EMBRYONIC GROWTH AND INNERVATION OF RAT SKELETAL MUSCLES

II. NEURAL REGULATION OF MUSCLE CHOLINESTERASE

By A. J. Harris

Department of Physiology, University of Otago Medical School, P.O. Box 913, *Dunedin, New Zealand*

(*Communicated by R. Miledi, F.R.S. – Received* 15 *January* 1980 – *Revised* 11 *November* 1980)

[Plates 1–6]

CONTENTS

The development of cholinesterase (ChE) was studied in embryonic rat diaphragm muscles, and aneural and control muscles compared to determine the influence of innervation on its development.

(i) ChE deposits developed in their normal positions in aneural muscles, giving a fainter histochemical reaction than in controls.

(ii) Muscles denervated at day 16 of gestation, the time at which ChE deposits are first seen normally, and examined at later times had 'junctional' deposits with a more normal histological appearance, but there were also many 'extra-junctional' deposits scattered across the muscle.

(iii) Velocity sedimentation analysis of muscle homogenates revealed that the nerve-specific 16*S* form of the enzyme was present in aneural muscles.

(iv) It is suggested that the special relationship between ACh receptor clusters, ChE deposits and nerve terminals may reflect the presence of an organizing sub-

structure which coordinates their differentiation within developing skeletal muscles. In the absence of the nerve, potential postsynaptic structures begin to develop autonomously.

Introduction

This is the second in a series of papers (see also: Harris 1981 *a*, *b*) discussing the importance of innervation in directing the embryonic development of rat skeletal muscle. This paper describes studies on the development of muscle cholinesterase (ChE) in aneural and in control embryonic rat diaphragm muscles.

Methods

Pregnancies were dated, embryos paralysed or denervated, and embryonic tissues dissected and prepared for study as described previously (Harris 1981 *a*).

Histology

Muscles were fixed in buffered glutaraldehyde–formaldehyde (Harris 1981 *a*). Whole muscles, sections or single fibres were stained with the Karnovsky technique (Karnovsky & Roots 1964). Frozen sections were cut at 20 μm and dried onto slides before staining. Single fibres were prepared by mechanical disaggregation of fixed tissue and dried onto slides. Weak staining was enhanced by incubating fixed tissues in 0.5 % saponin (BDH) in buffered saline (Fambrough & Devreotes 1978) and then staining for 1 h at 37 °C. Not only endplates but also autonomic nerve fibres and, when present, motor nerve fibres and their glial sheaths were strongly stained in tissues treated this way.

Velocity sedimentation

Muscles were assayed for multiple forms of cholinesterase by means of velocity sedimentation in sucrose gradients (Hall 1973). Embryos were injected with β-BTX to destroy muscle innervation (Harris 1981 *a*) as required, and control and treated embryos were dissected at later times. The mother was killed with a blow on the head, and embryos were quickly removed and placed on ice. They were then perfused through the heart with an ice-cold Ca-free solution containing NaCl 150 mM, sodium phosphate buffer pH 7.3 20 mM, KCl 5 mM and $MgCl_2$ 1 mM to remove blood. The diaphragm was dissected and carefully detached from the ribs and liver. The vertebral segment of the diaphragm was removed, and the remaining left and right costal and sternal segments blotted, weighed, and placed in ice-cold homogenization buffer (NaCl 1 M, Nonidet NP40 detergent (Shell) 5 ml/l, Tris buffer pH 7.3 50 mM, EDTA 0.2 mM). Protease inhibitors, soybean trypsin inhibitor or phenylmethylsulphonyl fluoride (Sigma) were present in all solutions.

Diaphragms were homogenized in glass homogenizers in 20–100 µl of homogenization buffer, depending on their size. Homogenates were centrifuged in a microcentrifuge at 20000 *g* at 4 °C for 15 min. The supernatant was removed for velocity sedimentation and the pellet was discarded.

Velocity sedimentation was performed by means of linear sucrose gradients (50–200 g/l) containing homogenization buffer. The entire sample was layered onto a 14 ml gradient and spun in an SW40 rotor in a Spinco Model L-2 centrifuge at a revolution rate of 38000 min^{-1}

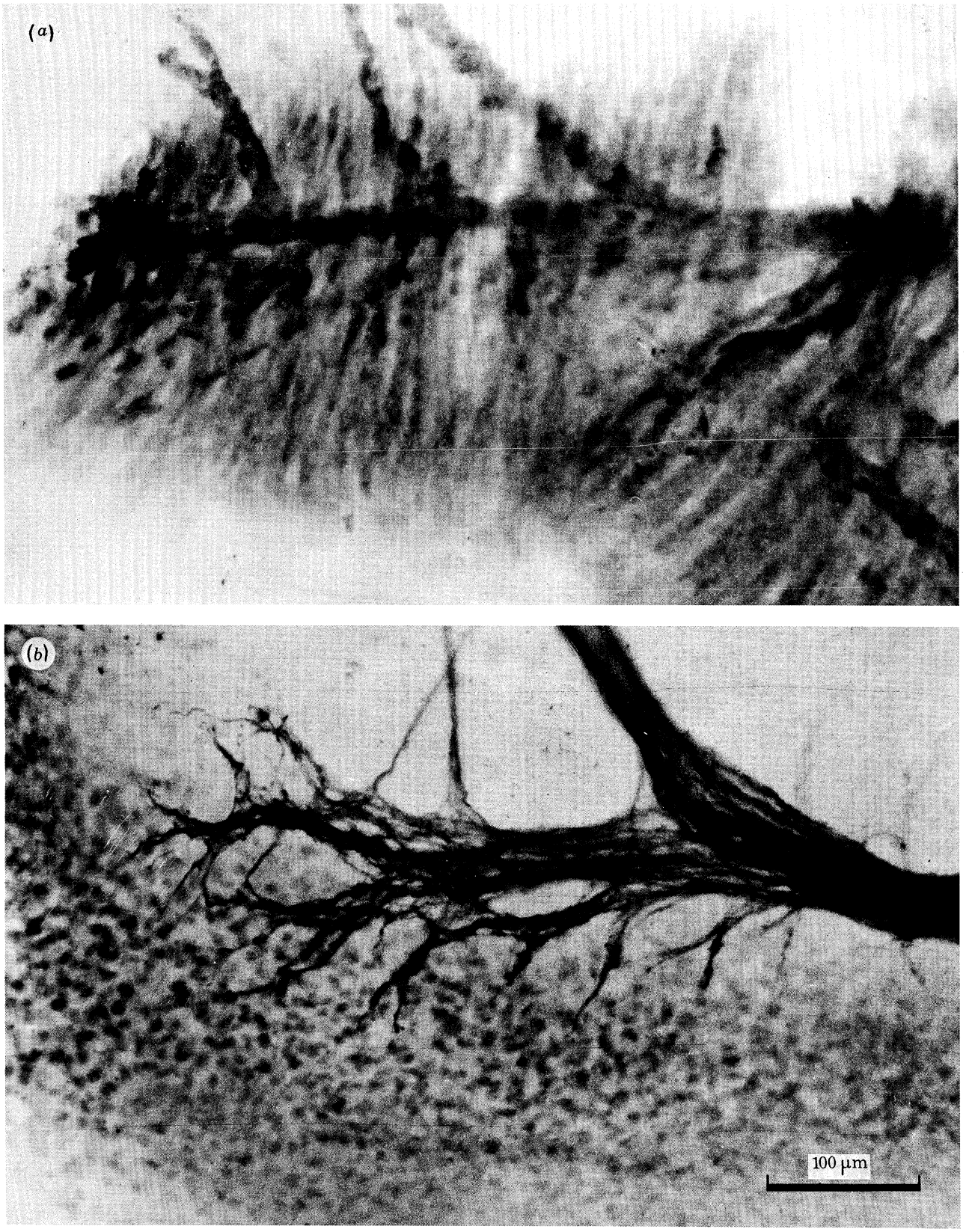

FIGURE 1. Innervation of embryo diaphragm muscles at d15. (*a*) Dorsal half of right hemidiaphragm, stained for ChE. Myofibres are diffusely stained, and the pattern of distribution of nerve sheath cells within the muscle is revealed. No junctional deposits of esterase can be resolved. (*b*) The same region of diaphragm from another embryo; nerve fibres stained with silver (Bodian technique; double exposure at two depths of focus). Nerves leaving the top of each photograph go to innervate the vertebral segment of the diaphragm.

(*Facing p.* 280)

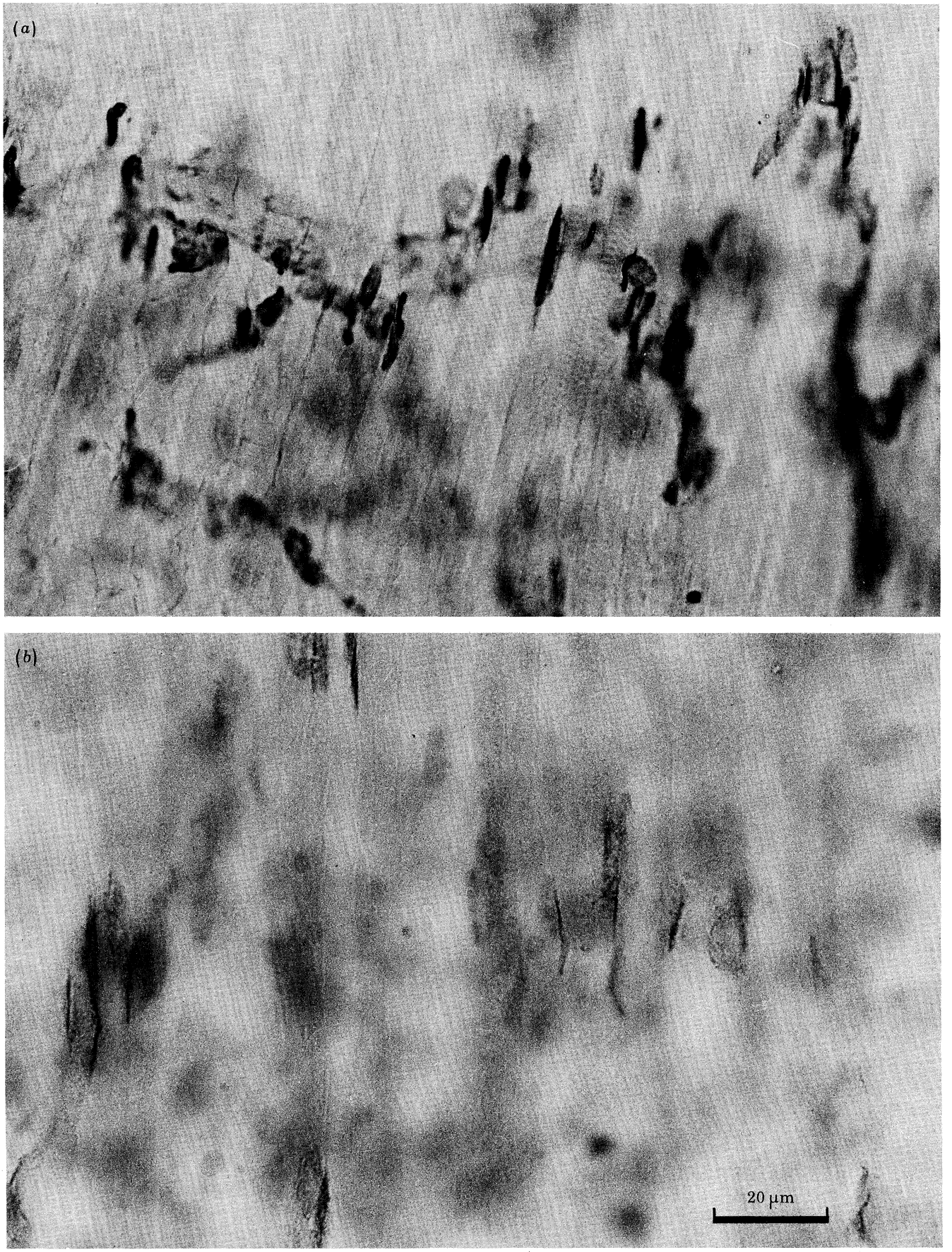

FIGURE 2. Histochemical staining for ChE in a muscle treated with β-BTX at d12: (*a*) d21 control muscle, showing deposits of reaction product at endplates and along nerve trunks (out of focus); (*b*) reaction product in β-BTX treated muscle. Whole mounts of muscles pretreated with 0.5% saponin; control incubated in Karnovsky stain for 15 min; β-BTX treated incubated for 1 h; 37 °C.

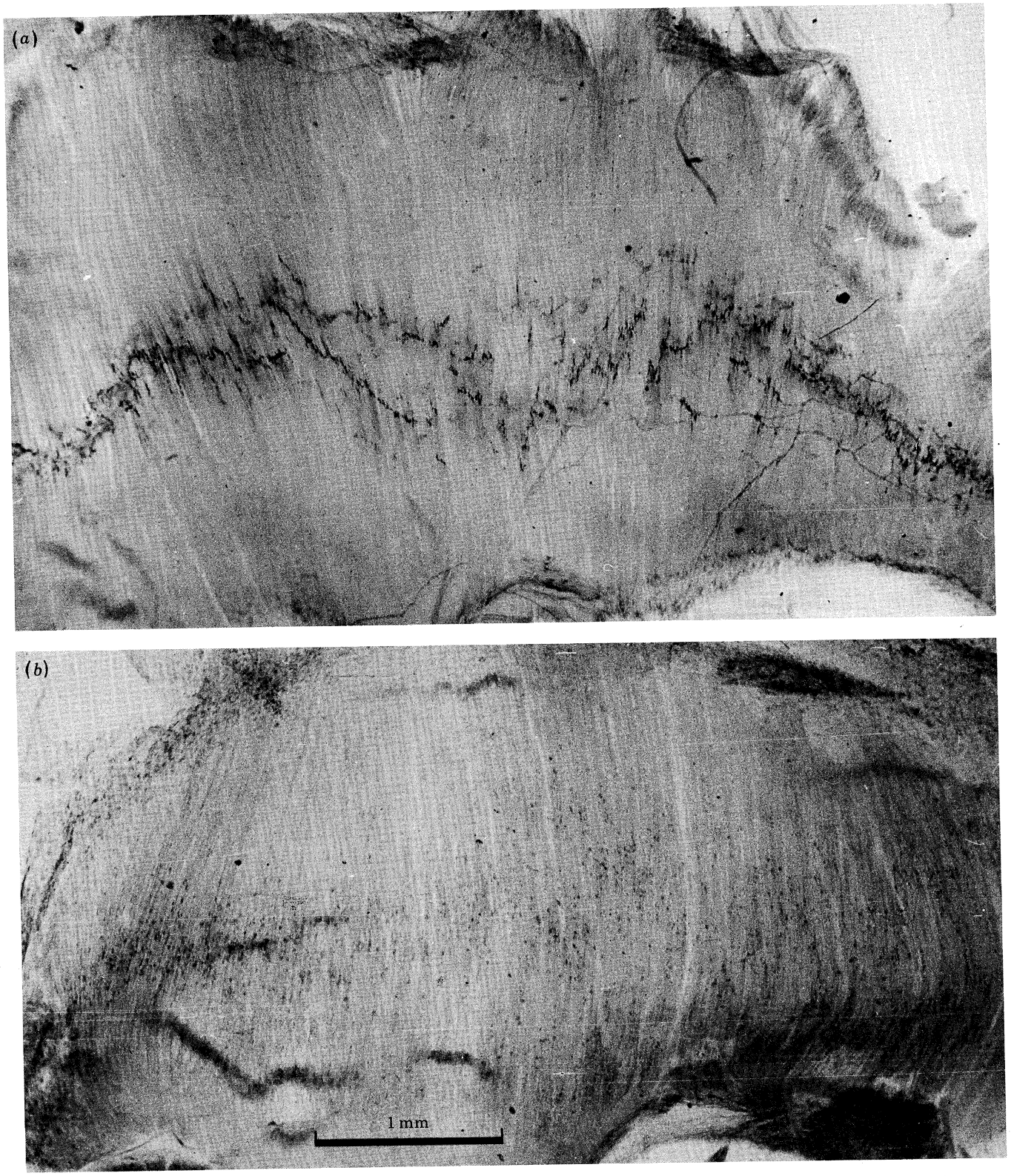

FIGURE 3. Cholinesterase distribution in aneural muscles; low-power views of right hemidiaphragms: (*a*) d20 control muscle; (*b*) muscle treated with β-BTX on d14 and examined at d20. There is considerable scatter in location of deposits of ChE reaction product.

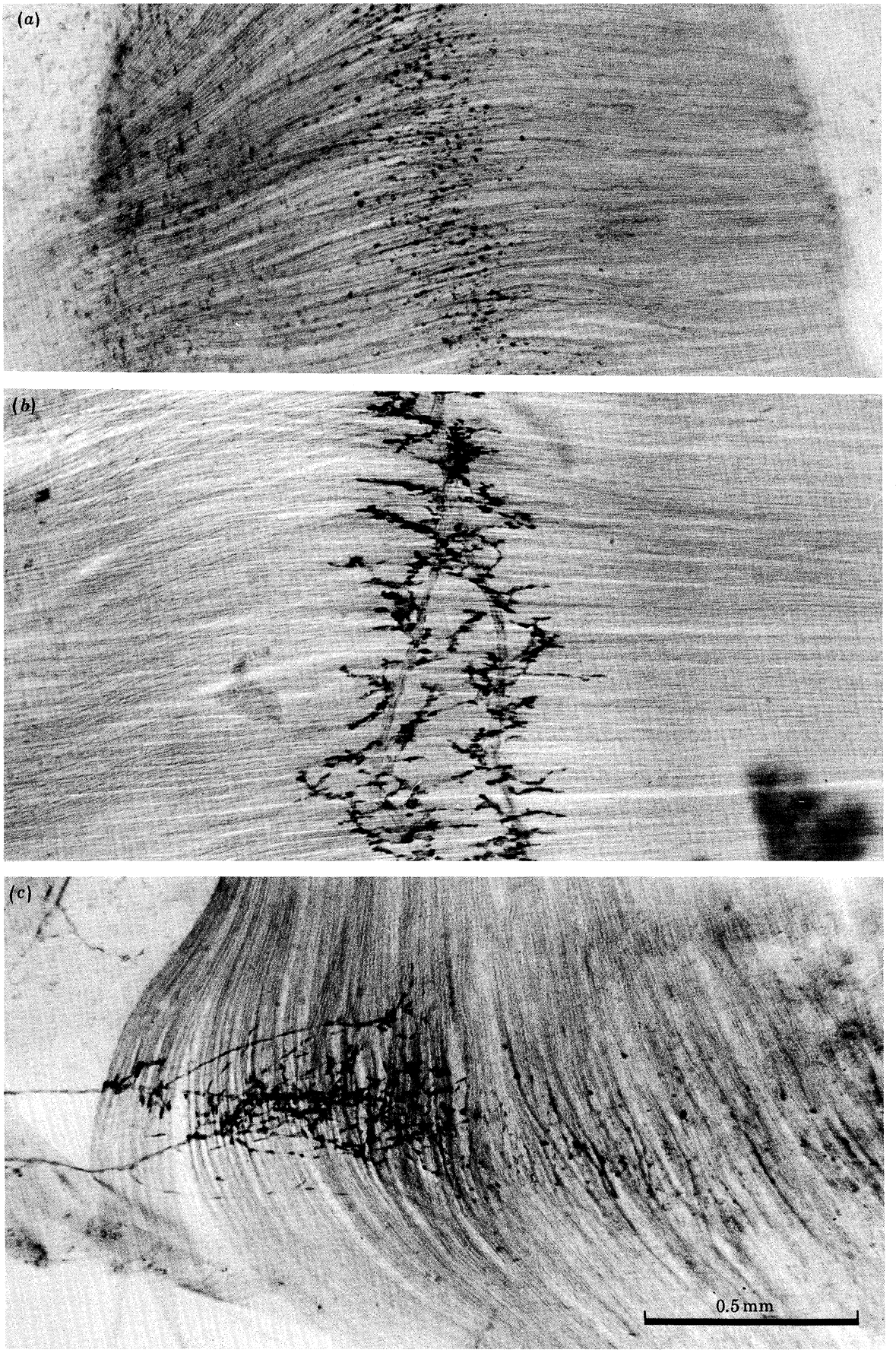

FIGURE 4. Cholinesterase in muscles from embryos twice injected with β-BTX, at d12 and d14, examined on d21 (*a*) Deposits of ChE reaction product in an aneural left hemidiaphragm; (*b*) d21 control, left hemidiaphragm; (*c*) dorsal end of right hemidiaphragm from a treated embryo, to illustrate ingrowth of aberrant innervation.

for 24 h at 2 °C. Fractions were collected by pumping 500 g/l sucrose solution through a hypodermic needle inserted through the bottom of the centrifuge tube so as to expel fractions from the top; 4*S*, 10*S* and 16*S* forms of the enzyme from control muscles were recognized by comparing elution profiles with those of Hall (1973) and Koenig & Vigny (1978), and served as standards for the experimental muscles. (Note that numbering of the fractions is reversed, due to collection from the top of the tubes.)

ChE activity was assayed by means of the technique of Ellman *et al.* (1961). Assays were performed at 20 °C, and controls run with each assay showed that activity remained stable at this temperature for at least 8 h. DTNB solution (20 μl) was added to 400 μl of enzyme preparation 10 min in advance of running the assay. This mixture was transferred to a cuvette and 200 μl of homogenization buffer was added. The reaction was started by adding 5 μl of acetylthiocholine solution and mixing well. Reaction rates were recorded with a Gilford Model 240 spectrophotometer and a Tohshin chart recorder.

Results

Histology

Analysis of the time of first appearance of ChE at the nerve–muscle junction was complicated by the strong reaction of the nerve sheath cells to the Karnovsky stain (Zelená & Szentagothai 1957; Csillik 1965; figure 1, plate 1). In muscles of d16 and older a dense line of reaction product lying beneath the diffusely stained terminal glial cell marked the junctional enzyme. These deposits could not be resolved earlier, but may have been obscured by the glial reaction.

The role of innervation in ordering the appearance of junctional ChE was studied by examining muscles from embryos injected with β-BTX. Aneural muscles from embryos treated at d12 and examined on d16 or later had 'junctional' deposits of enzyme reaction product. The reaction was faint, and required long incubation (30–60 min at 37 °C) to be clearly demonstrated. Pretreatment with saponin (see methods) enhanced the staining. The deposits, illustrated in figure 2, plate 2, could be identified only by a careful search under high magnification. They consisted of a faint increase in background staining, with a line of greater density superimposed. Their position in muscles examined on d18 was highly ordered. They occurred only near the midpoints of muscle fibres, forming a band along the midline of the muscle. By d21 their positions were less regular (figure 3, plate 3) although it was confirmed by examination of disaggregated fibres that there still was only one deposit per muscle fibre.

Treatment with β-BTX on d12 gave rise to embryos with aneural diaphragm muscles circulated with blood that had been exposed to innervated muscles elsewhere in the embryo (Harris 1981*a*). It could be argued that ChE was not synthesized by aneural muscles, but that a soluble form of the enzyme released from innervated muscles could become attached to particular sites on aneural muscle fibres, perhaps after local processing. In an attempt to resolve this question, embryos were twice injected with β-BTX, at d12 and again at d14. This treatment should have destroyed the innervation of the diaphragm before differentiation into myotubes had begun, and the injection at d14 should destroy all motoneurons generated since that time. Diaphragm muscles would not have been exposed to even the most distant effects of muscular innervation. This was not so, as doubly injected embryos were not fully paralysed, whereas embryos from the same litter receiving a single injection at d14 lost all motor innervation. Doubly injected embryos, examined at d21, had an extremely abnormal pattern of

voluntary movements and of reaction to electrical stimulation of the nose. Their diaphragm muscles were very thin and had regions with no multi-nucleate myotubes, but they did possess a band of deposits of ChE reaction product along their midline (figure 4, plate 4). This figure also illustrates the rare occurrence of aberrant innervation of an aneural diaphragm (see Harris 1981*a*); here a nerve has grown into the dorsal end of the right hemidiaphragm. One similar example was seen in a series of silver-stained preparations of embryos injected with β-BTX on d12 and examined at d21.

Diaphragm muscles from embryos injected with β-BTX on d14 reacted more strongly to ChE staining than did those injected on d12 (figure 3), although staining was still much fainter than in controls. Embryos injected on d16 or later gave a different picture (figure 5, plate 5). Here, the midline band of deposits of enzyme reaction product was very prominent, and there also were 'extra-junctional' deposits throughout the whole muscle. The nature of these scattered deposits is unclear as they were rarely seen on single fibre preparations from the same muscles. The muscle–tendon junctions also stained for ChE, as in normal controls but not in muscles injected with β-BTX at earlier times.

Velocity sedimentation

Velocity sedimentation profiles of ChE activity from control and aneural muscles are illustrated in figures 6 and 7, plate 6. Enzyme activity per muscle is expressed in arbitrary units. Activity in muscles from embryos injected with β-BTX on d14, and examined on d18, is illustrated in figure 6, and in muscles from embryos injected twice, on d12 and d14 and examined at d21, in figure 7. In both, the 16*S* form of the enzyme, which is specific for regions containing nerve–muscle junctions in adult muscles (Hall 1973), is present.

The experiments were not designed to obtain a quantitative recovery of enzyme from the muscles. In particular, the homogenate was spun only once so that enzyme trapped in the pellet was lost. With this reservation, the amount of each species of enzyme was calculated from the areas under the curves, and enzyme activity per muscle fibre calculated with use of the data for fibre numbers given in Harris (1981*a*). On d18, the ratios of ChE activity per fibre, aneural:control, were 4*S*, 1.2, 10*S* 0.6, 16*S* 0.7. On d21 the ratios were 4*S* 1.4, 10*S* 1.0, 16*S* 0.8. Results from embryos injected with β-BTX on d12, d12+d14 and d14 have been pooled in making these calculations (from a total of three experimental and three control muscles at d18, and 14 experimental and 7 control muscles at d21).

Effects of paralysis

Muscles from embryos chronically paralysed with TTX from d16 onwards (Harris 1981*a*) were reacted histochemically to demonstrate ChE. They appeared essentially the same as normal muscles. Staining of nerve sheaths and endplate ChE was normal, and there were no ectopic deposits of ChE. The only abnormality was that some endplate zones in muscles paralysed until d20 or d21 were wider than normal; this finding is further discussed in the following paper (Harris 1981*b*).

Discussion

Muscles from embryos injected with β-BTX on d12, which by all criteria available to this study had developed entirely without motor innervation, possessed deposits of reaction product of appropriate size and location to correspond to potential endplate sites when stained with the Karnovsky technique for histochemical demonstration of ChE. Careful examination with a

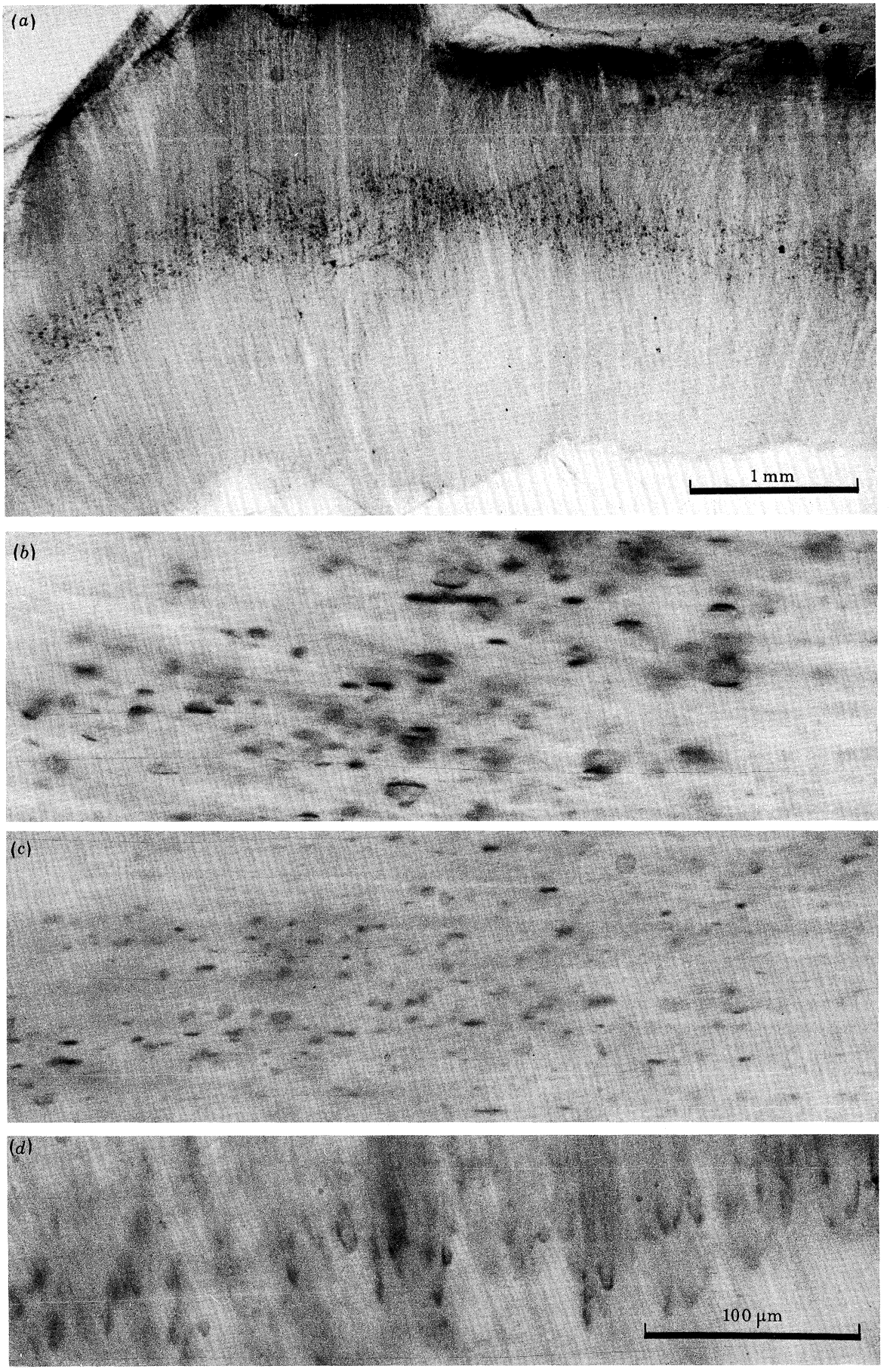

FIGURE 5. Cholinesterase in a muscle treated with β-BTX at d16, examined on d21. (*a*) low-power view, to show distribution of strongly staining deposits (scale bar, 1 mm). (*b*) View of central region of muscle to show 'junctional' deposits; (*c*) view of peripheral region to show 'extra-junctional' deposits; (*d*) deposits on muscle–tendon junctions. Scale bar labelled 100 μm applies to (*b*)–(*d*).

(*Facing p.* 282)

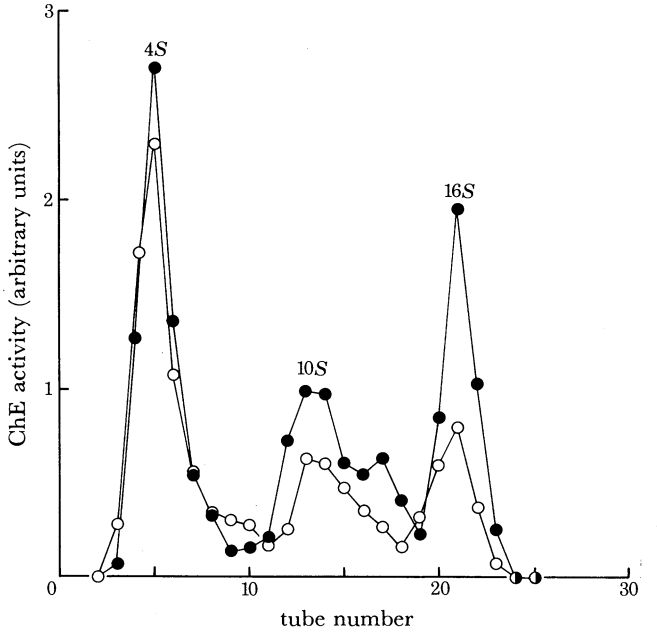

FIGURE 6. Velocity sedimentation profiles of ChE activity in d18 control (●) and aneural (○) muscles. Aneural muscles from embryos injected with β-BTX on d14. Peaks at 4*S*, 10*S* and 16*S* in the control data are identified by reference to Hall (1973) and Vigny *et al.* (1976).

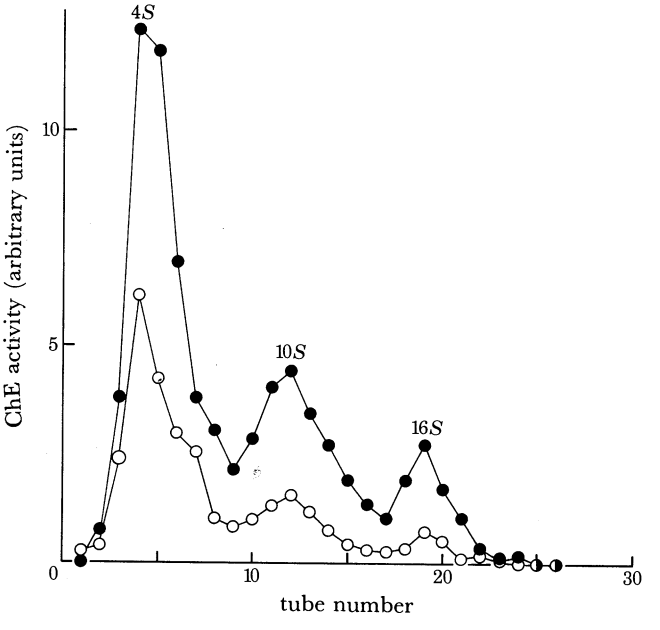

FIGURE 7. Velocity sedimentation profiles of ChE activity in d21 control (●) and aneural (○) muscles. Aneural muscles from embryos twice injected with β-BTX, on d12 and on d14.

high-powered objective lens revealed that every fibre appeared to possess a deposit, albeit much fainter than in control muscles. Muscles examined on d18 had deposits in a narrow band along their midline. By d21 the deposits were less regularly arranged, appearing in groups away from the midline in some parts of the muscle, but studies of disaggregated fibres still revealed only one deposit per muscle fibre.

Treatment with β-BTX on d16 produced muscles with a very different pattern of staining. There was a strongly stained band of endplate-like deposits of ChE reaction product along the midline, and by d21 there were many extra-junctional deposits scattered to either side of the midline. Although two out of three of the fibres in the d21 muscles formed after injection of β-BTX on d16 (Harris 1981*a*), they all had a uniform response to the ChE stain.

Molecular forms of ChE

Velocity sedimentation analysis of rat muscle cholinesterase has revealed the existence of three molecular forms: 4*S*, 10*S* and 16*S* (Hall 1973; Vigny *et al.* 1976; Koenig & Vigny 1978). The 16*S* form is found exclusively in regions of muscle containing endplates and disappears after denervation, and so is considered to be endplate-specific. Vigny *et al.* (1976) could not detect the 16*S* form before d15 in rat muscles. In adult rat muscles at least 90 % of the cholinesterase activity is due to specific acetylcholinesterase (AChE) (Hall 1973).

In the present study three peaks of activity were detected on velocity sedimentation of d18 and d21 embryonic control muscles, and these were identified as 4*S*, 10*S* and 16*S* on the basis of the works referred to above. Peaks with precisely the same sedimentation characteristics were obtained from analyses of aneural muscles, although the absolute amounts of activity per muscle were less. Values for the amount of the endplate-specific form remained lower than controls even when corrected for the reduction in number of muscle fibres in aneural muscles, but were greater than anticipated from the results of the histological studies.

ChE *and formation of neuromuscular junctions*

Cholinesterase deposits are first seen in normal embryonic muscles at least two days after nerve–muscle transmission can first be detected, indicating that synthesis of the enzyme, or at least its focal localization, may be induced by innervation. Few studies of the relationship between innervation and development of ChE have been made in mammals. Koenig & Vigny (1978) found that myoblasts cultured from d14 rat embryos developed into myotubes that did not synthesize 16*S* AChE, but that cultures from d18 embryos did synthesize this molecular form of the enzyme. Cultures from d14 embryos were induced to synthesize it by coculturing with spinal cord neurons; the location of the enzyme was not determined. The experiments of Koenig & Vigny showed that brief exposure to innervation can induce the synthesis of 16*S* AChE in myogenic cells, and commit their progeny to continue this synthesis in the absence of innervation. The relationship between primary and secondary myotubes in embryos, and myotubes grown in this tissue culture, is not known. Further information comes from studies of denervated muscles. Ectopic deposits of ChE (of undefined molecular type) appear in rat muscles denervated at birth (Lubińska & Zelená 1966; Bennett & Pettigrew 1974), despite the absence of nerves, as shown by electron microscope examination of sections through the ectopic sites (M. J. Dennis & A. J. Harris, unpublished results). Weinberg & Hall (1979) demonstrated that denervated adult rat muscles reinnervated at ectopic sites were induced to resume synthesis of 16*S* AChE, and that the enzyme became focally located not only at the

point of reinnervation but also at the old nerve–muscle junction despite the absence of nerve contact at that point. Lømo & Slater (1980), using a similar preparation, showed that AChE deposits formed after brief nervous contact with the ectopic site, provided that muscles were electrically stimulated.

Rather more work has been done with developing chick muscle, both *in ovo* and in tissue culture. Giacobini *et al.* (1973) and Gordon *et al.* (1974) studied the development of histochemically defined AChE in chick embryos paralysed with α-*Naja* toxin or curare. Deposits of AChE developed in the paralysed muscles, more or less normally in m. anterior latissimus dorsi but in reduced numbers in m. posterior latissimus dorsi. Rubin *et al.* (1980) used chick muscle tissue cultures innervated with disaggregated spinal cord neurons to show that synthesis of 19.5*S* AChE (the chick equivalent of rat 16*S* AChE) was reduced but not abolished by paralysis with curare, and that the development of focal deposits of ChE (of undefined molecular type) occurred only when nerve cells were present and was much reduced in curarized cultures. It is difficult to assess the relevance of this work to the study of mammalian development. Paralysis of chick muscle *in ovo* produces a profound atrophy, more than simply retarding development (Drachman 1967). Whether a distinction can be made between primary and secondary myotubes, and if so which type is grown in tissue culture, is not known. Nor can it be clearly stated whether tissue culture provides a model for embryonic development or for regeneration, which may employ different mechanisms (Dennis & Harris 1980).

The results of the mammalian studies show that direct interaction between a nerve and a particular site on a muscle is not required for a deposit of ChE to become focally located there. Only in cases where a nerve has briefly made contact with the site have these deposits been shown to consist of the 16*S* molecular form of AChE. Brief exposure to innervation can permanently induce the synthesis of 16*S* AChE in myogenic cells and their progeny.

Two aspects of the results of the present study should be considered. First, histologically defined ChE, which indirect evidence suggests was 16*S* AChE, became focally located in a position appropriate for endplate formation, in the absence of innervation. This shows that there is a mechanism intrinsic in developing muscle to define the sites at which innervation is to occur. In the following paper (Harris 1981*b*) evidence is presented showing the development of focal clusters of ACh receptors at sites appropriate for endplate formation, also in the absence of innervation. Electrical coupling between developing myotubes (Dennis & Harris 1979; Dennis *et al.* 1981) may be important in coordinating cell activity so as to organize and maintain this pattern in the tissue. Secondly, evidence from the experiments where β-BTX was injected on d12 strongly suggests that primary myotubes can synthesize 16*S* AChE even when their precursor cells have never experienced direct contact with a nerve. Focally located ChE was demonstrated histochemically, in muscles that had developed entirely without innervation, although it had a lower activity than when the precursor cells had experienced a temporary period of innervation, as shown in the experiments where β-BTX was injected on d16. The limitation to interpretation of these experiments is that no embryos developed without some expression of motor innervation. Application of β-BTX on d14 destroyed all motoneurons, but neurites were present in presumptive diaphragm muscle, and functional innervation of myotubes may have left a permanent imprint on the tissue. Application of β-BTX on d12 destroyed phrenic motoneurons but innervation developed elsewhere in the embryos and could possibly have had some remote influence on the developing diaphragm.

Muscles denervated from d16 onwards were compared with muscles paralysed for the same

period. The major differences were that junctional deposits of enzyme in the paralysed muscles were more dense and more elongated than in the aneural ones, and that there were ectopic deposits of reaction product in the aneural muscles. Nerves were able to regulate the dimensions of junctional deposits, and to suppress formation of ectopic deposits, despite being paralysed.

Double injections of β-BTX

Embryos injected with β-BTX on both d12 and d14 were not fully paralysed. Their pattern of movements was highly abnormal, indicating some deficiency in organization of the nervous system. One possible explanation is that motoneurons were still differentiating at the time of the d12 injection. All present may have been killed, disturbing whatever process regulates the initial number of motoneurons, so that motoneurons were still forming at d14 and continued to form after the second injection at that time. It will be of interest to study this question further, as definition of the stage in development at which a motoneuron becomes susceptible to β-BTX may be helpful in the pursuit of a variety of other studies.

I thank Alastair Buchan and Kathryn Hattersley for expert technical help. John Windsor and Charles Hollings participated in some experiments. This work has been supported by the New Zealand Medical Research Council.

References

Bennett, M. R. & Pettigrew, A. G. 1974 The formation of synapses in striated muscle during development. *J. Physiol., Lond.* **241**, 515–545.

Csillik, B. 1965 Functional structure of the post-synaptic membrane in the myoneural junction. *Akadémiai Kiadó, Budapest.*

Dennis, M. J. & Harris, A. J. 1979 Elimination of inappropriate nerve-muscle connections during development of rat embryos. *Prog. Brain Res.* **49**, 359–364.

Dennis, M. J. & Harris, A. J. 1980 Transient inability of neonatal rat motoneurons to reinnervate muscle. *Devl Biol.* **74**, 173–183.

Dennis, M. J., Ziskind-Conhaim, L. & Harris, A. J. 1981 Development of neuromuscular junctions in rat embryos. *Devl Biol.* **81**, 266–279.

Drachman, D. B. 1967 Is acetylcholine the trophic neuromuscular transmitter? *Archs Neurol., Chicago* **17**, 206–218.

Ellman, G. L., Courtney, K. D., Andres Jr, V. & Featherstone, R. M. 1961 A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmac.* **7**, 88–95.

Fambrough, D. M. & Devreotes, P. N. 1978 Newly synthesised acetylcholine receptors are located in the Golgi apparatus. *J. Cell Biol.* **76**, 237–244.

Giacobini, G., Filogamo, G., Weber, M., Boquet, P. & Changeux, J. P. 1973 Effects of a snake α-neurotoxin on the development of innervated skeletal muscles in chick embryo. *Proc. natn. Acad. Sci. U.S.A.* **70**, 1708–1712.

Gordon, T., Perry, R., Tuffery, A. R. & Vrbova, G. 1974 Possible mechanisms determining synapse formation in developing skeletal muscles of the chick. *Cell Tiss. Res.* **155**, 13–25.

Hall, Z. W. 1973 Multiple forms of acetylcholinesterase and their distribution in endplate and non-endplate regions of rat diaphragm muscle. *J. Neurobiol.* **4**, 343–361.

Harris, A. J. 1981*a* Embryonic growth and innervation of rat skeletal muscles. I. Neural regulation of muscle fibre numbers. *Phil. Trans. R. Soc. Lond.* B **293**, 257–277.

Harris, A. J. 1981*b* Embryonic growth and innervation of rat skeletal muscles. III. Neural regulation of junctional and extrajunctional acetylcholine receptor clusters. *Phil. Trans. R. Soc. Lond.* B **293**, 287–314.

Karnovsky, M. J. & Roots, L. 1964 A 'direct-colouring' thiocholine method for cholinesterases. *J. Histochem. Cytochem.* **12**, 219–221.

Koenig, J. & Vigny, M. 1978 Neural induction of the 16*S* acetylcholinesterase in muscle cell cultures. *Nature, Lond.* **271**, 75–77.

Lømo, T. & Slater, C. R. 1980 Control of junctional acetylcholinesterase by neural and muscular influences in the rat. *J. Physiol., Lond.* **303**, 191–202.

Lubińska, L. & Zelená, J. 1966 Formation of new sites of acetylcholinesterase activity in denervated muscles of young rats. *Nature, Lond.* **210**, 39–41.

Rubin, L. L., Schuetze, S. M., Weill, C. C. & Fischbach, G. D. 1980 Regulation of acetylcholinesterase appearance at neuromuscular junctions *in vitro*. *Nature, Lond.* **283**, 264–267.

Vigny, M., Koenig, K. & Rieger, G. 1976 The motor endplate specific form of acetylcholinesterase: appearance during embryogenesis and reinnervation of rat muscle. *J. Neurochem.* **27**, 1347–1353.

Weinberg, C. B. & Hall, Z. W. 1979 Junctional form of acetylcholinesterase restored at nerve-free endplates. *Devl Biol.* **68**, 631–635.

Zelená, J. & Szentágothai, J. 1957 Verlagerung der Lokalisation spezifischer cholinesterase während der entwicklung der muskel innervation. *Acta histochem., Jena* **3**, 284–296.

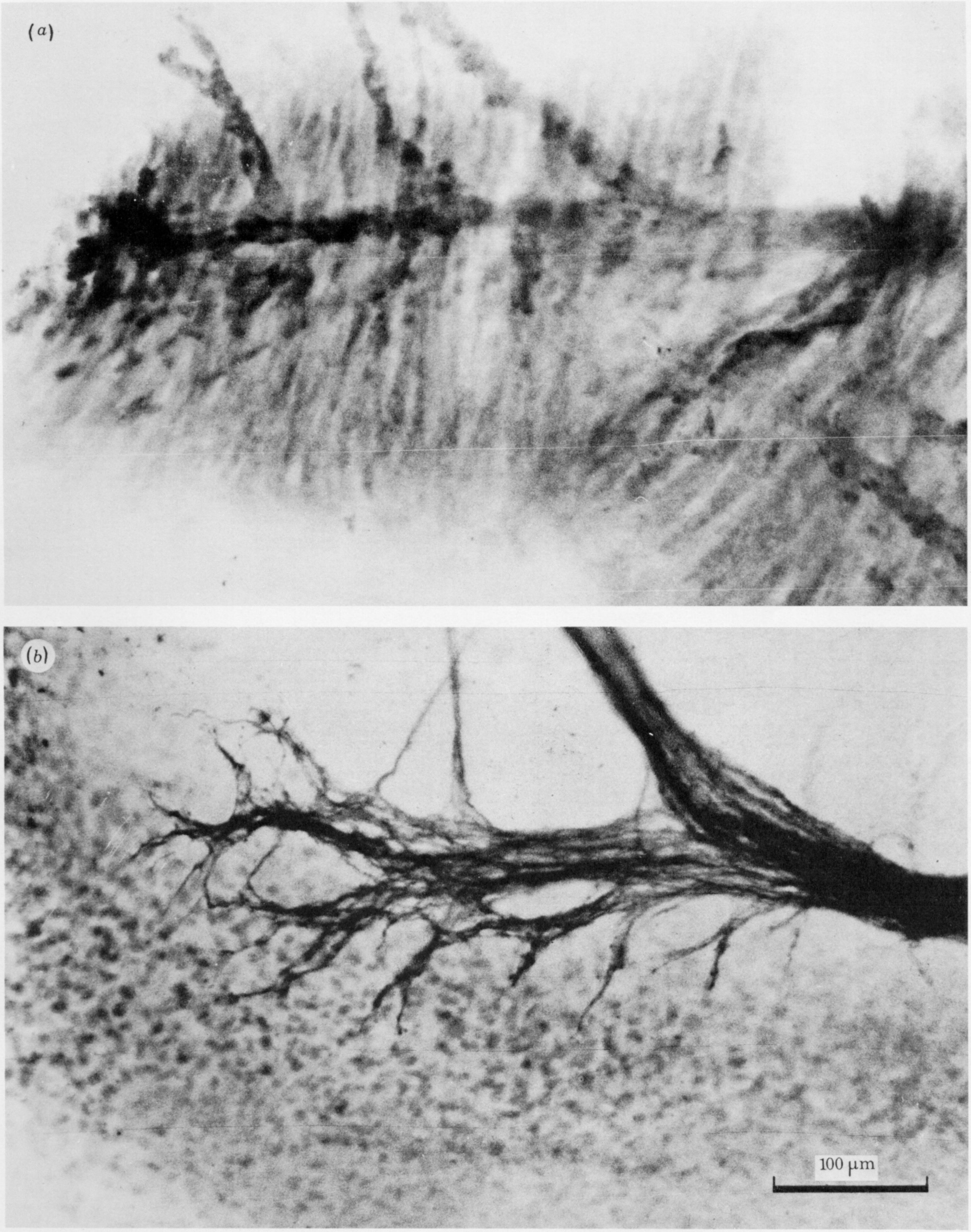

FIGURE 1. Innervation of embryo diaphragm muscles at d15. (*a*) Dorsal half of right hemidiaphragm, stained for ChE. Myofibres are diffusely stained, and the pattern of distribution of nerve sheath cells within the muscle is revealed. No junctional deposits of esterase can be resolved. (*b*) The same region of diaphragm from another embryo; nerve fibres stained with silver (Bodian technique; double exposure at two depths of focus). Nerves leaving the top of each photograph go to innervate the vertebral segment of the diaphragm.

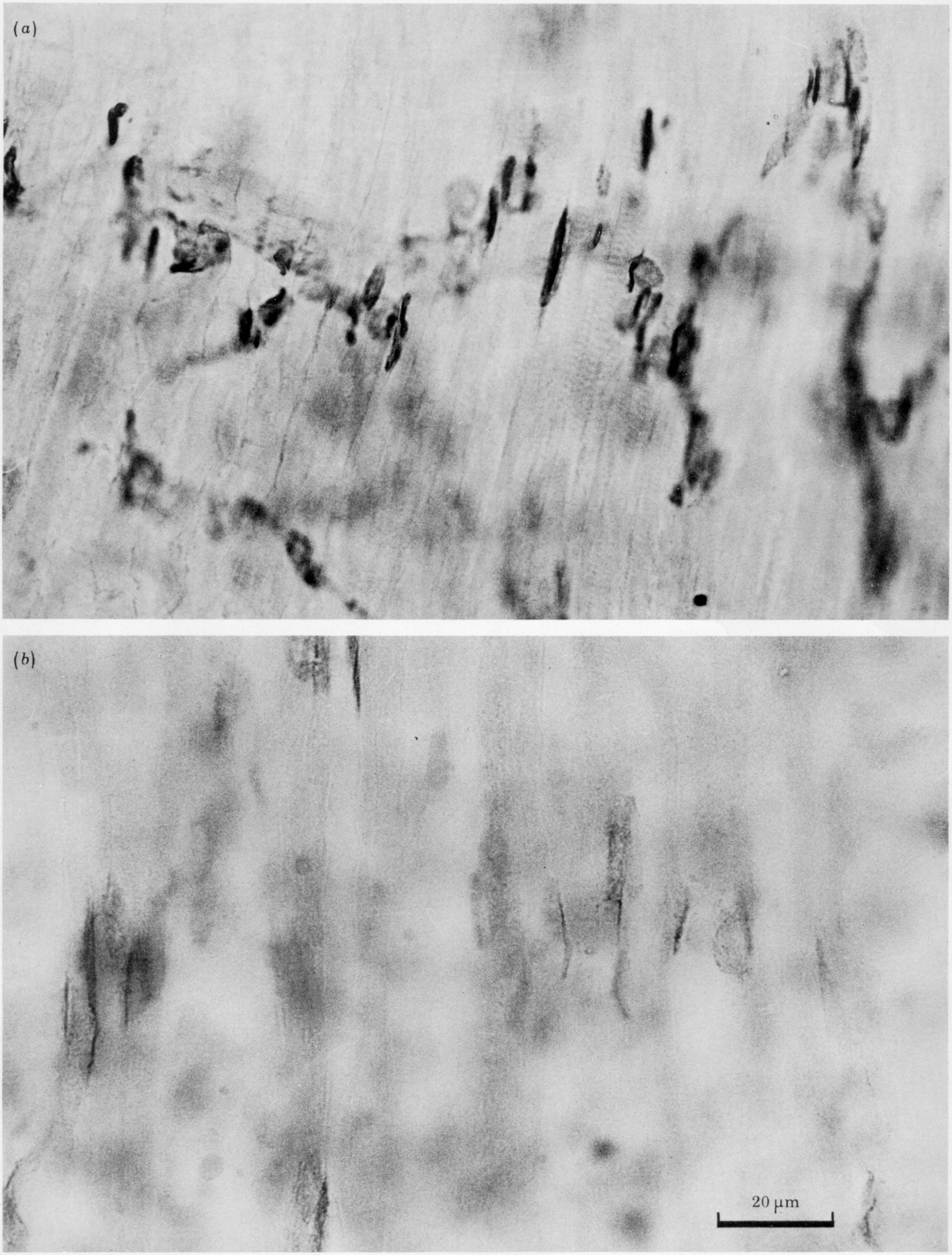

FIGURE 2. Histochemical staining for ChE in a muscle treated with β-BTX at d12: (*a*) d21 control muscle, showing deposits of reaction product at endplates and along nerve trunks (out of focus); (*b*) reaction product in β-BTX treated muscle. Whole mounts of muscles pretreated with 0.5% saponin; control incubated in Karnovsky stain for 15 min; β-BTX treated incubated for 1 h; 37 °C.

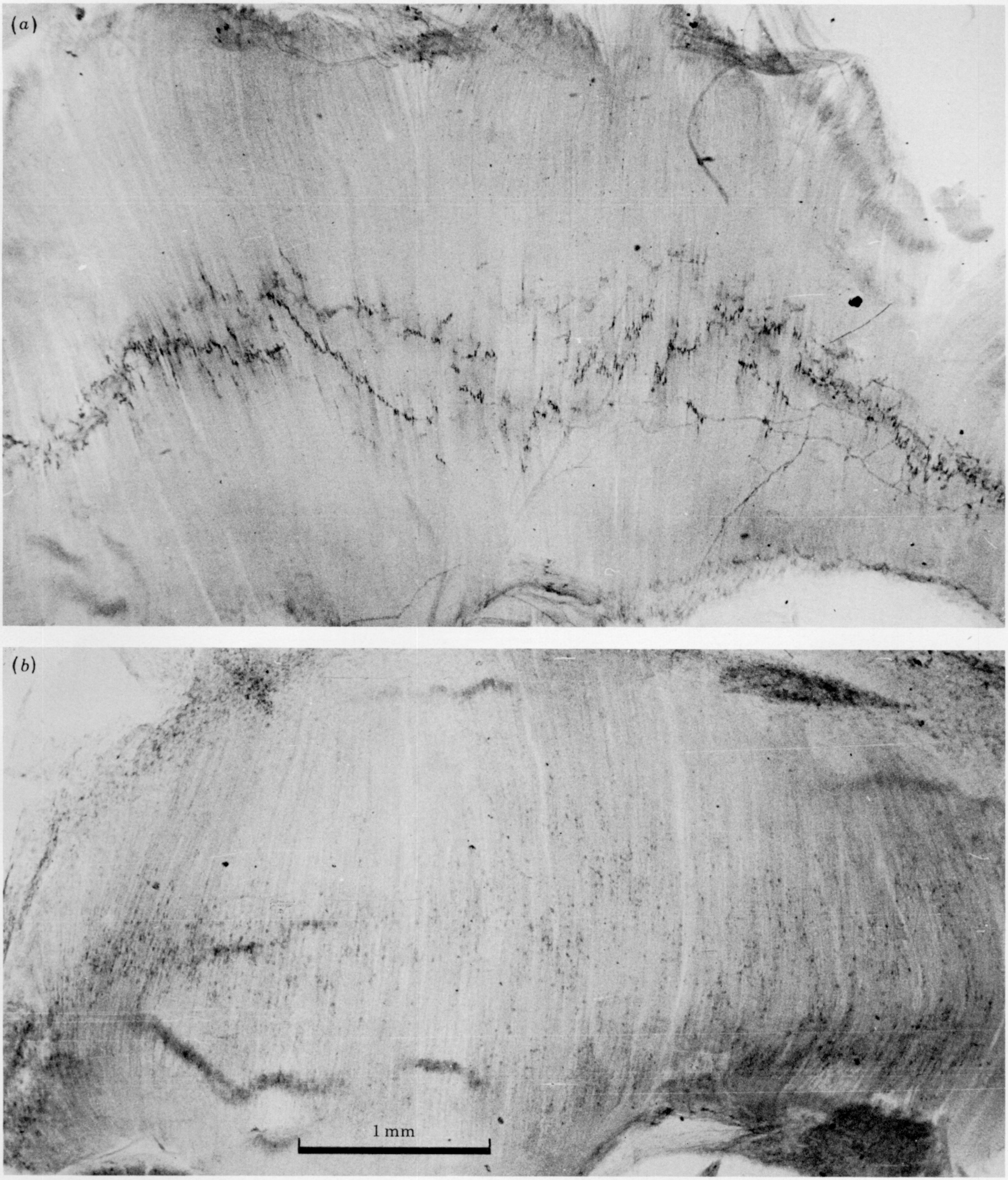

FIGURE 3. Cholinesterase distribution in aneural muscles; low-power views of right hemidiaphragms: (*a*) d20 control muscle; (*b*) muscle treated with β-BTX on d14 and examined at d20. There is considerable scatter in location of deposits of ChE reaction product.

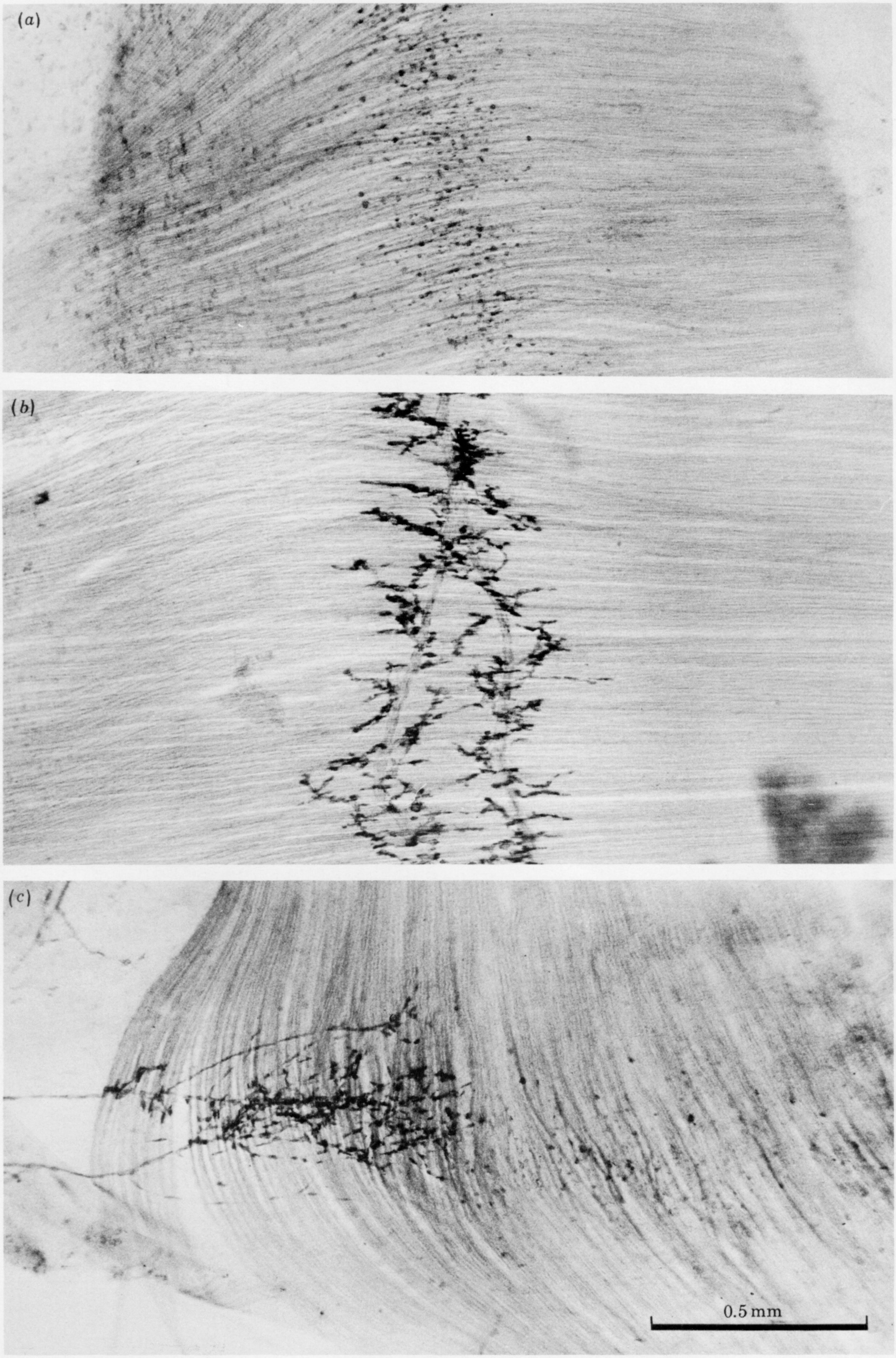

FIGURE 4. Cholinesterase in muscles from embryos twice injected with β-BTX, at d12 and d14, examined on d21
(*a*) Deposits of ChE reaction product in an aneural left hemidiaphragm; (*b*) d21 control, left hemidiaphragm; (*c*) dorsal end of right hemidiaphragm from a treated embryo, to illustrate ingrowth of aberrant innervation.

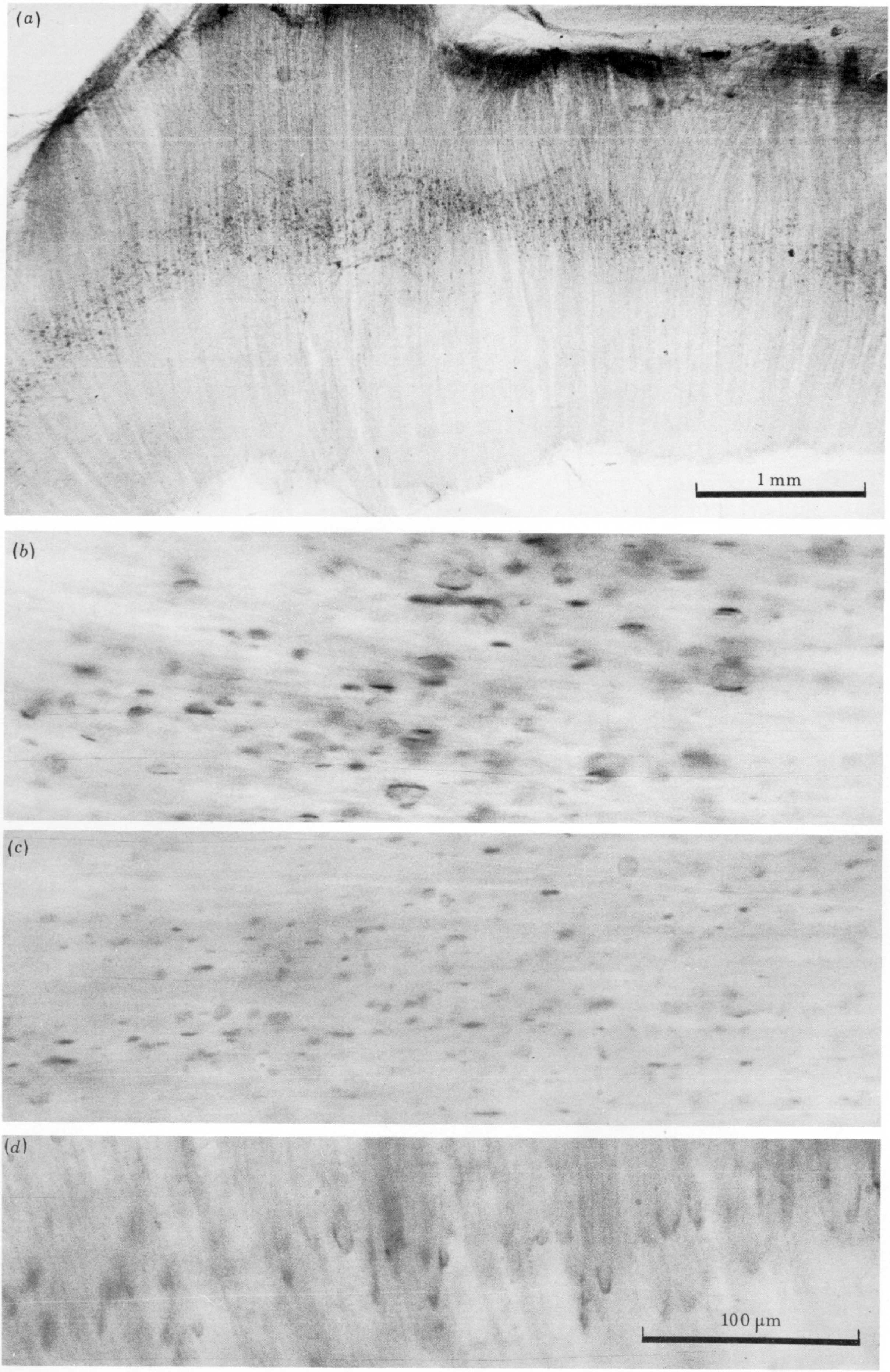

FIGURE 5. Cholinesterase in a muscle treated with β-BTX at d16, examined on d21. (*a*) low-power view, to show distribution of strongly staining deposits (scale bar, 1 mm). (*b*) View of central region of muscle to show 'junctional' deposits; (*c*) view of peripheral region to show 'extra-junctional' deposits; (*d*) deposits on muscle–tendon junctions. Scale bar labelled 100 μm applies to (*b*)–(*d*).