12, silver stain (Bodian). Nerve trunks supplying the internal intercostal muscles can be seen around the costal border of the diaphragm, and the nerve plexus surrounding the oesophagus is present near the bottom of the photograph. A single aberrant nerve branch leaves this plexus to enter the diaphragm; no other nerve fibres have been stained within the diaphragm, which by all physiological criteria was completely aneural. Scale bar, 0.5 mm.

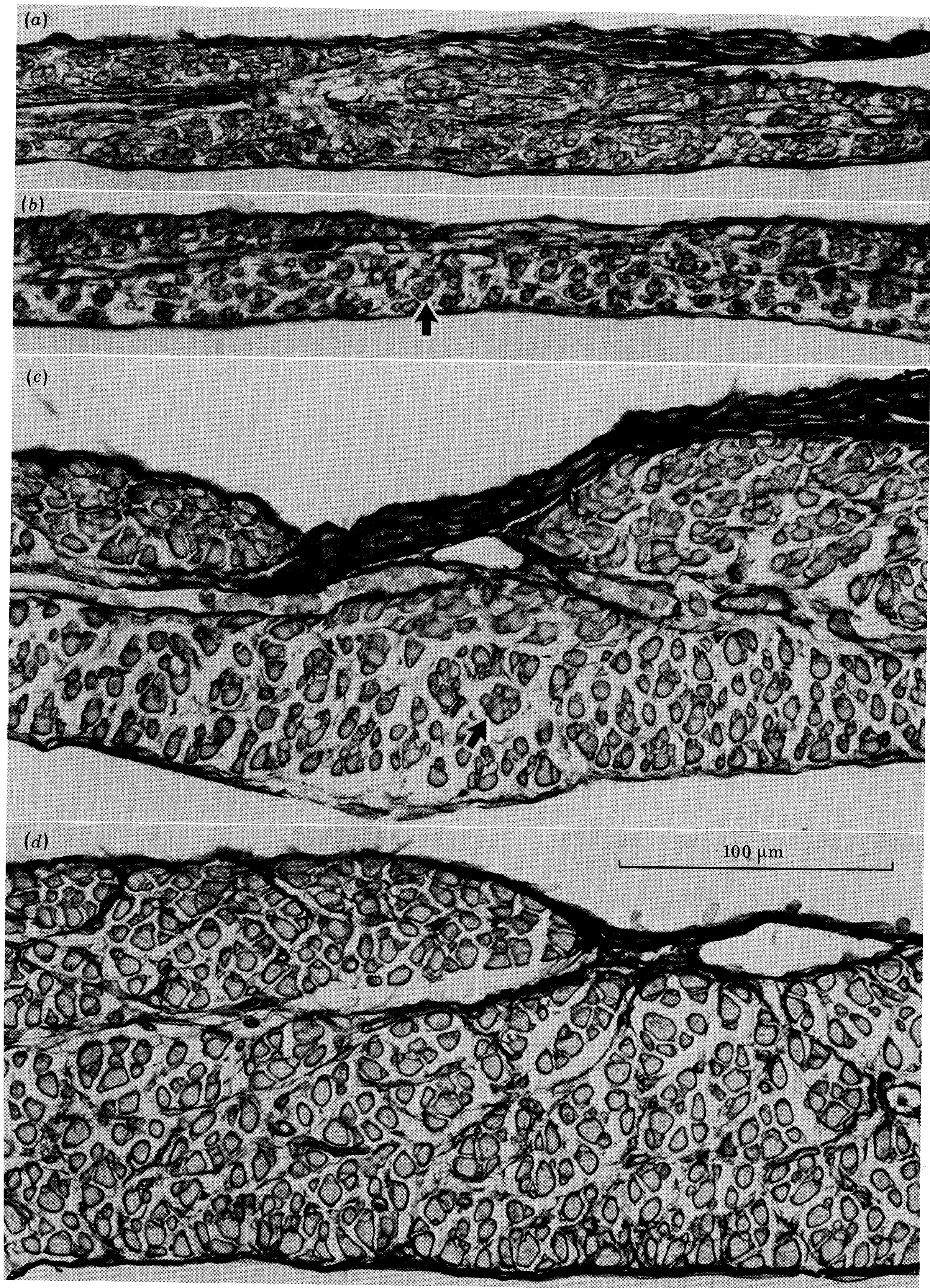

FIGURE 4. Transverse sections of normal embryonic muscles stained with Sirius Red, and viewed at the point of entrance of the phrenic nerve: (*a*) d16; (*b*) d17½; (*c*) d19; (*d*) d21. Note the clusters of secondary myotubes present on d17½ and later (arrowed).

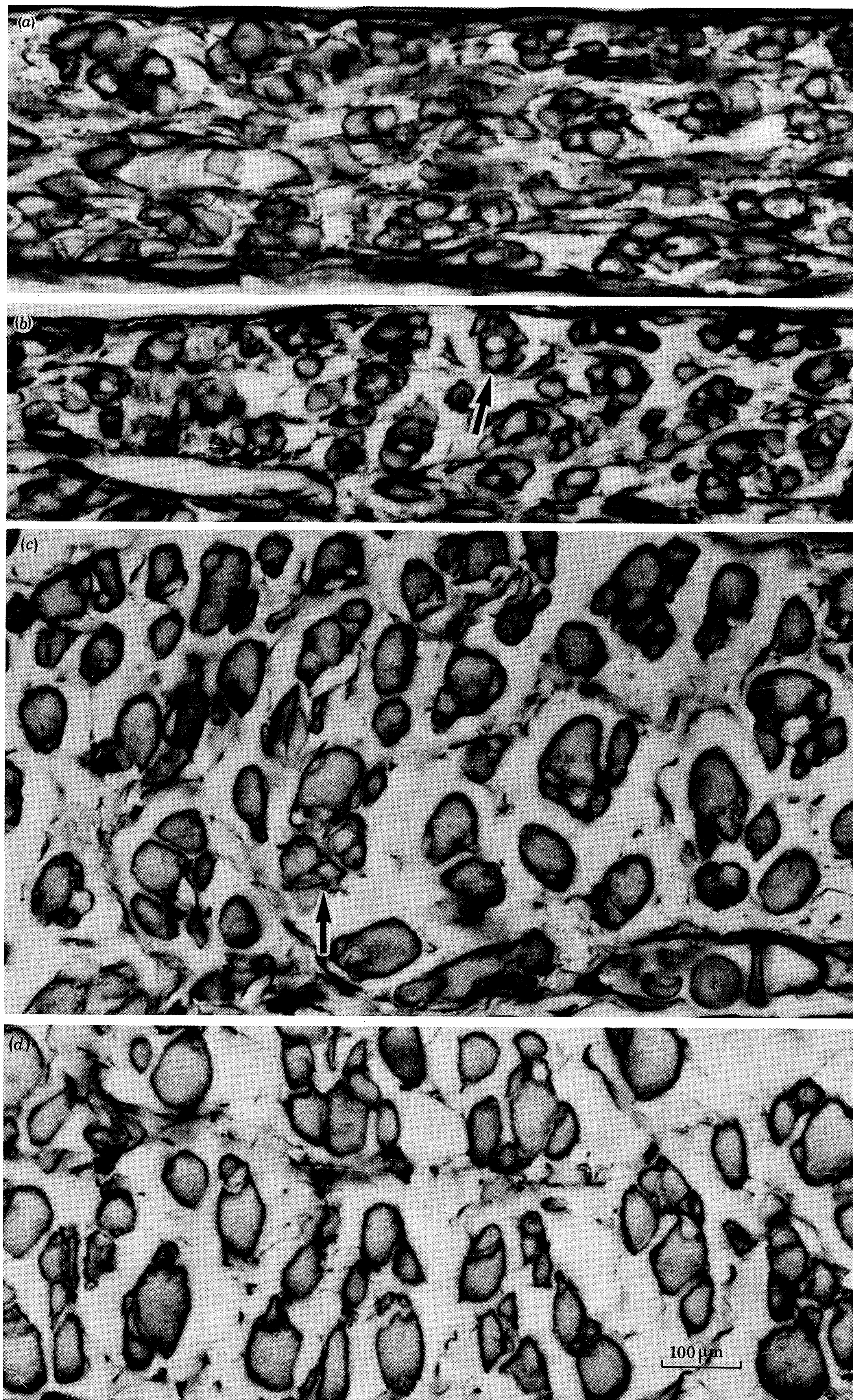

FIGURE 5. High magnification view of transverse sections through normal muscles: (*a*) d16; (*b*) d17½; (*c*) d19; (*d*) d20. Clusters of secondary myotubes in association with a primary myotube are marked by arrows. Note staining of red blood cells (r) in (*c*).

Muscle growth

Pattern of growth

Muscle nuclei were birth-dated with [^{3}H]thymidine in an attempt to see if new fibres were formed in any special region of the muscle. Label was injected into embryos of d12, d14, d15, d16 and d18, and their muscles examined at various later times. There were no heavily labelled muscle nuclei following injection at d12, but there was always a proportion of densely labelled nuclei throughout muscles labelled at the later times. Heavily labelled nuclei always were uniformly distributed throughout the whole muscle. This experiment provided no evidence to support the existence of a special site for generation of new fibres, such as along the lateral borders of the diaphragm.

Several difficulties, both physiological and technical, limit the usefulness of this experiment. It is assumed that a heavily labelled nucleus reflects the existence of a myoblast that was generated shortly after injection of label and then left the mitotic cycle to fuse into a myotube. Such a myoblast might become part of a newly generated myotube, or might fuse with an existing one. Thus the technique gives information on the sites of generation of myoblasts (assuming no extensive intramuscular migration), but does not specifically mark only new-formed myotubes. Conversely, however, a myotube with no heavily labelled nuclei almost certainly was formed after the time of labelling. A further difficulty was due to the presence of dorsal–ventral or medial–lateral gradients in the density of labelling of individual maximally labelled nuclei, which probably reflects failure of the label to equilibrate well in the extracellular space of the embryos, perhaps because of the small amount injected and its rapid uptake by cells. Thus this direct injection technique, although economical in the use of label, is not as satisfactory as the more conventional intravenous infusion into the mother's blood (Sidman *et al.* 1959). Another limitation is that labelled nuclei cannot rigorously be assigned to myotubes; the technique could not distinguish satellite cell nuclei, for example.

Histology

Cross sections of embryonic muscles fixed in formalin–sucrose and stained with Sirius Red are illustrated in figures 4, plate 3, and 5, plate 4. This dye stained the borders of units within the muscle a bright red, and their cytoplasm light yellow or light pink. Counts were made of units defined as the contents of a continuous deep-staining border. If a number of such units were clustered together (figures 4, 5, arrowed) they were counted as multiple units. Sirius Red is said to stain collagen and basement membrane (Sweat *et al.* 1964), but it also stained the surface of red blood corpuscles (figure 5*c*). If, when applied to muscle cells, it stained only basal lamina, then its use should produce profiles precisely equivalent to the Ontell & Dunn (1978) 'muscle units'. To test this, serial section reconstructions were made in which single muscle units were seen to split into multiple units over a distance of a few sections. Cross sections of apparent 'single' units should have contained several cells, each bounded by sarcolemma but acquiring a basement membrane sheath only at the point of branching (Ontell & Dunn 1978). Sirius Red stained only the outer profile of the 'single' unit, but stained individual profiles after branching had occurred. A further check was to view material stained with Sirius Red with phase microscopy through a red filter, so that the staining could not be seen. Counts of phase-dark profiles gave the same numbers as did counts of units defined by stained borders

when viewed with bright-field illumination through a green filter. The results of these two series of experiments support the assumption that only basal lamina was stained, and not the sarcolemma.

Fixation and staining of muscles from embryos of d18 onwards was very satisfactory, and unambiguous counts of units were obtained. Cells in muscles from d16 and d17 embryos tended to become vacuolated (figure 5) and it was helpful to examine the section with phase microscopy at the same time as units on the photomontage were counted. Cross sections of diaphragm muscles from embryos respectively of d16 and d18 showed a marked contrast in the pattern of unit profiles. In d16 muscles, profiles had rather constant dimensions and were arranged in small groups (figure 4*a*, 5*a*). In d18 and older muscles it was common to find either a single profile, or a larger profile with several smaller units clustered about it. This pattern was first seen in diaphragm muscles from embryos of d17, and was very clear in a series of embryos examined in the evening of d17 (d17½), as illustrated in figures 4*b* and 5*b*. Such multiple units were still obvious in muscles from d21 or new-born animals, but less frequent in muscles from week-old animals and almost totally absent from the series examined at 12 d postnatal. Kelly & Zacks (1969*a*) described secondary myotubes in rat intercostal muscle on d18, but did not examine tissues from d17 embryos. The close resemblance of the light microscope sections to their electron micrographs indicates that the small unit profiles first seen on d17 in the diaphragm also reflect the development of secondary myotubes.

Numbers of muscle fibres

Diaphragms were fixed so as to make it possible to cut cross sections that followed the course of the intramuscular nerve along the midline of the muscle, and sections at this level were selected for photography and counting. The results of unit counts from muscles ranging in age from d16 gestational up to 12 d postnatal are presented in table 1, and illustrated in figure 6*a*. The value for 12 d postnatal, 11918 ± 255 (s.d.) is arbitrarily taken as representing the adult number of muscle fibres in the discussion that follows.

The values for 5 d postnatal do not fit a smooth curve, and appear anomalous. The animals were from a single litter whose mean mass, 6.5 g, was less than usual for that age. Some values from other litters examined during the period 5–10 d postnatal were greater, but were part of an early series of experiments whose results were discarded because of possible technical inadequacies which could have led to an underestimate of the correct values. The values for 5d postnatal are thought to be accurate, but untypical.

There are several points of interest in these results. Secondary myotubes were first clearly identified on d17; so the number of units present at that time should represent an upper limit for the number of primary myotubes. These, therefore, account for less than 20 % of the adult number of muscle fibres.

It is assumed in the definition of primary myotubes that they do not continue to form once development of secondary myotubes has begun; evidence supporting this assumption comes from the results of the experiments on aneural muscles described below. Further consideration of the usefulness of distinguishing primary and secondary myotubes is given in the discussion.

The number of new units generated over 24 h intervals during the last week of gestation is given in figure 6*b*. The most rapid rate of increase in unit numbers, a doubling in 24 h, occurs from d17 to d18, corresponding to the time at which secondary myotubes were first seen. By

the time of birth (d21) 70% of the adult number of units has been reached, but new fibres continue to form during the first two postnatal weeks.

Growth of aneural muscles

Embryos were injected with β-BTX at d14, and their diaphragm muscles examined on d18, d20 or d21 (figure 7, plate 5). Muscles in this series examined on d18 were similar in appearance to d16 controls (figure 7*a*). Units were of similar dimensions, one to another, and arranged in small groups. There was however, a very small number of groups consisting of one larger unit surrounded by several small units, as first seen on d17 in normal controls. In the muscles examined later (see, for example, figure 7*b*) units were discrete and did not form groups.

The results of unit counts from these muscles are given in table 1 and illustrated in figure 8, where they are compared with mean values in control muscles. The means at d20 and d21 were not significantly different from the mean at d18 ($p > 0.1$). These values were the same as in d17 controls ($p > 0.3$), but significantly different from d16 controls ($p < 0.0001$) and d17½ controls ($p < 0.0025$). The results of the histology and unit counts indicate that primary

TABLE 1. UNIT COUNTS IN CONTROL AND EXPERIMENTAL ANIMALS

	controls								postnatal day		
	d16	d16⅓	d17	d17½	d18	d19	d20	d21	5	7	12
	1504	1605	2338	2515	5368	6487	6747	7604	9991	11779	12071
	1350	1158	2190	3709	4115	7097	8312	7486	8685		11684
	1762	1247	2431	2634	5150	6279	7574	8985	9382		11720
	1495	1331	—	4054	4748	6665	7287	8821	8409		12198
	1519	1706	—	2091	—	6689	8738	9526			
	—	1811	—	—	—	—	—	—	—	—	—
mean	1526	1476	2320	3162	4845	6643	7731	8484	9117	11779	11918
	± 148	± 267	± 122	± 682	± 550	± 302	± 199	± 897	± 712		± 255

aneural muscles

	d14–d18	d14–d20	d14–d21	d16–d21	d17½–d21	d18–d21
	1825	2484	2103	5369	6997	6803
	1855	2005	2911	3878	5781	7090
	1140	—	1452	4707	6174	7260
	2665	—	2576	5205	—	7584
	2295	—	2544	3937	—	—
	2589	—	2249	5163†	—	—
	—	—	2692	5331†	—	—
mean	2061 ± 574	2245 ± 339	2361 ± 483	4799 ± 646	6317 ± 621	7184 ± 326

paralysed muscles

					recovered from paralysis	
	d16–d21	d16 + d18–d21	d17–d21	d18–d21	d16–d21	d18–d21
	5357	3144	4846	4413	7420	7661
	5347	3752	5126	5626	7877	8513
	6611	4998	—	5629	7235	—
	5720	4759	—	3774	—	—
	5008	—	—	7944	—	—
	—	—	—	7281	—	—
mean	5608 ± 614	4163 ± 868	4986 ± 198	5778 ± 1605	7511 ± 330	8087 ± 602

† Injected with 5 μg of β-BTX.

myotubes formed with normal timing, but that secondary myotubes failed to be generated in the aneural muscles.

In a second series of experiments, β-BTX was injected at d14, d16, d17½ and d18, and units were counted at d21 (table 1 and figure 9). As a control for the efficacy and speed of action of the toxin two d16 embryos were injected with 5 μg instead of the standard 1 μg; the results were not significantly different ($p > 0.25$). All the muscles in this series had fewer units than had d21 controls, in proportion to the time elapsed since injecting toxin. On the other hand, all had more units than were present at the time of injection. The final number of units (table 2) was that which would be expected if new units continued to be generated at the usual rate for 1½–2 d after injecting toxin, and then stopped.

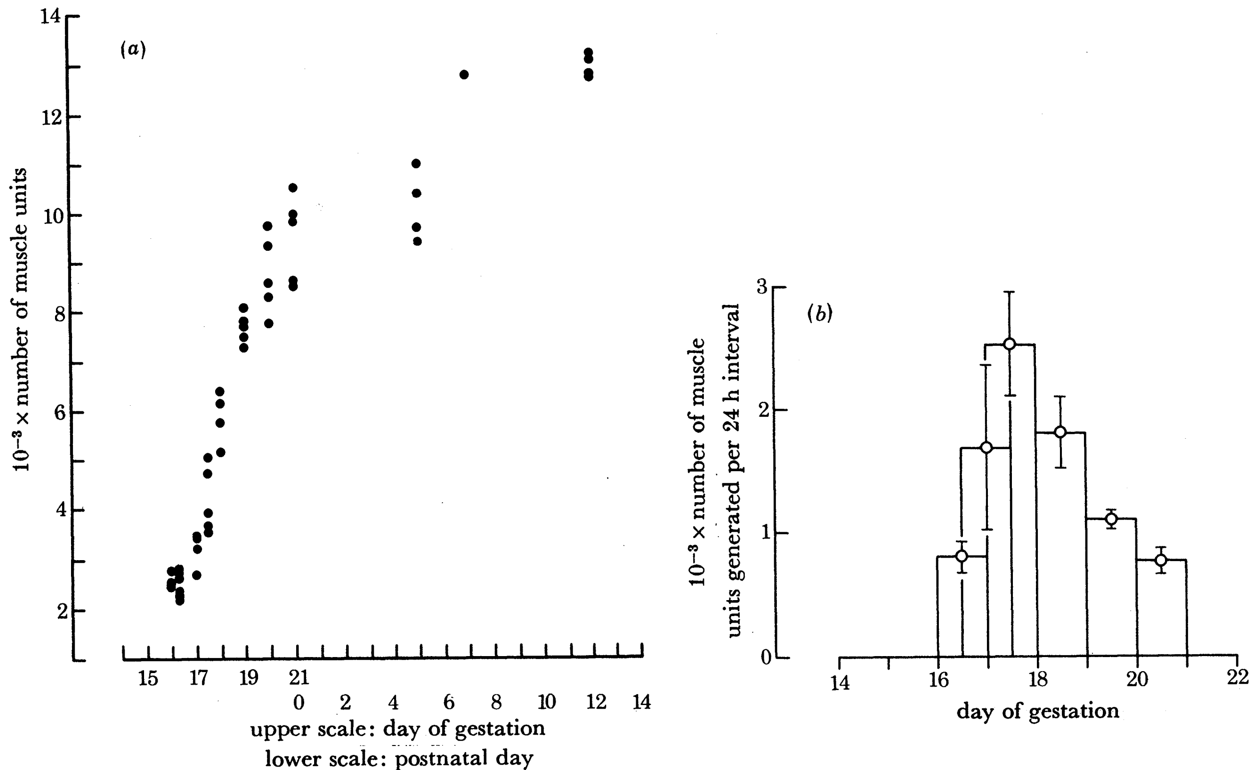

FIGURE 6. (*a*) The number of muscle units present in normal embryonic and early postnatal muscles. (*b*) The number of new muscle units generated over 24 h intervals from d16 to d21 (mean ± s.d.).

Growth of paralysed muscles

Embryos were paralysed by inserting TTX capsules at d16, d17 and d18, and their muscles were examined at d21. Cross sections of TTX-treated muscles are illustrated in figure 10, plate 6. No evidence can be seen of the continued generation of secondary myotubes, which is still a feature of d21 controls (figures 4*d*, 5*d*) although reduced in comparison with earlier times.

The most striking feature of counts of units in this experiment (table 1; figure 11) is the large scatter between different muscles. This ranges from muscles that are little different from d21 controls to muscles affected more severely than any treated with β-BTX over the same interval. Great care was taken to be certain that embryos were completely paralysed, before their tissues were selected for analysis, and this scatter is not due to failures in efficacy of the technique.

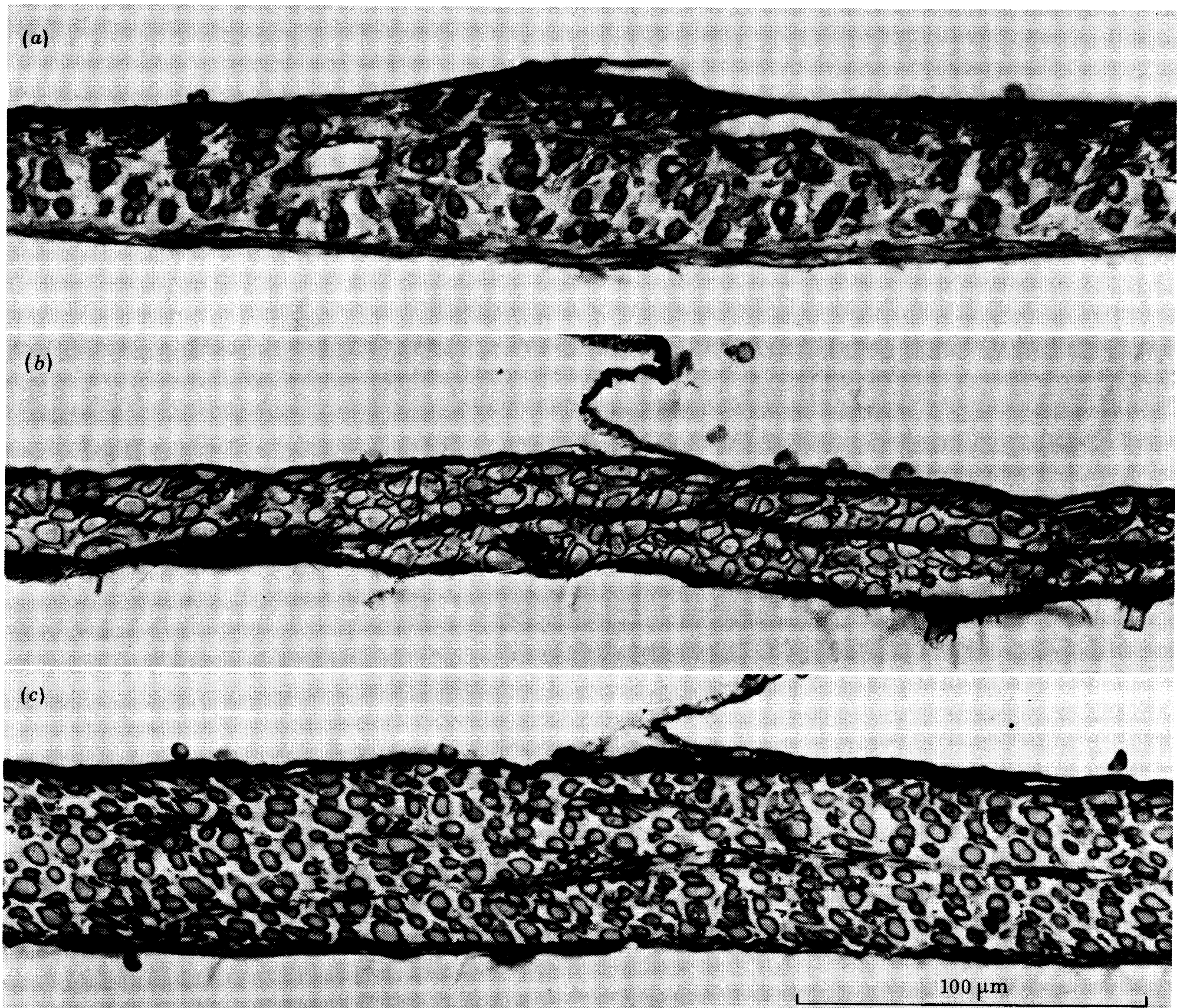

FIGURE 7. Cross sections of aneural muscles from embryos injected with β-BTX. (*a*) Injected on d14 and examined on d18; (*b*) d14–d21; (*c*) d16–d21. Sections are taken near the midpoint of the muscles, at their thickest points, so that fibre numbers can be compared with those in figures 5 and 10.

(*Facing p.* 268)

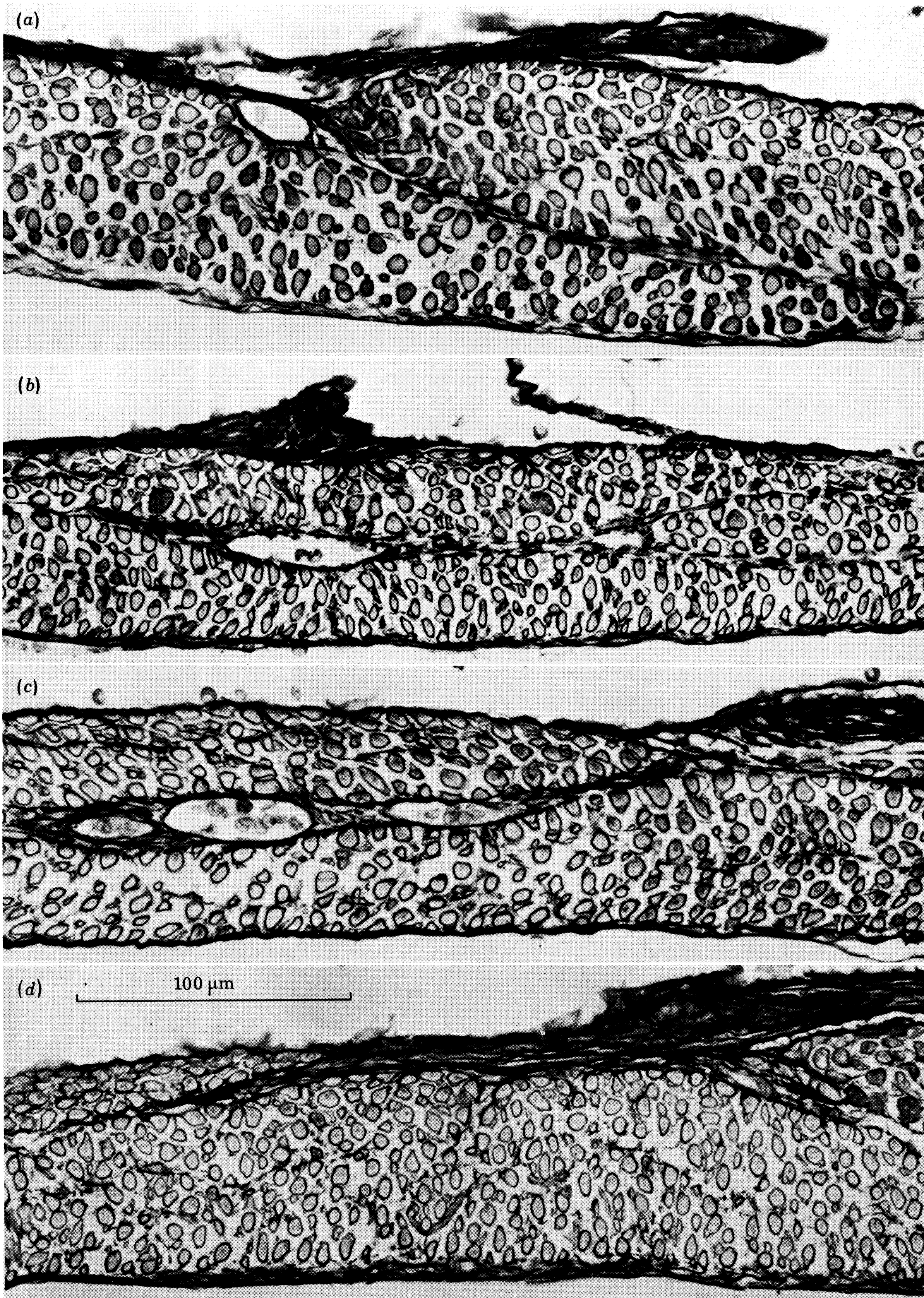

FIGURE 10. Cross section of muscles from embryos paralysed with TTX and examined on d21. Sections are from the midpoint of the muscle, in the region of the nerve entry. (*a*) Two capsules inserted, at d16 and d18; (*b*) capsule inserted on d16; (*c*) capsule inserted on d17; (*d*) capsule inserted on d18.

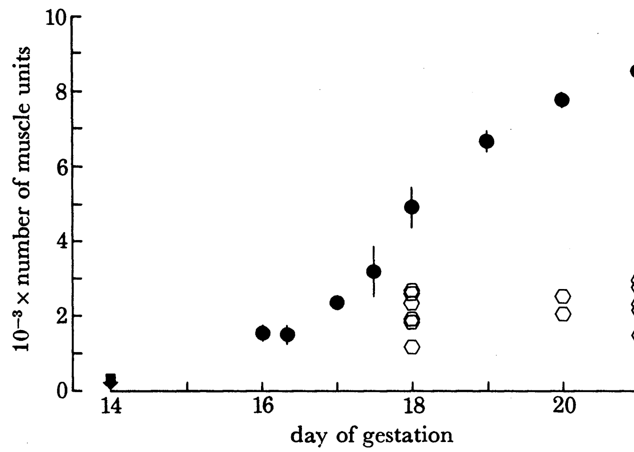

FIGURE 8. Open symbols (○), number of muscle units in aneural muscles from embryos injected with β-BTX on d14 and examined at later times; ●, number of muscle units in control muscles (mean ± s.d.).

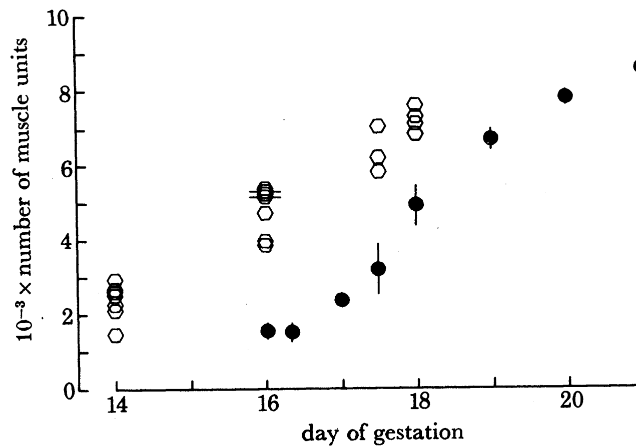

FIGURE 9. Open symbols (○), number of muscle units in aneural muscles from embryos injected with β-BTX at the times shown and examined on d21; ●, normal controls (means ± s.d.)

TABLE 2. GENERATION OF NEW MUSCLE UNITS IN ANEURAL AND PARALYSED MUSCLES

treatment ...	TTX	β-BTX	control
treatment interval	number of units generated		
d16+d18–d21	2637	3093	6958
d16–d21	4082	3093	6958
d17–d21	2648	3548†	6164
d17½–d21	1767‡	3155	5322
d18–d21	933	2339	3639

† By extrapolation, R^2 = 0.94.
‡ By extrapolation, R^2 = 1.00.

As mentioned previously, paralysed muscles started to fibrillate shortly after the TTX was washed out. Little is known about the actions of TTX on embryonic myotubes, and it is not unreasonable to suggest that the concentration required to block nerve action potentials and hence paralyse the embryo might be less than that blocking spontaneous muscle action potentials. As the rate of release of TTX from the capsule falls with time (Mills & Bray 1980) a point

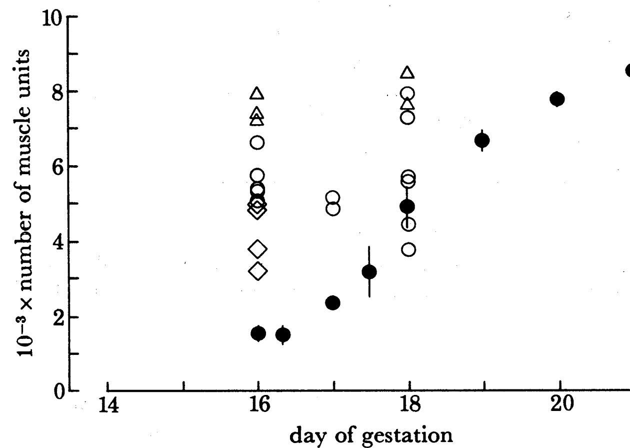

FIGURE 11. Number of muscle units in muscles from paralysed embryos, with TTX capsules inserted at the times given, and examined on d21, compared with normal control muscles (means ± s.d.): ○, one capsule inserted; ◇, two capsules inserted (second on d18); △, low-dose capsule, so that embryo recovered from paralysis before being examined.

might be reached at which nerve-induced paralysis was maintained, but the disused muscles began to contract spontaneously and this in turn could affect the generation of new myotubes. To test this, embryos had TTX capsules inserted at d16, and again at d18. These embryos had significantly ($p < 0.02$) fewer fibres at d21 than did embryos paralysed for the same interval with a single capsule. Two of the four doubly paralysed embryos had fewer units than had any aneural embryo, although mean values from these two groups were not significantly different ($p > 0.2$).

The results are summarized in table 2, and compared with the results of the β-BTX experiments. With the exception of muscles exposed to a single capsule of TTX over the interval d16–d21, paralysis was more effective than denervation in inhibiting the production of new muscle units.

A second experiment was designed to search for the presence of any 'critical period' in muscle development, during which paralysis might irreversibly inhibit the development of a proper number of muscle fibres. This was done by inserting capsules too short to maintain paralysis for the full period of the experiment. For example, it was noted that capsules with a 20 μm pore containing 0.7 μl of 0.03 M TTX and inserted into d16 embryos could maintain paralysis through d19, but a proportion of such treated embryos possessed sluggish reflex responses to stimulation of the head at d21. Muscles from five embryos treated at d16 or d18 with short capsules and showing reflex responses on d21 were counted. The mean number of units was only slightly less (9%) than the mean in d21 controls ($0.1 > p > 0.05$) (figure 11; table 1). Embryos completely lacking spontaneous movements and exhibiting only the faintest of reflex responses had unit numbers within the normal range.

Discussion

The principal reasons for using the diaphragm muscle for these studies were: familiarity, it has been subject to the most extensive physiological studies of any rat muscle; ease of dissection, the borders of the muscle can unambiguously be defined even in early and atrophic embryos, which was not true of limb muscles; and the feasibility of performing unit counts, which are not possible in some other easily dissected embryonic muscles such as intercostals. The preparation presented some difficulty because of its size, so that fibre counts were technically demanding in terms of histology and also were very tedious. It will now be of interest to extend these studies to other muscles, and in particular to see if the time course of development is the same throughout the embryo. The time of maximum rate of formation of new muscle units (figure 6*b*) should provide a useful basis for this comparison.

Histology of embryonic skeletal muscle

The experiments described here were made possible by the discovery that formol–sucrose fixation and Sirius Red staining of embryonic skeletal muscles produces 'units' that can reliably be counted with light microscopy. Kelly & Zacks (1969*a*, *b*) in their electron microscope study of the histogenesis of rat skeletal muscles described muscles at d16 as consisting of primary myotubes arranged in groups. At d18, muscles contained groups of cells consisting of a well differentiated primary myotube with less differentiated secondary myotubes and undifferentiated cells clustered around it, with each such cluster ensheathed by basal lamina. Careful examination of their illustrations reveals traces of basal lamina in the d16 material, but it is sparse when compared to d18. The light microscope material from the present study matches well the description by Kelly & Zacks, with the addition that secondary myotubes were also present in muscles from d17 embryos.

Ontell & Dunn (1978) studied neonatal rat skeletal muscle using 6 μm sections of Epon-embedded material and phase microscopy, and stress that light microscopy cannot resolve individual filamented cells when two or more are clustered together within a common basal lamina. Sirius Red was selected as an agent said to stain basal lamina and collagen, but it also stained the surface of red blood cells (figure 5*c*) and the borders of units in d16 embryonic muscle (figure 4*a*, 5*a*). This gave rise to concern as to whether the counts might include more than Ontell & Dunn's 'muscle units', and perhaps represent numbers of individual cells. This doubt cannot entirely be eliminated, but the possibility was not supported by the results of comparing phase and bright-field views of the same material, or of making serial section reconstructions of large apparently monocellular units that split into several units with individually stained sheaths.

The pattern of muscle growth

Muscle nuclei were labelled with [^{3}H]thymidine to see if there were special areas of formation of new muscle fibres within the diaphragm muscle. If, for example, new fibres were added laterally, at the edges of the muscle, then nuclei birth-dated early in development should mostly be found near the middle of an older muscle. This was not seen; if label was injected at d14 or later, heavily labelled nuclei were present throughout muscles examined on d21. This pattern was consistent with the description of muscle growth given by Kelly & Zacks (1969*a*) and Ontell & Dunn (1978), where new fibres form in clusters throughout the muscle. This has implications

for understanding regulation of the pattern of innervation, as two endplates that were adjacent to one another in a d15 muscle will be separated by three or four new muscle fibres in a d21 muscle, and two endplates that were 1 mm apart in a d15 muscle will be 8 mm apart in a d21 muscle. There is further discussion of this topic in the third paper of this series (Harris 1981 *b*).

Paralysis and denervation of embryos in utero

Treatment with β-BTX or TTX, to induce denervation or paralysis, respectively, did not grossly interfere with embryonic growth. Animals were not dwarfed, there were no teratogenic effects, the 0–20 % relative mass loss could be accounted for by retardation of muscle development alone, and the only abnormality seen was a tendency of β-BTX-treated embryos to exhibit subdermal oedema. Insertion of the glass capsules did not have effects independent of TTX; this was shown not only for water-filled control capsules, but also when low-dose capsules were used to induce temporary paralysis. The site of injection of β-BTX did not appear to be important (it was routinely injected intra-abdominally), as attempts to produce local paralysis by injection of small amounts of toxin into particular areas, for example limbs, proved unsuccessful, the embryos being affected generally even when the dose was too small to induce total paralysis. Similarly, whether TTX capsules were placed intra-thoracically or intra-abdominally was of no significance to the results.

β-BTX is a polypeptide, relative molecular mass 22000 (Abe *et al.* 1977), which acts presynaptically to block neuromuscular transmission (Chang *et al.* 1973). Isolated nerve-muscle preparations need only brief exposure to the toxin to reach full intoxication, which will occur after the usual 30–60 min delay despite repeated washing in toxin-free bathing medium (Kelly & Brown, 1974), indicating that the toxin is rapidly and irreversibly bound to nerve terminals. There is little change in nerve terminal ultrastructure in the early stages of transmission block, but later there is complete destruction of the terminals (Abe *et al.* 1976; Strong *et al.* 1977). Hirokawa (1977, 1978, 1979) has shown that injection of β-BTX into chick embryos *in ovo* completely destroys certain classes of neuron. He also has demonstrated that the toxin binds to cell bodies of embryonic motoneurons, and postulates that their destruction may be local and not dependent on uptake of toxin by nerve terminals. The toxic action of β-BTX depends on its phospholipase A_2 activity (Strong *et al.* 1976); its specific action on certain nerve cells appears to be due to the toxin binding to nerve cell membrane receptors and then enzymatically destroying the membrane (Strong *et al.* 1977).

Regulation of muscle fibre numbers

There were two principal aims for this investigation: to quantify the role of innervation in regulating skeletal muscle development; and to determine how this regulation is achieved. The tools employed were chemical denervation with β-BTX, and chronic paralysis of embryonic nerves and muscles with TTX. In comparing the effects of these two treatments it should be noted that muscles removed from aneural embryos and placed in oxygenated bathing solution spontaneously contracted and relaxed, whereas muscles removed from paralysed embryos were quiescent for a few minutes until the TTX had washed off. While the state of muscles in the embryos can only be assumed, it is likely that aneural muscles were fibrillating whereas TTX tended to suppress fibrillation. Because of the maintained electrical coupling between muscle cells in both classes of treated embryo, spontaneous activity in any part of a muscle

spread through the whole tissue to cause coordinated contractions. This is different from denervation fibrillation in the adult, where single fibres contract individually (with minor exceptions) (Purves & Sakmann 1974; Harvey & Kuffler 1943).

Muscle innervation developed normally in the presence of TTX. Isolated paralysed muscles contracted in response to nerve stimulation as soon as the toxin had washed off, junctional ACh receptor clusters and deposits of ChE developed normally, and silver-stained or ChE-stained nerve fibres were normally distributed throughout the muscles (Harris 1981 *a*, *b*).

The aim of the experiments employing β-BTX was to define the numbers of muscle fibres at given times that were committed to develop without further support from the nervous system. Their design assumes that β-BTX promptly removed all nervous influences from the muscles. Evidence that this was so includes the observations that 1 μg of toxin killed young rats of 7.5 g body mass within an hour (d14 embryos weigh 0.13 g; d18 embryos weigh 1.25 g), increasing the dose fivefold had no influence on the number of fibres developing in embryos injected on d16, developing nerve trunks no longer stained with silver or showed a histochemical reaction to ChE stain within 24 h of injection of toxin (Harris 1981 *a*, *b*), and physiological examination of isolated β-BTX-treated muscles revealed no signs of innervation.

Animals injected with β-BTX on d14 were examined on d18 gestation, with the finding that their diaphragm muscles contained the same number of units as d17 control muscles. Embryos examined at later times showed no further increase in the number of units. As d17 is the time at which secondary myotubes were first seen histologically this result is interpreted as meaning that primary myotubes can develop autonomously, but that initiation of secondary myotube development normally depends on innervation. Diaphragm muscles also developed in animals injected with β-BTX on d12, or on both d12 and d14, where it can be presumed that innervation was destroyed before muscle tissue had begun to differentiate.

A series of embryos was treated with β-BTX on d16, d17½ and d18, and their muscles were examined on d21. Comparison with controls from the time of injection made it possible to estimate the number of units that could still form but were undetectable histologically at those times (table 2). For example, d16 control diaphragms possess 1526 fibres, and a further 3093 of the 6958 that would normally form by d21 appear in the absence of innervation. One interpretation of these results is that 'committed myotubes' were already present as parts of muscle units, but the histology failed to reveal them. This argument might appear plausible for d17½ and d18 embryos, but is difficult to make for d16 muscles, where only primary myotubes should have been present. Furthermore, the finding that some TTX-treated embryos were affected to a greater extent than β-BTX-treated ones shows that there was no irreversible commitment to form a particular number of myotubes. An alternative explanation is that the temporary innervation induced a response in muscle tissue that persisted for a time after β-BTX treatment. The persistence of this hypothetical inductive action has been calculated by means of data from tables 1 and 2, and the results of this calculation are presented in table 3. If new units continued to be generated for a time after β-BTX treatment, and then generation stopped, this time was 1.57 ± 0.27 d. A similar conclusion as to the effects of β-BTX on formation of junctional ACh receptor clusters is reached in the third paper in this series (Harris 1981 *b*), together with evidence that their formation was maintained for a time and then stopped, rather than slowed. It also is shown (Harris 1981 *a*) that muscles treated with β-BTX on d16 maintain a commitment to form ChE deposits (see also Koenig & Vigny 1978).

In the series of embryos implanted with TTX capsules on d16, d17 and d18, one-half had muscles with fewer units than any in the β-BTX series. In two out of six embryos treated with TTX on d18, development of more units was halted. This was not an artefact due to mechanical disturbance of growth, as there was little deficit in numbers of units following implantation of low-dose capsules when the same operative techniques are used but only a temporary paralysis is produced. Further investigation, with use of two successive implantations to maintain a high level of TTX in the embryos, indicated that the effects of TTX were dose related over and above their capacity to cause paralysis. This may reflect an ability of TTX to block spontaneous activity of muscle at a dose level higher than that sufficient for paralysis of nerves.

TABLE 3. PERIOD OF CONTINUED GENERATION OF MUSCLE UNITS AFTER INITIATING β-BTX OR TTX TREATMENT

treatment ...	TTX	β-BTX	control
treatment interval		time/day	
d16+d18–d21	1.77	—	5
d16–d21	2.37	1.94	5
d17–d21	1.03	1.56†	4
d17.5–d21	0.5†	1.31	3.5
d18–d21	0.46	1.48	3

† By extrapolation; see table 2.

It is concluded that two conditions must be met to allow normal generation of new muscle fibres. First, the developing muscle must be innervated. Without innervation, only primary myotubes are formed, and secondary myotubes, which give rise to about 80% of the fibres in an adult muscle, fail to appear. Secondly, the muscle must contract. If muscles are paralysed, and even if they are innervated, generation of secondary myotubes is promptly halted. The effect of paralysis on primary myotubes has not yet been investigated.

The existence of dual prerequisites, innervation and muscle contraction, suggests that innervation is required to generate contraction. If, however, innervation is present and then removed the muscle tissue retains a commitment to generate secondary myotubes for about $1\frac{1}{2}$ d. It is presumed that myogenic contractions, which are present even in innervated embryonic muscle at least until d18, provide the necessary muscle activity. The graded effects of TTX over and above its ability to paralyse nerves, and the restoration of normal muscle unit numbers in the presence of even the slightest signs of recovery from paralysis, show that myogenic contractions in the presence of a paralysed nerve are sufficient to ensure normal generation of new myotubes. The actual rate of generation in these circumstances must for a short time be greater than normal to account for recovery to the normal total number of units. Myogenic activity in aneural muscles would be at least as strong as in muscles with TTX-induced nerve block, and yet did not maintain normal development. Thus innervation may have a trophic action as well as activating muscle contraction; this point is discussed in the third paper in this series (Harris 1981 *b*). These results do not preclude the possibility that an adequate amount of artificial electrical stimulation might maintain normal embryonic development of muscle.

Primary and secondary myotubes

The interpretations given above imply that there are qualitative differences between primary and secondary myotubes. As mentioned in the introduction, primary myotubes are said to develop early, and to provide a structural framework about which secondary myotubes form. Various biochemical and structural differences have been noted between primary and secondary myotubes (see, for example: Ashmore *et al.* 1972; Beerman & Cassens 1977; Szentkuti & Cassens 1979) and it is suggested that primary myofibres differentiate to a more mature state and become indistinguishable from secondary myotubes late in development, e.g. just before birth in the foetal pig (Swatland & Cassens 1973). It still is debatable whether two distinct classes of developing skeletal muscle cells really exist, but the concept is attractive, and is supported by the results of the experiments presented here.

Critical periods in muscle development

There is a period in the development of the visual system during which a short episode of disuse may lead to a permanent deficit in function (Hubel & Weisel 1970). Experiments were done to see whether the effects of disuse on rat skeletal muscle were reversible, as irreversibility would have important clinical implications. Temporary paralysis beginning at d16 or d18 had no long-maintained effect on fibre numbers. The effect of temporary paralysis at earlier times was not examined. Whether temporary paralysis had long-term effects on muscle fibre size or contractile strength is not known.

Denervation at birth gives rise to a permanent deficit in fibre numbers even in muscles that later become reinnervated (McArdle & Sansone 1977; Betz *et al.* 1980). For technical reasons it was not possible to look for effects of temporary disuse in the early postnatal period.

A more general discussion of the interactions between developing nerves and skeletal muscles is given in the third paper in this series (Harris 1981*b*).

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FIGURE 2. Autonomic nerve fibres surrounding blood vessels near the costal borders of β-BTX-treated muscles (embryos injected at d12 examined at d21). (*a*) Silver stain (Bodian); (*b*) AChE stain.

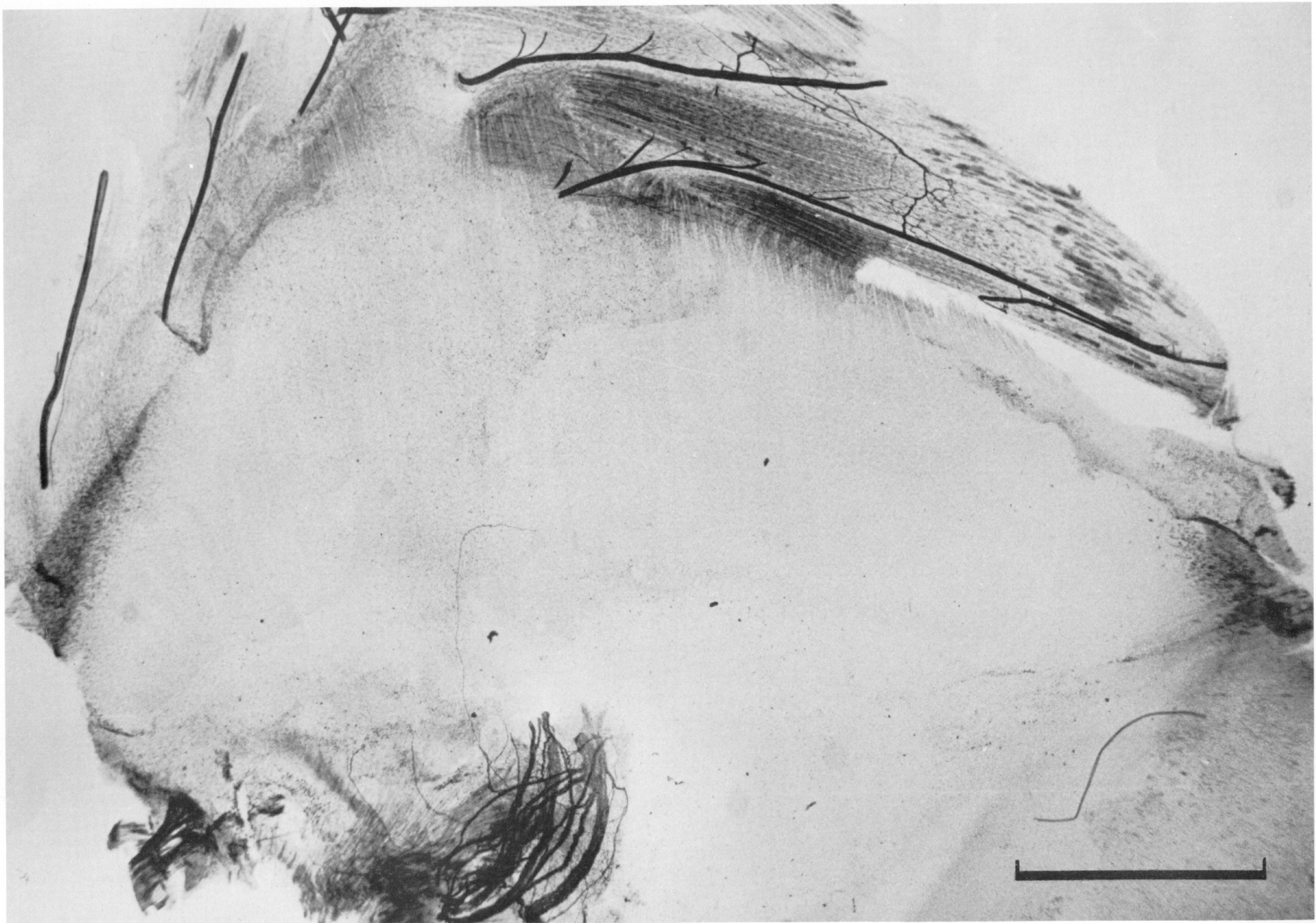

FIGURE 3. Selective denervation of diaphragm muscles by injecting β-BTX at d12. Left hemidiaphragm muscle from d18 embryo treated at d12, silver stain (Bodian). Nerve trunks supplying the internal intercostal muscles can be seen around the costal border of the diaphragm, and the nerve plexus surrounding the oesophagus is present near the bottom of the photograph. A single aberrant nerve branch leaves this plexus to enter the diaphragm; no other nerve fibres have been stained within the diaphragm, which by all physiological criteria was completely aneural. Scale bar, 0.5 mm.

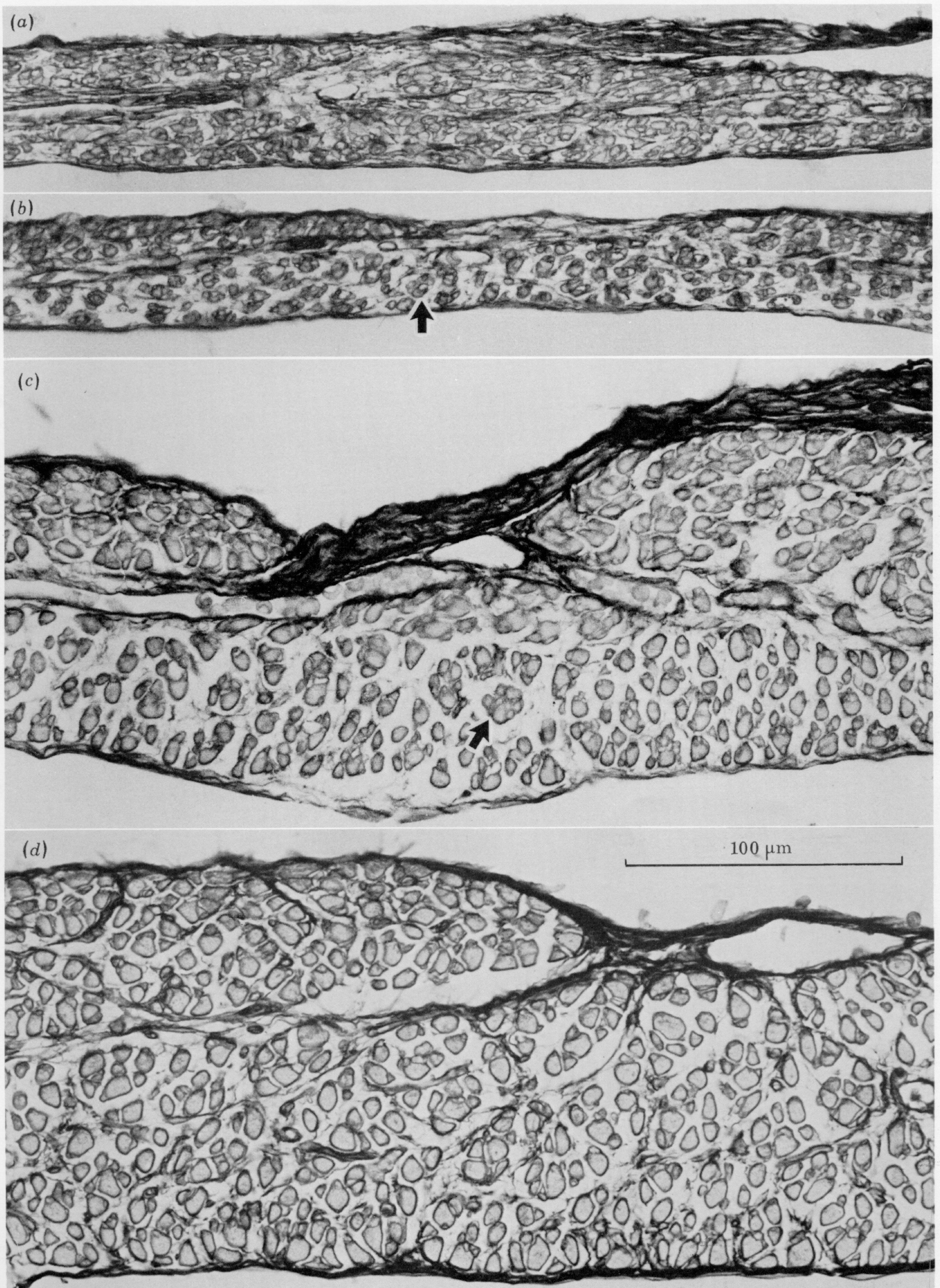

FIGURE 4. Transverse sections of normal embryonic muscles stained with Sirius Red, and viewed at the point of entrance of the phrenic nerve: (*a*) d16; (*b*) d17$\frac{1}{2}$; (*c*) d19; (*d*) d21. Note the clusters of secondary myotubes present on d17$\frac{1}{2}$ and later (arrowed).

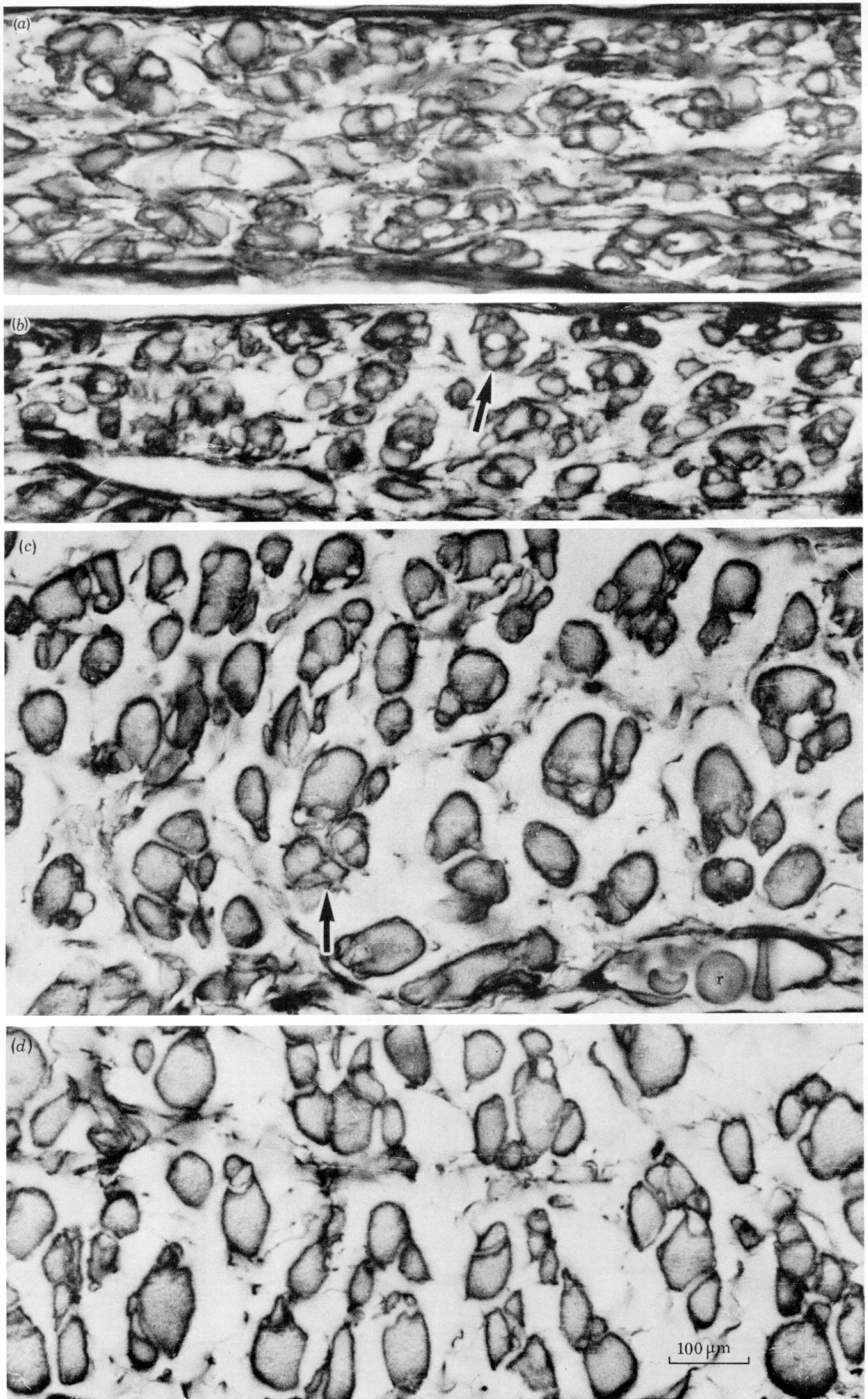

FIGURE 5. High magnification view of transverse sections through normal muscles: (*a*) d16; (*b*) d17½; (*c*) d19; (*d*) d20. Clusters of secondary myotubes in association with a primary myotube are marked by arrows. Note staining of red blood cells (r) in (*c*).

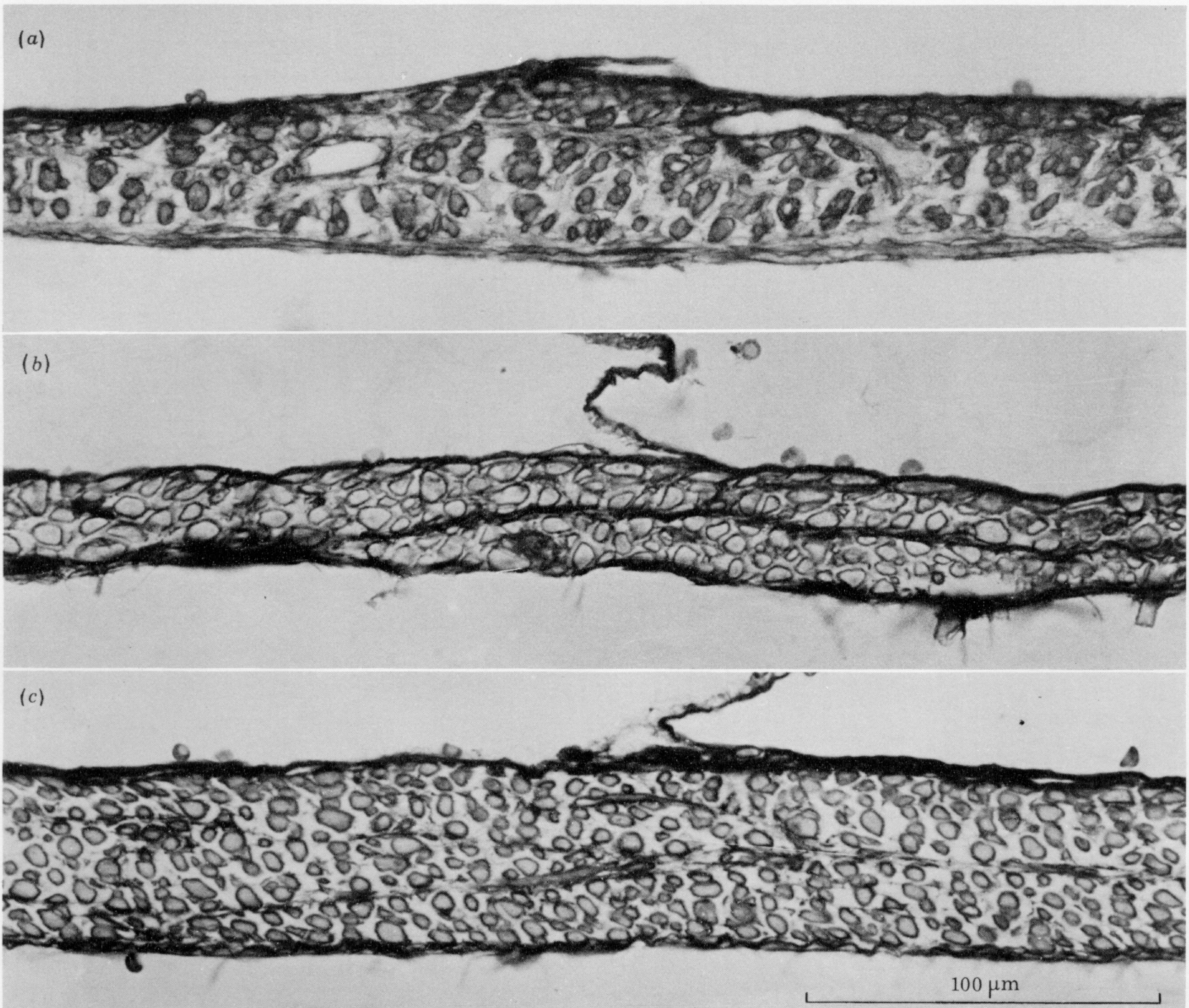

FIGURE 7. Cross sections of aneural muscles from embryos injected with β-BTX. (*a*) Injected on d14 and examined on d18; (*b*) d14–d21; (*c*) d16–d21. Sections are taken near the midpoint of the muscles, at their thickest points, so that fibre numbers can be compared with those in figures 5 and 10.

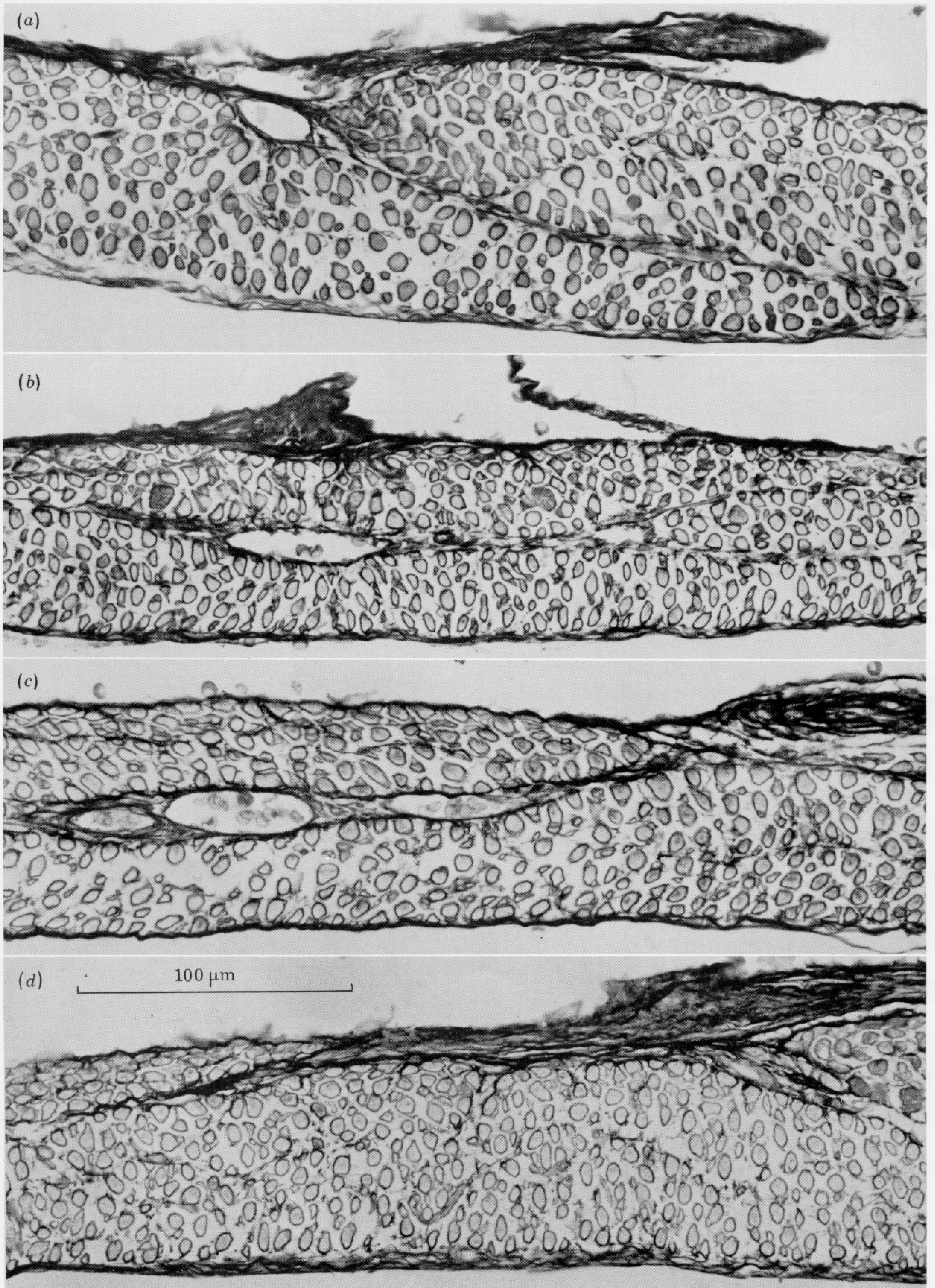

FIGURE 10. Cross section of muscles from embryos paralysed with TTX and examined on d21. Sections are from the midpoint of the muscle, in the region of the nerve entry. (*a*) Two capsules inserted, at d16 and d18; (*b*) capsule inserted on d16; (*c*) capsule inserted on d17; (*d*) capsule inserted on d18.