The structure and innervation of tortoise muscle spindles

Abdel Hemid M. F. Ragab

THE STRUCTURE AND INNERVATION OF TORTOISE MUSCLE SPINDLES

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by

Abdel Hamid M.F. Ragab, M.B., B.Ch. (Ain Shams, Cairo)

Department of Zoology, University of Durham

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Durham

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PUBLICATIONS

Preliminary observations on (a) capsular structure as seen in serial sections, and (b) the capsular structure and innervation of the tortoise spindle have been communicated to the Physiological Society:

CROWE, A., & RAGAB, A.H.M.F. (1967). Some preliminary observations on spindle-like structures in tortoise muscle. J. Physiol., Lond. 192, 22 - 23P.

CROWE, A. & RAGAB, A.H.M.F. (1969). The innervation and capsular structure of the tortoise muscle spindle. J. Physiol., Lond. 201, 5 - 6P.

Detailed papers on the anatomy of the tortoise spindle, including a large part of the work presented in this thesis have been accepted for publication:

CROWE, A. & RAGAB, A.H.M.F. (1970). The structure, distribution and innervation of spindles in the extensor digitorum brevis I muscle of the tortoise Testudo graeca. J. Anat. 106, 521 - 538.

CROWE, A. & RAGAB, A.H.M.F. (1970). Studies on the fine structure of the capsular region of the tortoise muscle spindles. J. Anat. (in press).

CONTENTS

I INTRODUCTION

The early work on muscle spindle has been ably reviewed by Regaud & Favre (1904) and the subsequent developments have recently been brought together and discussed by Barker (1968). Therefore only the briefest historical summary of the general outlines will be presented here, together with a more detailed summary of the more pertinent studies viz. those concerning the reptilian muscle spindles. Where relevant, details of studies on the muscle spindles of other species will be discussed in relation to the results of this study.

The term muscle spindle ('Muskelspindel') was first introduced by Kühne, (1863) to describe the structures consisting of groups of relatively small (intrafusal) muscle fibres, enclosed within a connective tissue capsule. Cajal's (1888) original supposition that the extracapsular nerve endings and the intracapsular nerve endings were motor and sensory respectively was confirmed by Onanoff (1890), and Sherrington (1894) as a result of their degeneration experiments.

The number of intrafusal muscle fibres per spindle and the size and number of spindles per muscle, varies widely in different species and in different muscles of the same animal. Muscle spindles are more abundant in limb muscles than in trunk muscles (Hines, 1927).

Most of the work on the morphology of muscle spindles has been done on mammals and amphibia, particularly cat and frog because they are convenient laboratory animals.

The muscle spindles of reptiles have received comparatively little attention. Apart from occasional 'difibrillar' spindles in lizards the spindles of the snakes and lizards are monofibrillar (Cipollone, 1897), but are of two types (Giacomini, 1898; Szepsenwol, 1960), one with a long thin capsule, the other with a short thick capsule. The intrafusal muscle fibre of the former is more or less uniform in structure, well striated at the sensory region and without a nuclear aggregation in that region. This type of spindle is supplied by a single sensory nerve fibre which divides to supply an extensive ending. In contrast the intrafusal muscle fibre of the latter type of spindle lacks the cross striations at the sensory region which has a nuclear aggregation. The single sensory nerve fibre supplies this type of spindle with a restricted type of ending.

The above mentioned findings have been recently confirmed and amplified in respect of structure and innervation (Proske, 1969) and fine structure (Fukami & Hunt, 1970). According to the former author, the intrafusal muscle fibres of the lizard spindle are about 10 μm in diameter which is 20% of the diameter of the extrafusal muscle fibres. The latter study shows that there are no differences in the fine structure of the intrafusal muscle fibres from the two types.

of snake spindle apart from those noted above.

Two types of fusimotor nerve endings (plate and grape) are present and supplied by skeletomotor collaterals. According to Szepsenwol (1960), the long thin capsulated spindles receive plate endings only and the short thick capsulated spindles receive grape endings. Proske (1969) agrees that the short thick capsulated spindles are always supplied by grape endings, and points out that they are absent from muscles lacking grape innervation, and the long thin capsulated spindles may have either plate or grape motor endings.

Hines (1930) reported that muscle spindles of the alligator are multifibrillar (2-5) and each spindle is supplied by only one sensory nerve fibre which terminates in a single morphological type of sensory ending. She reported collateral grape endings on the extracapsular region of the spindles. Cole (1955) reported that the diameter of the intrafusal muscle fibres of the alligator muscle spindles are approximately the same as those of the extrafusal muscle fibres.

There are only two reports on chelonian muscle spindles, the first by Huber and de Witt (1897) on those of the tortoises Emys meleagris and Chrysemys picta, the second by Giacomini (1898) on those of the tortoise Testudo graeca. According to the former study, all the spindles are multifibrillar, while according to the latter both monofibrillar and multifibrillar spindles exist. Both authors agree that tortoise spindles

have only one morphological type of sensory ending, described by Giacomini as flower sprays. Giacomini (1898) reported the presence of both plate and grape motor endings on the intrafusal muscle fibres of the tortoise spindle.

The major changes in concepts of structure and function of the muscle spindles of other species, as well as great improvements and innovations in the techniques available for anatomical studies since these early reports make the study of the anatomy of the muscle spindles of the tortoise both interesting and worthwhile at the present time. Further, because the tortoise occupies a peculiar isolated evolutionary position and has few close relatives, it would be of interest to compare its muscle spindles with those of other species.

The present investigation was made using both the light microscope (LM) and the electron microscope (EM) to study the muscle spindles in the tortoise Testudo graeca. The LM was used to determine the general morphology of the spindle, as well as the innervation and histochemistry of both the intrafusal and extrafusal muscle fibres. Samples of frog and rat muscles were used as a control and for comparative study of their spindles. The EM was used to study the fine structure of the capsular region of the spindles and its sensory nerve endings. Finally, an attempt has been made to correlate the anatomical findings with the present knowledge of the anatomy and physiology of the muscle spindles of

other species.

II MATERIALS AND METHODS

1. The tortoise material

1.1 Choice of muscle

After surveying the different muscles of the tortoise, the hind limb muscle extensor digitorum brevis I (EDBI) was chosen for detailed study. It has virtually parallel fibres which run the whole length of the muscle from origin to insertion. This allows accurate cutting of transverse and longitudinal sections. It is a small, thin, flattened muscle and therefore suitable, not only for LM studies including complete spindle counts, but also for EM preparation. Furthermore the EDBI muscle has other properties which make it suitable for a proposed neurophysiological study. In particular, it is readily accessible and easily separable from the adjacent tissues without causing damage to its nerve and blood supply. It has a good tendinous insertion to the claw and the nerve supplying it can easily be traced to the spinal cord.

The major part of the present investigation was carried out on the EDBI muscle, but for the sake of generalization, the sartorius, biceps brachii and retractor capitis muscles of the tortoise were also used.

1.2 Size of tortoise used

In the present investigation most of the tortoises used were adult females (300-750g) with eggs inside. Although the weights of all the tortoises used varied widely, all the animals selected for a given method were of approximately the same weight. Since smaller animals have comparatively thinner muscles with less connective tissue, these were used whenever the method demanded greater penetration of reagents.

Thus EDBI muscles from small animals (300-450g) were found more suitable for supravital staining with methylene blue. For this method thin muscles with thin epimysia were both effectively oxygenated ensuring better staining, and easier to tease. Small animals were also preferred for EM preparations since this necessitated the use of cytological fixatives known for their poor tissue penetration. The small muscles made it possible to fix the whole muscle, successfully, in situ by immersion.

The larger EDBI muscles from larger animals (600-750g) were easier to orientate for cutting accurate transverse sections, and easier to cut in both paraffin embedded and fresh (unfixed) frozen material (Section 3.1 and 3.31). They were also more suitable for the block silver impregnation method, which overstained the superficial muscle fibres, so that in thinner muscles, virtually all the muscle fibres were overstained.

1.3 Number of tortoises used

In the present investigation a total of 40 tortoises were used. Six EDBI muscles from 5 tortoises (600-750g) were fixed and embedded in paraffin wax for the preparation of serial transverse sections. Four of these muscles were used for a detailed study of their spindles and the remaining 2 were stained and examined for the elastic fibres in the spindles. Two EDBI muscles from a sixth tortoise were used for the determination of shrinkage due to the histological methods (section 5.11).

The muscles of the hind and fore limbs from 3 tortoises (600-750g) were impregnated with silver and provided six EDBI muscles for the study of their innervation. Twenty two EDBI muscles from 11 tortoises (300-400g) were perfused with methylene blue. Eight EDBI muscles from 4 tortoises (600-750g) were used for the study of muscle fibre histochemistry. These animals also provided 1 sartorius, 1 biceps brachii and 4 retractor capitis muscles from which fresh frozen sections for capsular count were prepared, and the single biceps brachii stained by methylene blue by immersion for the study of its innervation. Twenty EDBI muscles from 10 tortoises (300-400g) were processed for the EM study. In addition 6 tortoises (300-450g) were used to determine the optimum conditions for getting good supravital staining of nerves and nerve endings with methylene blue, but no data from this material will be presented.

2. Rat and frog material

Two soleus muscles from 2 albino rats weighing approximately 200g, and 2 gastrocnemius muscles from 2 frogs weighing approximately 20g were fixed and embedded in paraffin wax in exactly the same way as that used for preparing the tortoise muscles. Transverse sections were cut for comparative study.

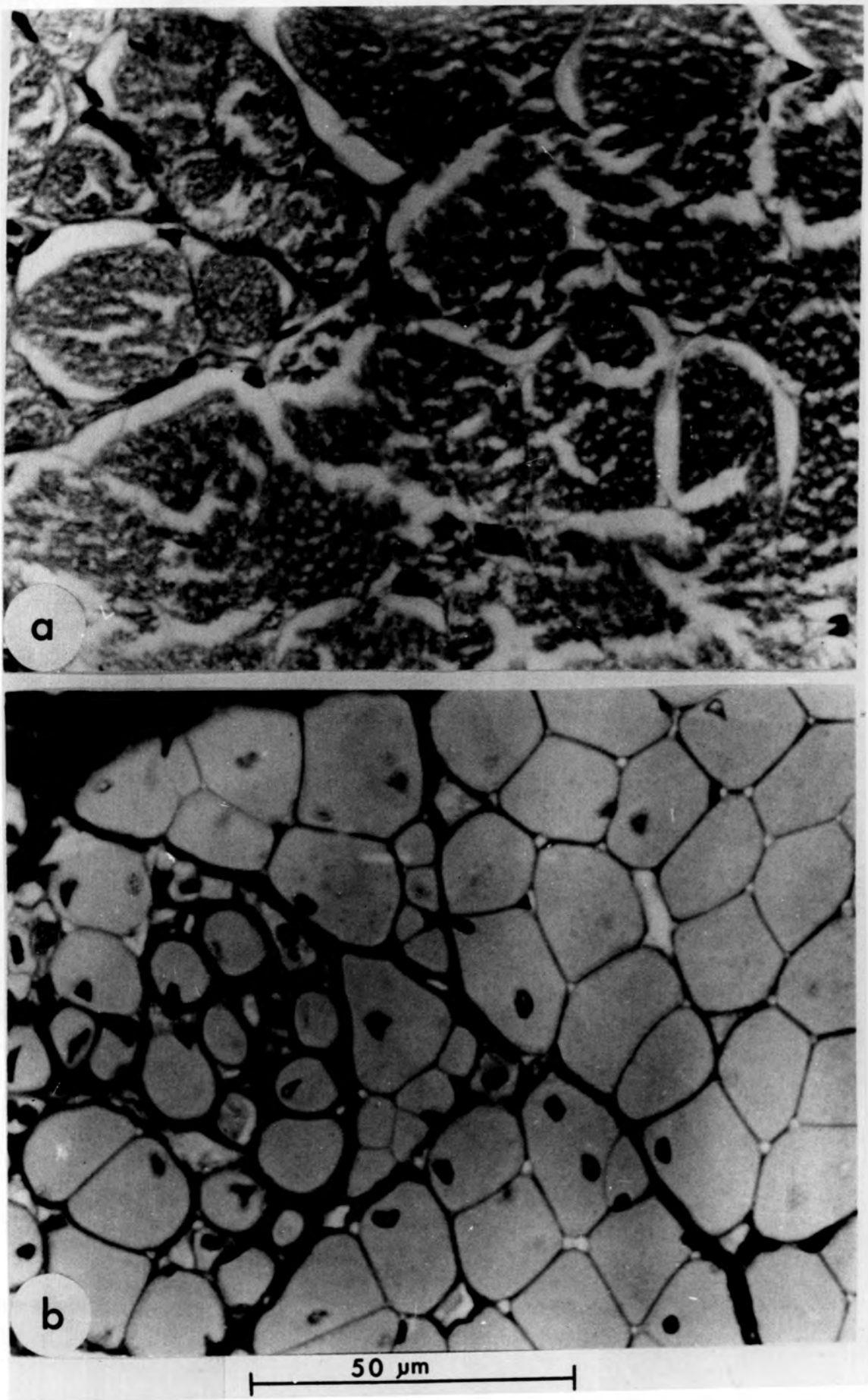
3. Methods for light microscopy

3.1 Preparation of serial transverse sections

3.11 Fixation and embedding techniques

From the beginning of this part of the study difficulty was encountered in fixing and embedding tortoise muscles. Ten per cent formalin in saline did not cause obvious hardening of the tissue, but the extrafusal muscle fibres were broken in transverse sections. The intracapsular part of the intrafusal muscle fibres was poorly fixed and it was not uncommon to see an empty space in this region instead of the muscle fibres (Fig. 1). Bouin's fluid caused the sarcolemma to break up and the myofibrils were scattered outside it. Glutaraldehyde (5% in saline), a more delicate cytological fixative, preserved the superficial layers of the muscle extremely well but left the deeper regions unfixed, and it was therefore rejected. The tortoise muscle was well preserved with Zenker's acetic fluid, but a great deal of shrinkage occured. In some cases it created

FIGURE 1. Transverse sections of tortoise EDBI muscle, to demonstrate the effects of 2 fixatives, (a) 10% Formalin (24 hrs), (b) Susa (see text). To the left of both (a) and (b) is a group of smaller intrafusal fibres. In (a) the muscle fibres are swollen and have undergone partial disintegration and the nuclei, perimysium and endomysium are poorly preserved, in marked contrast to (b) where the muscle fibres are rounded and intact, with the nuclei, perimysium and endomysium well preserved. H & VG.


a
b
50 µm

a false space between the muscle fibres and their endomysium. Susa fixative gave the best results, provided that the tissue was not fixed for more than four hours. Longer fixation caused gradual hardening and shrinking of the tissue. In practice a fixation time of three hours was found to give optimum results (Fig. 1). Ethyl alcohol as dehydrating agent and Dioxan as a dehydrating and clearing agent were both unsuitable because they caused excessive hardening of the material before dehydration was complete. Cellosolve (2-ethoxyethanol) followed by toluene was found to dehydrate and clear the material without causing appreciable hardening.

Sections were obtained most satisfactorily from tissue embedded in a plasticised paraffin wax such as 'Paramat' (Gurr). The sections spread more easily and there was less tendency for them to fold than with material embedded in ordinary paraffin wax.

The following scheme was therefore adopted to prepare muscles for sectioning:-

The EDBI muscles of freshly pithed animals were exposed, the distal part of the hind limb separated at the knee-joint, and the muscles fixed in situ by immersion in Susa fixative for 3 hours. The muscles were dissected out, and dehydrated for 30 min in two changes of 95% cellosolve to which iodine crystals had been added until it was dark brown.

The dehydration was then completed using three changes of absolute cellosolve over 1 hr. The muscles were cleared in toluene for 20 min with two changes and finally embedded in 'Paramat' paraffin wax. The proximal ends of the muscle were labelled.

A rotary microtome (Spencer 820 microtome) was used to cut 10 μm serial transverse sections. Each albuminized microscope slide had ten consecutive sections attached to it.

3.12 Staining techniques

Four of the 6 muscles (2 from the same tortoise and 2 from two different tortoises) were used for an intensive study of their spindles. Alternate slides were stained with Weigert's Iron Haematoxylin and Curtis' Ponceau S substitute for van Gieson (H & VG) (Carlton and Drury, 1957), and Masson's Haematoxylin-Ponceau-Fuchsin-Light Green (Masson's trichrome stain; Humason, 1962). These methods were chosen because they stained the connective tissue a different colour from the other structures. This helped in the identification of spindles. Curtis' Ponceau S stain was preferred because, unlike the van Gieson stain, it was insoluble in both water and alcohol, so it did not come out during washing and dehydrating the sections prior to mounting in D.P.X. Masson's trichrome stain had the further advantage of showing the difference in myofibrillar density in the different types of intrafusal

muscle fibres (Boyd, 1962).

The remaining 2 tortoise muscles together with transverse sections from 2 rat soleus muscles and 2 frog gastrocnemius muscles were stained for elastic fibres using the Orcein stain, Verhoeff's Elastin stain or Weigert-Hart-Resorcin-Fuchsin stain (Humason, 1962) and counterstained with VG.

3.2 Methods for whole spindle preparations

Teased whole spindle preparations were used for the study of the innervation of the muscle spindles. Such preparations have the great advantage that nerve fibres can be traced for considerable distances with more certainty and precision than is possible in reconstruction from serial sections.

The de Castro silver block impregnation and supravital methylene blue techniques were preferred to the gold chloride method because they give better definition of the nerves and their endings. Furthermore it is necessary to tease the muscle into small pieces prior to gold chloride staining (Boyd, 1962), which would not allow the nerve fibres to be traced far enough to see whether they are skeletomotor collaterals.

For both silver and methylene blue methods, teasing was carried out in glycerol using fine mounted needles under a stereoscopic zoom dissection microscope (Bauch & Lomb) at a magnification of x7-x60 using both reflected and transmitted

light. Spindles were mounted in glycerol on microscopic slides, and the coverslips were 'ringed' with pitch before detailed examination.

3.21 Silver impregnation

Cajal (1903) introduced a method for block silver impregnation which was later modified by de Castro (1925). Barker and Ip (1963) modified de Castro's technique to obtain preparations of whole spindles in cat muscle in which the silver is taken up evenly by nerves and their fine endings. However, tortoise muscles that were prepared according to the Barker and Ip method, were found to be rubbery in consistency and difficult to tease, probably due to the different nature of the connective tissue. Furthermore the muscle fibres, their nuclei and the connective tissue nuclei were all too darkly stained to allow proper study of the nerve endings. The method was modified to give the following procedure:

The muscles were dissected out, from freshly pithed animals, labelled and immersed for 6 days in freshly prepared fixative (chloral hydrate, 1g; 95% alcohol, 45 ml; distilled water, 50 ml; conc. nitric acid, 1.25 ml), which was changed every 48 hours. The muscles were then washed for 24 hours in filtered running tap water, blotted, and immersed in 100 ml of 95% alcohol with three drops of ammonia (sp. gr. 0.88) for 48 hours. They were blotted and incubated in a 1.5%

solution of silver nitrate at 37°C for 6 days, renewing the solution every 48 hours. The muscles were then blotted and reduced by immersion for 48 hours in a freshly prepared solution of 2g hydroquinone in 100 ml of 30% formic acid. Finally they were washed for 6 hours in distilled water with 3 changes, cleared in glycerol for at least 7 days, and teased.

This modification of the Barker and Ip method, gave satisfactory results in that the muscle fibres, their nuclei and the connective tissue nuclei were all lightly stained, and nerve fibres and their endings were well stained. It was noticed that the increase in nitric acid concentration facilitated teasing by softening the tough connective tissue, but further increase above 1.25% caused undesirable effects i.e. breaking of the nerve fibres. It was also found that increasing the concentration of the formic acid to 30% facilitated teasing, but further increase made the muscle fibres gelatinous in consistency and was therefore undesirable. The reduction in ammonia concentration resulted in a reduction of the staining property of the muscle fibres and the nuclei, yet further reduction led to poor staining of the nerve endings and a greenish discolouration of the muscle fibres. The only drawback of the method was that it caused a great deal of shrinking.

3.22 Methylene blue

Methylene blue staining although somewhat capricious has the advantage of causing negligible shrinking of the

tissues (Jones, 1966b). Therefore, it was preferred in the studies of nerve fibre diameters. It has the further advantage of being more rapid in execution than the silver method. A modification of Boyd's (1958) perfusion technique was used as follows:

(a) Composition of the perfusate

A solution of methylene blue B, (E. Merck, Darmstadt) dissolved in the Kreb's solution with reduced sodium bicarbonate of pH 6.6 as described by Boyd (1958) failed to stain both the motor and sensory nerve endings, but stained all muscle fibres dark blue. A series of 6 tortoises was perfused with the dye dissolved in Kreb's solution having different pH values obtained by altering the concentration of either the sodium bicarbonate or the potassium dihydrogen phosphate or both. Reduction of sodium bicarbonate did not have any effect, but increasing the concentration of potassium dihydrogen phosphate to bring the pH down to 6.0 stained the sensory endings only, but the stain faded after a few hours. Further increase of the potassium dihydrogen phosphate concentration to bring the pH down to 5.5-5.6 stained the sensory endings better and in some cases stained the motor endings as well. The muscle fibres were lightly stained and the preparations kept for over a year in a refrigerator with only slight fading. The amount of potassium dihydrogen phosphate needed to reach this pH was 5.5 g/l. The other components of the reduced sodium bicarbonate Kreb's solution as described by Boyd (loc.cit.)

were not altered.

The muscles were perfused under as uniform conditions as possible, yet the presence or absence of stained nerve endings was unpredictable. In most cases only the sensory nerve endings were stained; sometimes both motor and sensory endings were stained, rarely no staining occured.

(b) Perfusion apparatus

The apparatus used for perfusion consisted of two 5l reservoirs, one containing normal saline* and the other containing the methylene blue solution. A mixture of 95% oxygen and 5% carbon dioxide was bubbled through both solutions starting 10 min before the commencement of perfusion and continued until perfusion was completed. Both reservoirs were connected via a 'Y' joint to a condenser and thence to a polythene cannula. The temperature of the water circulating through the outer jacket of the condenser was thermostatically controlled to maintain the perfusate at 38°C. The condenser was placed as close as possible to the animal to minimise the drop in the temperature of the perfusate. The height of the reservoirs was adjusted so that the pressure head at the tip of the cannula was equivalent to 90 mm Hg. The rate of flow through the system was regulated by Hoffman clips.

(c) Perfusion procedure

The tortoise was pithed and the plastron separated from the carapace by cutting the bridges uniting them.

The skin was cut where it joined the plastron which was then lifted and separated from the muscles attached to it. The neck and limbs were extended to facilitate the exposure of the dorsal aorta. The proximal 2 cm of the dorsal aorta was dissected away from the adjacent tissues, and two ligatures 1 cm apart were tied loosely around it. The proximal ligature was tightened and used to manipulate the aorta, which was then partially opened between the two ligatures. A polythene cannula was introduced into the lumen of the artery and the distal ligature tightened around it. Care was taken not to push the cannula too far into the artery otherwise its tip entered one of the common iliac arteries, in which case only one limb was perfused. The abdominal veins were severed and normal saline was perfused until the effluent was clear. The saline was shut off and perfusion with methylene blue solution was started and maintained for 20 min. The vessels were flushed again with normal saline for 1 min. The muscles were dissected out immediately and immersed in continuously oxygenated Kreb's solution for 30 min. The muscles were fixed in 8% ammonium molybdate at 4°C for 16 hr, cleared in glycerol for 2 hr and squashed under a glass slide before teasing.

3.3 Histochemical techniques

3.31 Preparation of fresh frozen sections

The animals were pithed, the muscles were dissected

out and slightly stretched to approximately their resting lengths on a piece of card. They were quickly frozen by immersion for 1 min in isopentane, cooled to about -160°C with liquid nitrogen (Maxwell, Ward & Nairn, 1966). They were then taken out, and separated from the card using cooled forceps and frozen to microtome chucks by means of a drop of water and carbon dioxide gas. The chucks with the attached muscles were stored in the cryostat (Slee cryostat with retracting microtome) at -20°C for an hour before sectioning to allow for their temperature to equilibrate with that of the cryostat.

Starting from the proximal end of the muscle, 10 consecutive 10 μm sections were taken and mounted in pairs on 5 microscope slides. Fifteen more 10 μm sections were cut and discarded before a further series of 10 sections was prepared. This procedure was repeated throughout the muscle.

The first slide of each group of 5 so obtained was fixed in 5% glutaraldehyde for 5 min, washed in distilled water and stained with H & VG for the identification and tracing of spindles. The second, third, fourth and fifth pairs were stained for phosphorylase, myofibrillar adenosine triphosphatase (ATPase), succinic dehydrogenase activities, and Sudan Black (for fat content), respectively. Great care was taken to stain all the slides for a given enzyme stain simultaneously and in the same solutions to eliminate apparent variations

in activity due to changes in the staining conditions. In a further attempt at standardization, the staining intensity of fibres was compared with other fibres on the same sections.

3.32 Staining Techniques

For the demonstration of phosphorylase activity, the Takeuchi & Kuriaki (1955) technique, as modified by Eränkö and Palkama (1961) was used. The optimum incubation time for the tortoise muscles was found to be 12 min. The last step in this technique, i.e. mounting the sections in Gram's iodine was omitted. Instead the sections were dried using a hair drier, then mounted in D.P.X. The contrast between the dark and pale fibres was thus greatly improved. Of the histochemical methods used, phosphorylase staining gave the most satisfactory differentiation of muscle fibre types. The method of Nachlas, Tsou, de Souza, Cheng & Seligman (1957) was used for the demonstration of succinic dehydrogenase activity. The optimum incubation time was 20 min. Myofibrillar ATPase activity was demonstrated using the modified Gomori technique of Padykula and Herman (1955a, b). The optimum incubation time was 5 min. It was found that it was of the utmost importance to take the sections out of the cryostat immediately, and to dry them with a hair drier at room temperature for at least 20 min. before staining. Failure to observe these two points resulted in folded sections and detachment

of the sections during washing. The propylene glycol-Sudan method of Chiffelle & Putt (1951) was used to demonstrate the lipid content of the muscle fibres.

4. Methods for electron microscopy

Fixation by perfusing the pithed animal was tried and proved unsuccessful due to either the vasoconstrictor or coagulant effect of the fixative. Instead, the animal was pithed, the muscles quickly exposed and the epimysium split open in several places parallel to the direction of the muscle fibres. The muscles were fixed in situ by immersing the severed distal part of the hind limb in 3% glutaraldehyde, buffered with 0.1 m sodium cacodylate (Mercer & Birbeck, 1966) at pH 7.3 for 4 hours. The individual muscles were dissected out of the limb and washed with 4 changes of buffer for two hours. Ten of the twenty EDBI muscles were then post fixed in chrome osmium (Dalton, 1955) and the remaining muscles were post fixed in 1% osmium tetroxide, buffered with 0.1 m sodium cacodylate at pH 7.3 for 2 hours. After post fixation they were washed in 4 changes of buffer for one hour and dehydrated through a graded series of ethanol; 50% (15 min); 70% (15 min); 95% (15 min) and absolute (3 x 30 min). Up to this stage of preparation the various solutions were at 0-4^{o}C. The next steps were carried out at room temperature.

The muscles were passed through 2 changes of epoxy-propane over a period of 15 min, transferred to a 50/50 mixture

and then a 25/75 mixture of epoxypropane and TAAB epoxy resin (TAAB laboratories) for 1 hour and overnight respectively. They were then transferred to absolute resin for 8 hours with 2 changes, and finally embedded in a third change of resin and kept in oven at 60°C for 2 days to harden. It was found that the most favourable cutting properties were obtained when the resin mixture consisted of: TAAB resin, 50 ml; dodecenyl succinic anhydride, 30 ml; methyl nadic anhydride, 20 ml; 2 ml 2,4,6-tri (dimethylaminomethyl) phenol.

Transverse sections were cut on an LKB ultrotome using glass knives. Transverse sections (1-2 μm) of the whole muscle area, were mounted on microscopic slides and stained with toluidin blue (toluidin blue, 0.1 g; borax, 0.1 g; pyronin, 0.05 g; distilled water, 60 ml) and examined under the LM to find and determine the position of the spindles in the block. The block was trimmed around the spindle and ultra-thin transverse sections (50-60 mμ) were cut. The block was then turned through 90° to prepare for cutting longitudinal sections of the spindle.

The ultrathin sections were picked up on either formvar coated or uncoated 200 mesh copper grids. They were stained for 3 min with lead citrate (Reynolds, 1963) either directly or following 20 min staining in a saturated solution of uranyl acetate in 70% alcohol.

The sections were examined under an AEI EM 6B electron microscope at an accelerating voltage of 60 kV.

On examination the material post fixed in Dalton's chrome osmium had less contrast, but it was noticed that the zone between the plasma membrane and the basement membrane of the cells was stained as well as the membranes themselves (Fig. 40a). With osmium post fixation this zone was unstained (Fig. 40b). Therefore post fixation in Dalton's chrome osmium was preferred because it may be assumed that it stains some cellular components which are not stained with osmium alone. In fact, material post fixed in Dalton's chrome osmium showed glycogen granules much better than material post fixed in osmium. All the electron micrographs presented in this study were from material post-fixed in Dalton's chrome osmium and stained with lead citrate unless otherwise stated.

5. Measurements

5.1 Light microscopy

All LM measurements were done to the nearest 0.5 μm using a x16 Zeiss micrometer eyepiece and x40 objective. The eyepiece micrometer was calibrated using a stage micrometer.

5.11 Correction for shrinkage

The degree of longitudinal shrinking was assessed for each of the 4 serially sectioned muscles by comparing their resting lengths before and after fixation and embedding. It was found to be about 12%. All the muscle and spindle lengths given in the results have therefore been corrected by this factor.

An assessment of transverse shrinking could not be determined for each individual fibre. Instead, 2 EDBI muscles were frozen and transverse fresh frozen sections from the mid-point of both muscles were taken. The remaining parts of the muscles were fixed and embedded in paraffin wax in exactly the same way used for preparing muscles for serial sections and transverse sections were cut. It was thus ensured that the fresh frozen and paraffin embedded sections were taken from the same region of the muscle. Five hundred extrafusal muscle fibre diameters were measured from each preparation, the mean values of the diameters were compared and shrinkage factor of 25% was calculated for the paraffin embedded material. This factor has been applied to all the muscle fibre diameters given in the results.

5.12 Spindle length

The length of the spindles and the individual intrafusal muscle fibres, in serial sections, were obtained by counting the number of the appropriate consecutive 10 μm sections and applying the correction factor for shrinkage.

5.13 Muscle fibre diameters

(a) Extrafusal muscle fibre diameter

The largest and the shortest diameters of 75 muscle fibres were measured in each of 4 sections taken from near the mid-point of each of the 4 serially sectioned muscles. The

mean diameter for each of the 300 muscle fibres was calculated, corrected for shrinkage and plotted on a histogram (fig. 4).

(b) Intrafusal muscle fibre diameter

The longest and shortest diameters of each intrafusal muscle fibre of 4 spindles were measured in every section of alternate sets of 10 consecutive serial sections and the mean diameter for each individual fibre was calculated. Great care was taken to identify each individual fibre in a spindle by numbering each fibre in a sketch drawn for every tenth section, or more often if the fibres changed their relative positions. In this way the changes in the diameters of the intrafusal muscle fibres throughout their entire length were measured, and reconstructions of the intrafusal muscle fibres were made (Figs. 6, 8, 10).

Due to the variation in the diameter of the intrafusal muscle fibres revealed by the above method (see Results 5.2) it was decided to measure the diameters of all 282 intrafusal fibres in the 27 spindles, used for the detailed study, at two standard points. The mid-polar points were selected because the variations in diameter were least at these regions. The diameter of each intrafusal muscle fibre was measured on ten consecutive sections at these two points and the corrected mean value calculated and a histogram plotted (Fig. 4).

5.14 Nerve fibre diameters

(a) Sensory

In methylene blue preparations only the sensory fibre diameters were measured, because not enough of the motor nerve fibres were sufficiently well stained to constitute an adequate sample for analysis. Sensory nerve fibres were measured 1 mm from spindle entry. Due to the irregularity of the thickness of the myelin sheath, 6 measurements of the total internodal diameters were usually taken for the same internode and the mean calculated. A total of 50 such means were plotted as a histogram (Fig. 36).

(b) Motor

Motor nerve fibres were measured in silver impregnated material which suffered from severe shrinkage. Stacey (1969) calculated a factor of 1.41 for converting measurements of myelinated nerve fibres stained with silver into their equivalent diameters in fresh material.

The total internodal diameters of 25 motor nerve fibres terminating in grape endings and 25 motor nerve fibres terminating in plate endings were measured just before the fibre branched to give rise to the fusimotor collaterals. The technique of measurements was the same as in the methylene blue preparations, and the means were corrected for shrinkage using Stacey's factor.

5.2 Electron microscopy

In order to make accurate measurements on electron micrographs, it is necessary to calibrate the magnification of the EM. This was carried out using a grating carbon replica (Ernest F. Fullam, Inc., New York) and the formulae provided with it to arrive at the precise magnification at which the micrographs were actually taken.

Measurements of mitochondrial diameters were carried out on electron micrographs of final magnification of x25,000. Only the diameters of those mitochondria in transverse section were measured. Measurements of finer structures such as microvesicles, microtubules, multivesicular bodies, and the gaps between membranes were measured on electron micrographs of final magnification of x100,000.

6. Identification of sensory and motor endings

Since differential denervation was not attempted, the identification of sensory and motor nerves and their endings was made according to the following criteria. Sensory nerve endings were assumed to resemble, in form and location, the established sensory nerve endings found in the spindles of other species, while motor nerve endings were assumed to resemble the motor nerve endings on the extrafusal muscle fibres. Sensory nerve fibres would not be branches of those supplying extrafusal muscle fibres, but motor nerve fibres

might be. In silver stained preparations, where the capsule was visible, nerve endings within the capsule were considered to be sensory; those on the polar regions were considered to be motor. Since the study of serial transverse sections had shown that the intrafusal muscle fibres had two points of maximum diameter, approximately coincident with the limits of the capsule (Results, section 5.2), these could be used to delimit the 'capsular' region. A nerve ending was considered to be sensory if it was seen to lie between the two positions of peak diameter, in those methylene blue preparations where the capsule was not clearly seen. In those methylene blue preparations in which skeletomotor nerve endings were not stained it was assumed that the fusimotor endings would not have stained either, and the endings of the single, thick, heavily myelinated nerve fibre entering the spindle were regarded as sensory.

With the EM, the criteria used to identify the sensory endings were those of Merrillees (1960), Katz (1961) and Landon (1966). Thus the sensory endings contain aggregations of mitochondria and few vesicles; lack a Schwann cell cover but might be associated with a satellite cell. Further, the sensory endings are enclosed within the basement membrane of the intrafusal muscle fibre (hypectolemmal; Katz, loc. cit.) in contradistinction to the motor endings which lie above the basement membrane (epectolemmal; Katz, loc. cit.). Finally, the sensory myoneural junction is usually smooth with no

junctional folds.

III RESULTS

1. Structure of the extensor digitorum brevis I muscle

The EDBI muscle lies under the extensor digitorum longus muscle except at its origin where it is covered by the peroneal muscles (Fig. 2). It takes origin from the lower quarter of the lateral surface of the fibula, passing downwards and medially over the tarsals, metatarsals, phalanges and the deeper muscles attached to them, and inserts into the dorsal surface of the distal phalanx and the claw of the first toe by a small tendon. The tendon has a short extension on the lateral side of the distal 15-20% of the muscle, so that the fibres on the medial side are therefore slightly longer than those on the lateral side. At its origin the muscle is wide, tapering as it approaches the insertion. The dorsal surface is convex and the plantar surface has a slight concavity where the muscle overlies the deeper structures, so that it is shaped like one of the halves of a truncated cone that has been split down its axis, and any transverse section of the muscle has a 'D' shape. The fibres run the whole length of the muscle from origin to insertion. All of the fibres, except those lying on the extreme medial and lateral sides, cross the axis of

FIGURE 2. The dissected left hind limb of the tortoise *T. graeca* showing the extensor digitorum brevis I muscle (EDBI) *in situ*. It can be seen that some of the muscle fibres cross the long axis of the muscle (arrow).

C - claw of the first digit (insertion of EDBI)

EDBI - extensor digitorum brevis I muscle

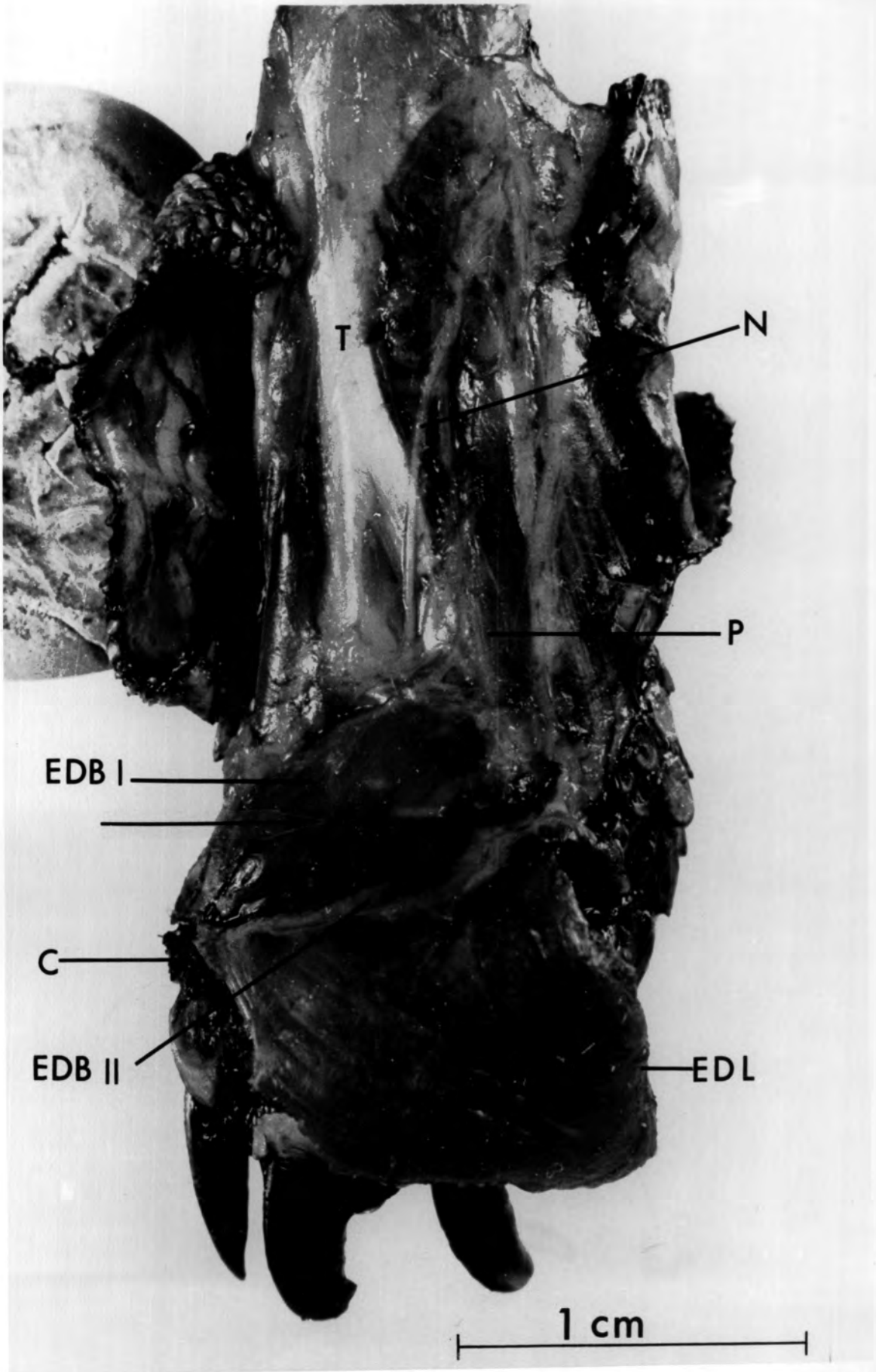
EDBII - extensor digitorum brevis II muscle

EDL - extensor digitorum longus muscle

N - branch of the sciatic nerve

P - peroneal muscles

T - tibia


T
N
P
EDB I
C
EDB II
EDL
1 cm

the muscle (Fig. 3).

The muscle is innervated by a branch of the sciatic nerve which divides into 2 or 3 branches just before its entry into the muscle. The nerve enters the plantar surface of the muscle, accompanied by a branch of the sciatic artery, at a point midway between origin and insertion.

In transverse sections the muscle has a distinct epimysial layer of connective tissue which sends in trabeculae of perimysium subdividing the muscle into several fasiculi. Each muscle fibre has a well defined endomysial layer.

Figure 4 is a histogram of the mean diameters of 300 extrafusal muscle fibres. The mean value was found to be 27.3 μm with a standard deviation of 5.71 μm.

2. Number, distribution, and position of the spindles

Table 1 shows the number of spindles in each of the 4 muscles studied in serial transverse sections, the number of intrafusal muscle fibres in each spindle, the lengths of the individual spindle, and length and position of the capsules. Although these muscles were taken from animals of similar size, there was a great variation in their spindle content. Particularly interesting was the result that the 2 EDBI muscles C and D, of the same animal, contained 4 and 10 spindles respectively. An assessment of the number of spindles

FIGURE 3. Diagram of EDBI to show the arrangement of the extrafusal muscle fibres (arrows) and spindles (Sp) relative to the long axis of the muscle. Only the fibres at the extreme medial and lateral sides of the muscle do not cross the long axis of the muscle. To the right of the figure transverse sections through different levels of the muscle show the change in the position of a spindle as it passes through the muscle. The proximal end of the spindle is attached to the upper surface of the muscle and the distal end to the lower surface.

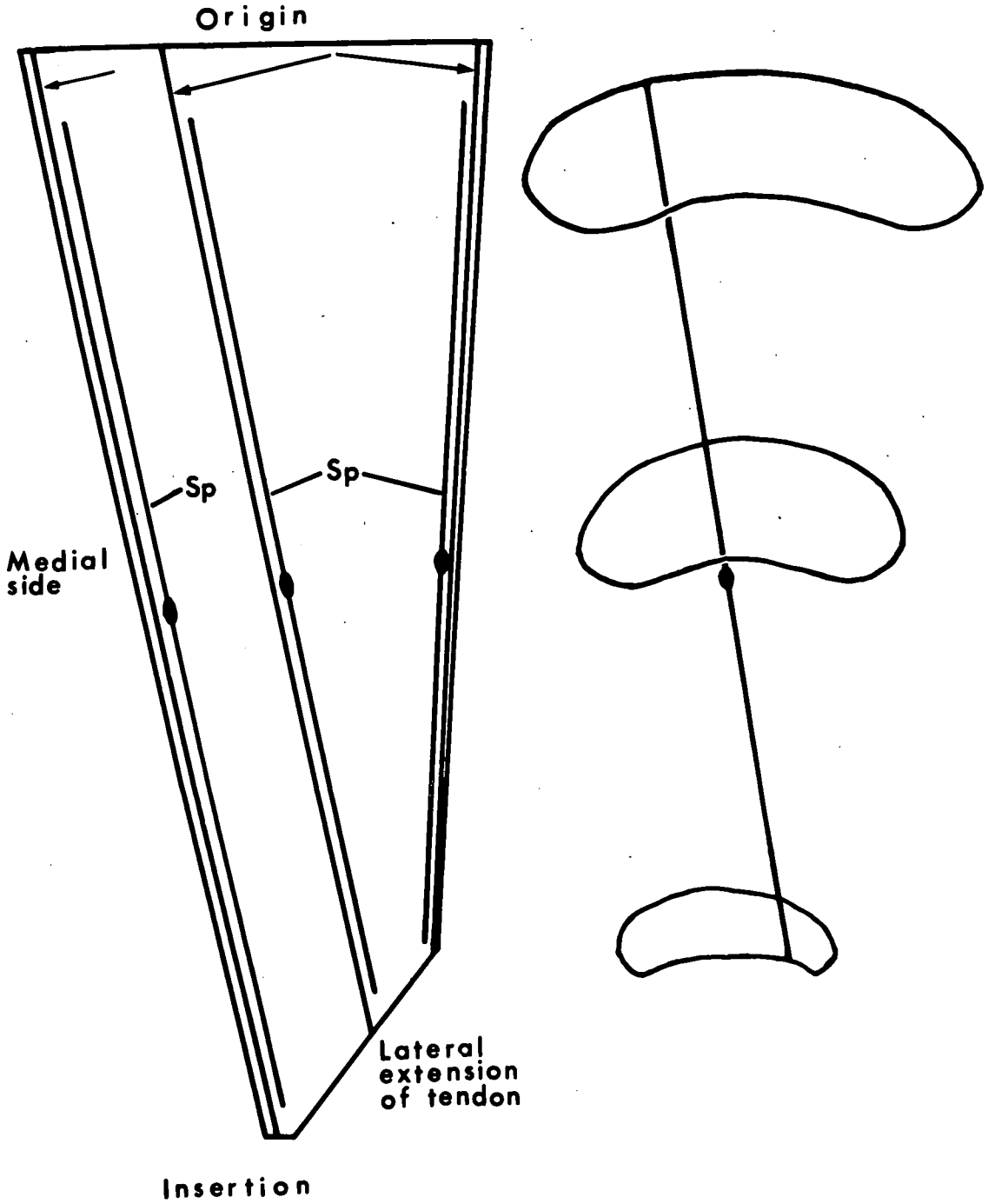

Origin
Sp
Sp
Medial side
Lateral extension of tendon
Insertion

FIGURE 4. Distribution of the mean diameters of 282 intrafusal muscle fibres (unshaded columns) and 300 extrafusal muscle fibres (shaded columns) measured in serial sections stained with Masson's trichrome or H & VG. The mean diameter of the intrafusal muscle fibres is 10.6 μm (S.D. 2.30 μm), and the mean diameter of the extrafusal muscle fibres is 27.3 μm (S.D. 5.71 μm).

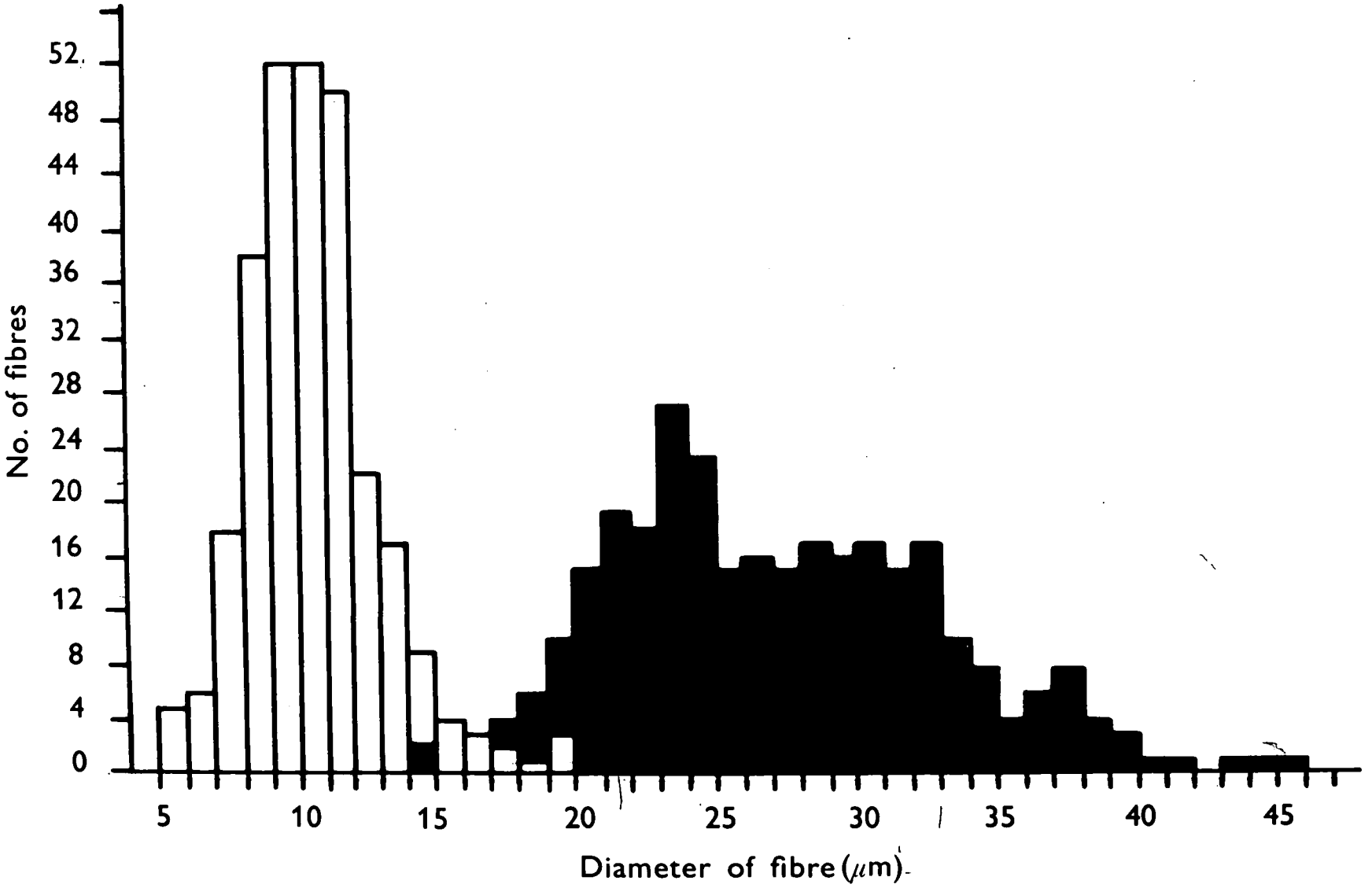

No. of fibres
52
48
44
40
36
32
28
24
20
16
12
8
4
0
5
10
15
20
25
30
35
40
45
Diameter of fibre (μm)

TABLE I

Principal data of 27 muscles spindles studied in the serial sections of four muscles

(In the case of the tandem spindles, data for both capsules are shown but not included in the calculation of mean capsular position (Column 6).

	Spindle Number	No. of Intrafusal muscle	Spindle length (mm)	Length of capsule (mm)	Distance of mid-capsule from proximal end of spindle (mm)
Muscle A. Length	A1	7	9.5	1.1	4.7
14.1 mm. From	A2	13	10.3	1.3	5.5
left leg of	A3	12	10.2	1.0	2.8
animal No. I	A4	10	10.2	0.9	1.9
Mean values		10.5	10.05	1.1	3.7
Muscle B. Length	B1	7	11.4	1.8	4.9
15.4 mm. From	B2	11	9.4	1.0	7.5
left leg of	B3	3	9.4	0.7	7.2
animal No. II	B4	8	9.3	1.3	4.0
	B5	8	10.5	0.5	3.5
	B6	14	11.0	0.8	2.5
	B7	16	9.9	1.3	5.8
	B8	7	10.2	1.4	6.3
	B9	4	10.1	1.1	6.2
Mean values		8.7	10.13	1.1	5.3
Muscle C. Length	C1	11	9.6	1.7	4.7
12.9 mm From	C2	13	10.2	1.9	4.9
left leg of	C3	11	9.6	0.8	3.8
animal No. III	C4 (tandem)	17	8.3	0.7, 1.0	1.1, 5.9
Mean values		13.0	9.43	1.22	4.4
Muscle D. Length	D1	9	8.2	0.9	6.5
12.5 mm. From	D2	2	8.7	0.6	6.2
right leg of	D3	17	6.9	0.8	5.6
animal No. III	D4	10	8.5	0.8	5.1
	D5	17	8.6	1.3	2.0
	D6 (tandem)	8	7.9	0.8, 0.6	2.2, 3.5
	D7	16	9.7	1.1	2.2
	D8	14	8.0	0.9	4.9
	D9	11	9.4	1.1	5.0
	D10	6	7.0	1.3	2.9
Mean values		11.0	8.29	1.02	4.4

in the muscles prepared for teasing could not be made, because it must be assumed that some of the spindles were lost during teasing, and others failed to take the stain. In any case, many of the muscles were taken from animals that were rather smaller than those used for the preparation of serial sections.

The spindles did not aggregate in a particular part of the muscles, but were evenly distributed throughout them. Each spindle ran the greater part of the length of the muscle, so that when the lengths of the spindles in each muscle were calculated as percentages of the total length of the muscle, the mean values for muscles A, B, C and D were 71.27%, 65.79%, 73.05% and 66.32%, respectively.

All the spindles in the muscles were arranged so that the intrafusal muscle fibres ran parallel to the extrafusal muscle fibres. Most of the spindles (74.0%) were contained within the extrafusal muscle fibres which crossed the axis of the muscle, the remaining spindles (26.0%) were located among those extrafusal muscle fibres on the extreme lateral and medial sides of the muscle which did not cross its axis (Fig. 3). This is precisely the result that one would expect if the spindles were evenly distributed throughout the muscle, since the majority of the muscle fibres crossed the axis of the muscle.

The proximal ends of the spindles were attached to

the epimysium covering the dorsal surface of the muscle, and their distal ends were attached to the epimysium covering the plantar surface. The mean distances between the origin of the muscles and the proximal ends of the spindles were 2.4 mm, 3.0 mm, 2.2 mm and 2.2 mm for muscles A, B, C and D respectively. The mean distances between the insertion of the muscle and the distal ends of the spindles were 1.7, 2.2, 1.1 and 1.9 mm for muscles A, B, C and D respectively. So it could be said that the spindles were placed very slightly nearer to the insertion of the muscles than to the origin.

3. General structure of the spindles

The following 4 sections will deal with the general features of the tortoise spindle based on a study of a total of 143 spindles. The data were mainly obtained from 27 spindles found in the 4 muscles that were serially sectioned and extensively studied (Table I), as well as from 22 spindles studied histochemically, 82 teased whole spindle preparations and 12 spindles used for the EM study.

3.1 Spindle length

Table I gives the mean spindle length for the 27 spindles as 9.3 mm (range 6.9 - 11.4 mm). It is seen that the spindles of the longest muscle, B (15.4 mm) had a mean length of 10.13 mm, whereas the shortest muscle, D (12.5 mm),

had a mean spindle length of 8.29 mm. Furthermore, the longest spindle (11.4 mm) was in muscle B, and spindles of less than 8 mm long were found only in muscle D.

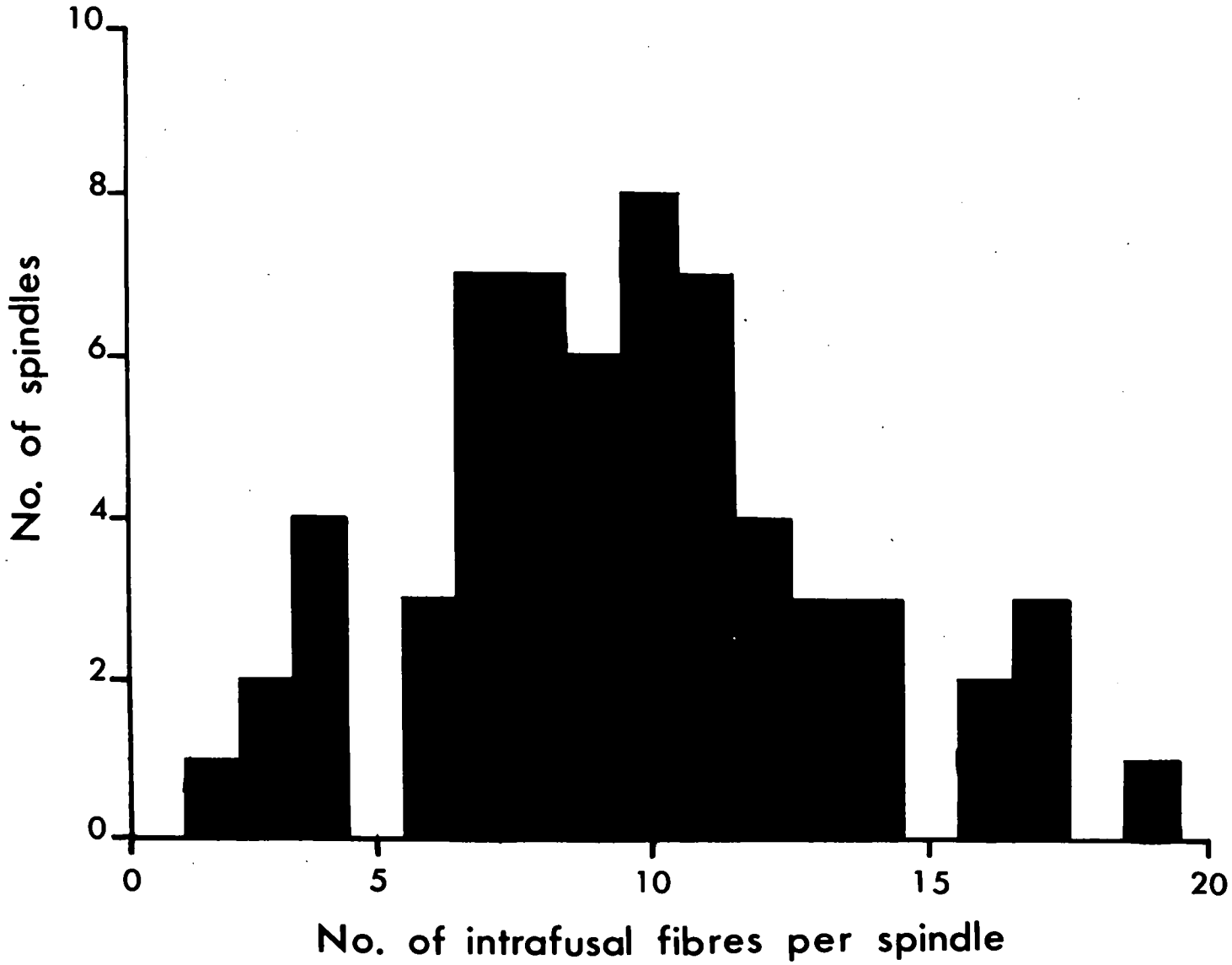
3.2 Number of intrafusal muscle fibres

The mean number of intrafusal muscle fibres in the 27 spindles in the muscles examined in serial sections was 10.4 (range 2-17, Table I). If the data from 22 spindles examined in the histochemical study, and the 12 spindles examined in the EM were included, the mean number of intrafusal muscle fibres of these 61 spindles was 9.7 (range 2-19). Figure 5 shows a histogram of the number of intrafusal muscle fibres per spindle plotted against the number of spindles. Although all the muscles were carefully examined for the presence of the monofibrillar spindles as described by Giacomini (1898), none was found in the tortoise muscles used in the present study.

3.3 Position of the capsules

Within the muscles the capsules were always continuous at one side with either a trabecula of perimysium or the epimysium (Figs. 7, 9, 11) depending on whether the capsule was lying deeply in the muscle or superficially. In fact, of the 27 spindles in serial transverse sections, 20 had their capsules deep in the muscle and were connected with the

FIGURE 5. Distribution of the number of intrafusal muscle fibres per spindle, for 61 spindles. The mean is 9.8 (S.D. 3.86).


10
8
6
4
2
0
No. of spindles
0
5
10
15
20
No. of intrafusal fibres per spindle

perimysium. The remainder were superficially placed and continuous with the epimysium.

Of these 27 spindles, 25 were of the 'single' type with one encapsulated region and only 2 spindles were of the 'double'tandem' type, i.e. with two successive encapsulated regions. A third 'double tandem' spindle was found in the 82 teased whole spindle preparations.

3.31 Single spindles

The mean distance between the mid-capsular point and the proximal end of the 25 single spindles was 4.6 mm. The relative position of the midpoint of the capsule on a spindle can be calculated by expressing the distance (X) between the mid-capsular point and the proximal end of the spindle as a ratio of the total length of the spindle (L). The mean of these ratios for the 25 single spindles was 0.49 with a standard deviation of 0.184. Thus although the mean position of the capsule is half way along the spindle, the standard deviation is high indicating a great degree of dispersion around the mean.

3.32 Tandem spindles

Tandem spindle D6 (Table I) was 7.9 mm long, the proximal and distal capsules were respectively 0.8 and 0.6 mm long and were 0.6 mm apart. The mid-points of the proximal and

distal capsules were 2.2 and 3.5 mm respectively from the proximal end of the spindle, so that both capsules were in the proximal half of the spindle, and the distal pole was twice the length of the proximal pole.

The other tandem spindle (C4, Table I), was 8.3 mm in length, the proximal and distal capsules measured 0.7 and 1.0 mm respectively, they were widely separated by an inter-capsular region of 3.9 mm. The mid-capsular point of the proximal and distal capsules were 1.1 and 5.9 mm respectively from the proximal end of the spindle, so that the proximal capsule was placed near to the proximal end, and the distal capsule near to the distal end of the spindle. This spindle will be described in detail in the next section.

3.4 Structure of individual spindles

In this section a detailed account on the general structure of two 'single' spindles and a 'tandem' spindle will be given to show the important features of the tortoise spindle with its variations. Reconstructions of these spindles (Figs. 6, 8, 10) show the corrected lengths and diameters of their intrafusal muscle fibres and the length and position of their capsules. Selected transverse sections from different levels show the important features of each spindle (Figs. 7, 9, 11).

Spindle B1 may be considered as a typical 'single' spindle which had 7 intrafusal muscle fibres, all of which

were of the same length except fibre 1 (Fig. 6), which was 0.8 mm (7.0%) shorter than the others which were 11.4 mm long. The capsule was 1.8 mm in length with a mid-capsular point 4.9 mm from the proximal end of the spindle and was continuous with a band of perimysium on one side. All the intrafusal muscle fibres maintained a reasonably uniform diameter from origin to insertion, except for the characteristic variation in the capsular region (see section 5.2), i.e. they increased in diameter reaching their maximum diameter near both poles of the capsule, and decreased as they traversed the capsule, to a minimum at the mid-capsular region. However fibre 7 did not show a marked decrease in diameter in its intracapsular region and in fibres 4 and 5 the decrease was irregular in the intracapsular region showing a third small peak. It can be seen that the two peak values in the diameter of an intrafusal muscle fibre were of approximately the same size, but fibre 6 showed a considerably greater increase in diameter at the proximal pole of the capsule as compared to that at the distal pole. The relationship of the diameter change to the capsule is illustrated in Fig. 7. In Fig. 7e fibres 1, 2 and 7 were still large and not yet encapsulated, while fibres 3, 4, 5 and 6 were encapsulated and small. In Fig. 7f all the fibres were small and encapsulated.

FIGURE 6. Reconstruction of spindle B1 typical of EDBI. The limits of the capsule are indicated on each intrafusal fibre by white lines. The corrected lengths and diameters of the fibres are to scale; the relative positions of the intrafusal fibres are diagrammatic. The lengths of the fibres from the proximal end of the spindle are shown on the vertical scale. The levels from which the transverse sections shown in Fig. 7 were taken are marked a - i.

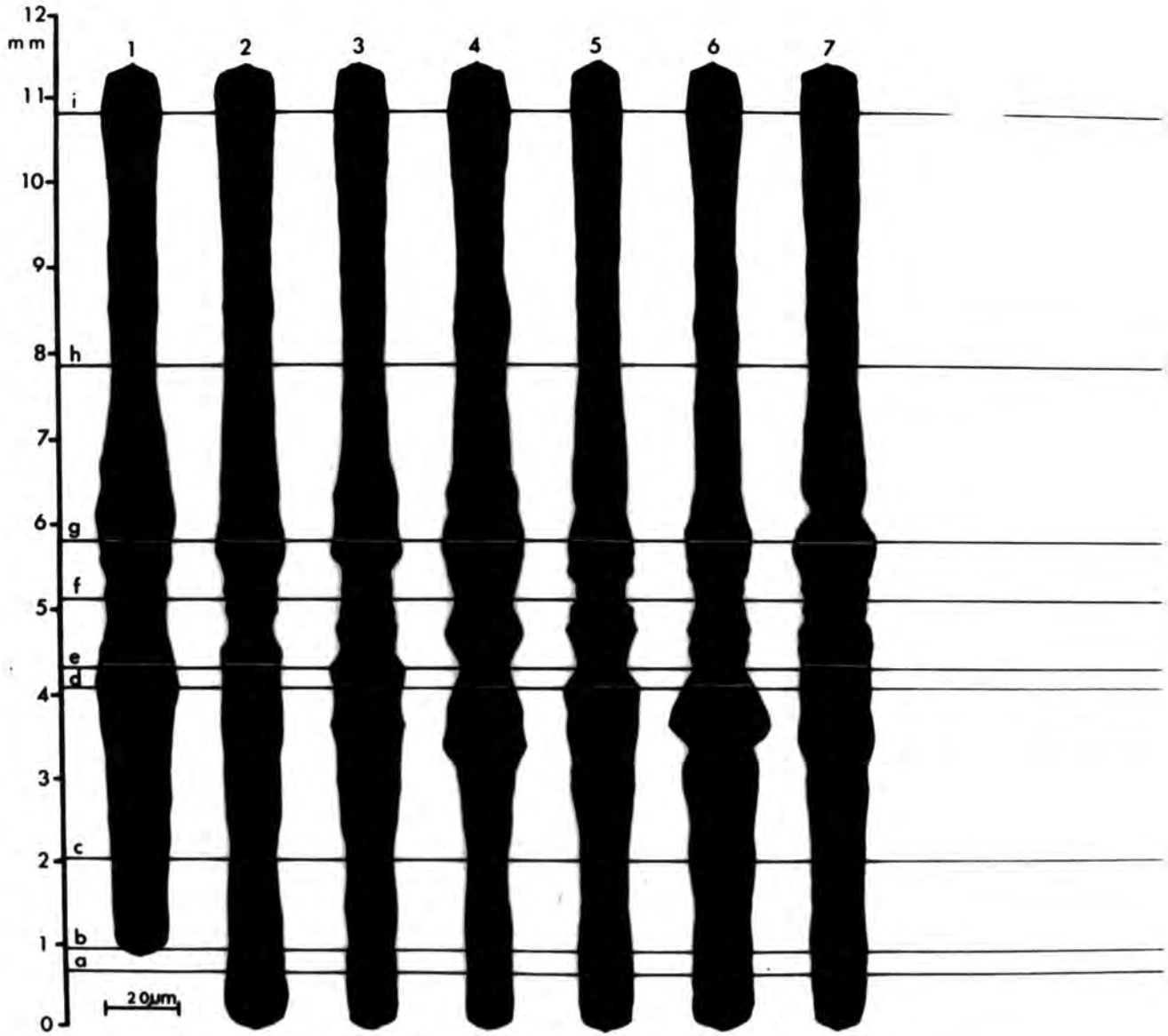
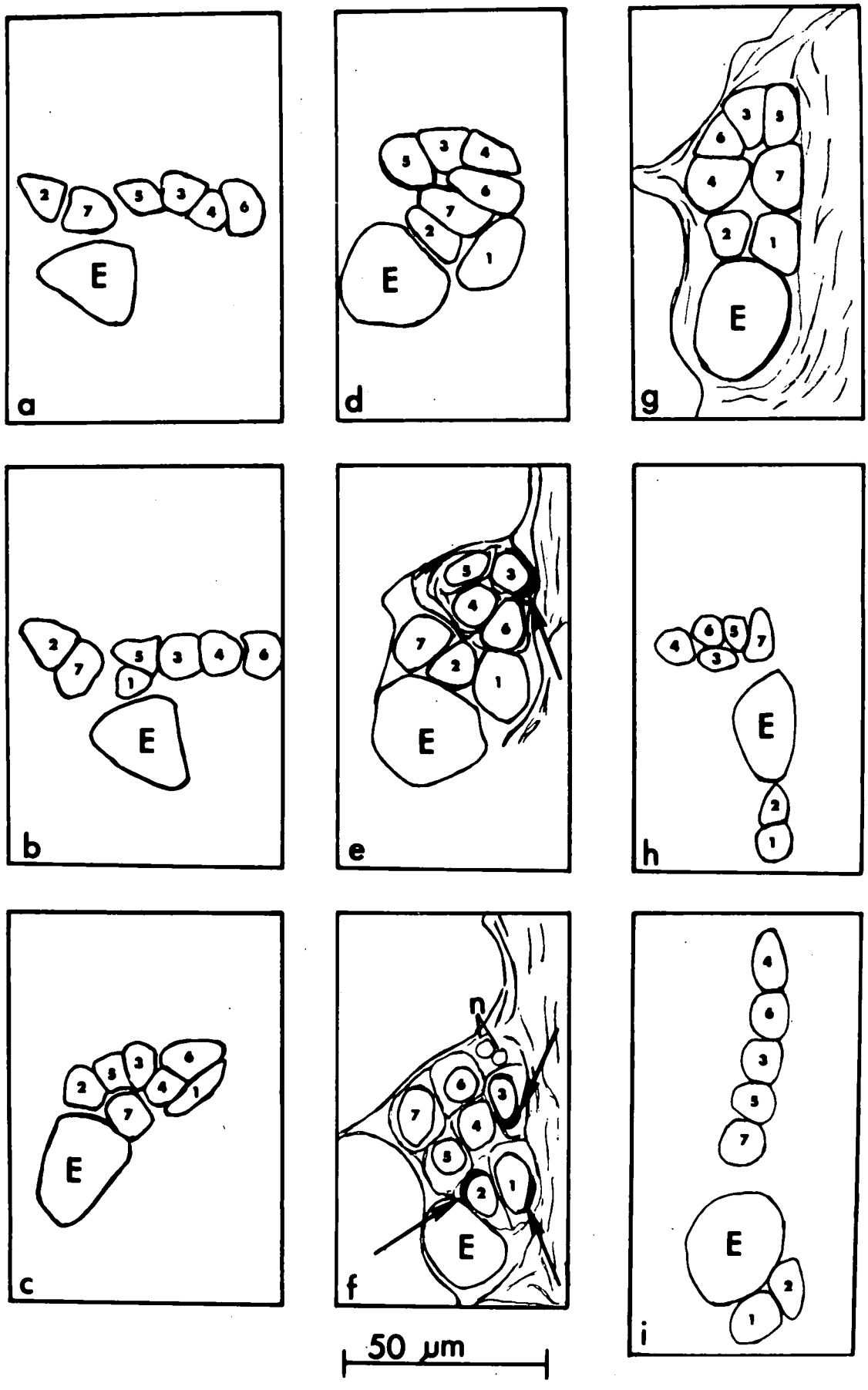
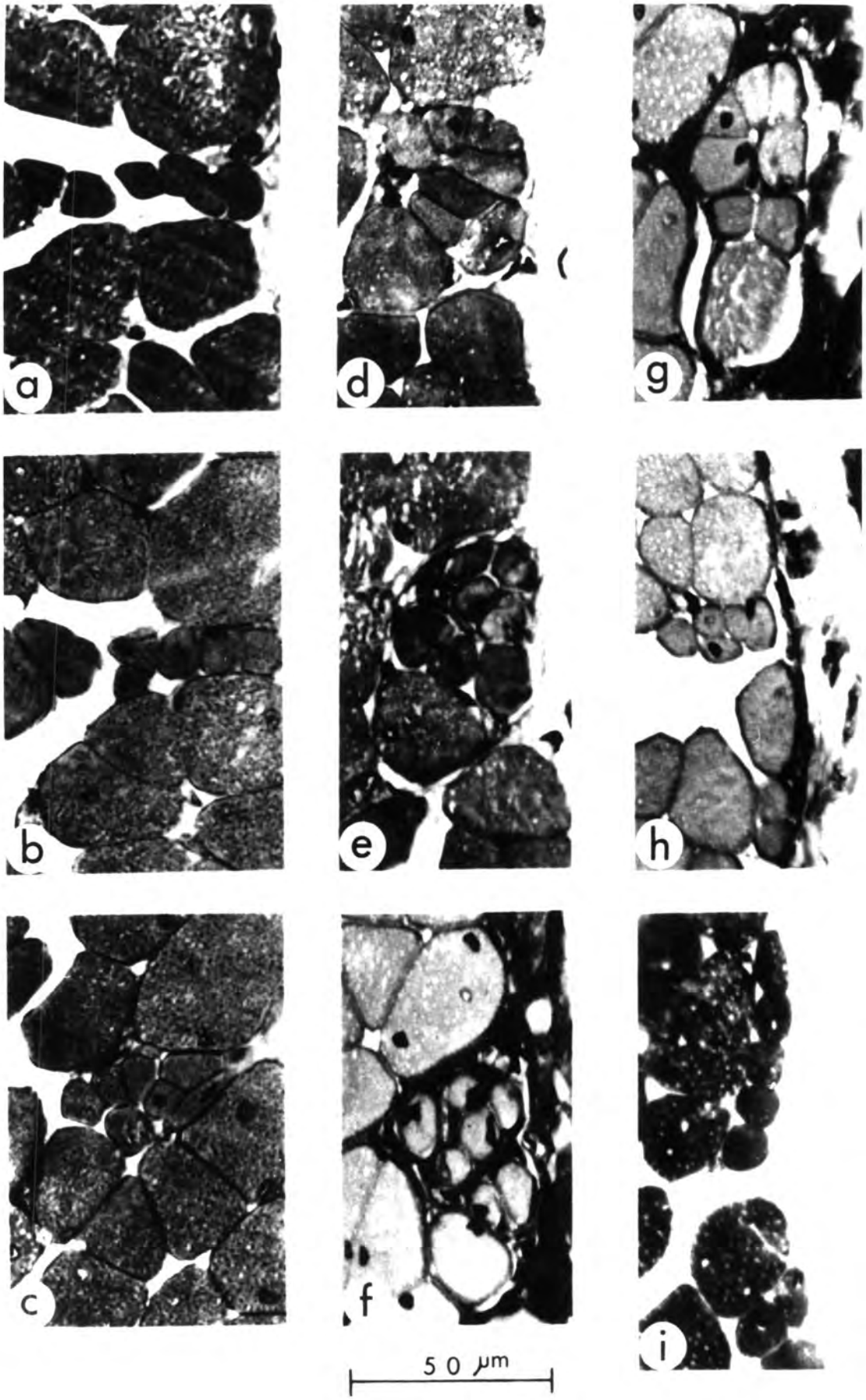

12
mm
11
10
9
8
7
6
5
4
3
2
1
0
i
h
g
f
e
d
c
b
a
1
2
3
4
5
6
7
20µm

FIGURE 7. Transverse sections of the 'typical' spindle B1. A key identifying the individual intrafusal fibres is given on the facing page. Note the changes in diameter, grouping and relative positions of the intrafusal fibres. An extrafusal fibre (E) is closely associated with the spindle. Nerves (n) and satellite cell nuclei (arrows) are present. Sections f, g and h are stained with H & VG, and sections a - e and i are stained with Masson's trichrome.


a
E
2
7
5
3
4
6
d
E
5
3
4
6
7
2
1
g
E
3
5
6
4
7
2
1
b
E
2
7
5
3
4
6
1
e
E
5
3
4
6
7
2
1
h
E
4
6
5
7
3
2
1
c
E
2
5
3
6
4
1
7
f
n
E
6
3
7
4
5
2
1
i
E
4
6
3
5
7
2
1
50 µm


a
d
g
b
e
h
c
f
i
50 µm

This spindle showed the characteristic separation of the intrafusal muscle fibres as they approached the spindle poles until finally they were no longer tightly grouped together, as in the capsular region, but were arranged in linear formation (Fig. 7).

The intrafusal muscle fibres were separated by an extrafusal muscle fibre in the distal pole (Fig. 7), which remained in close association with the spindle and its capsule for its whole length.

Spindle D2 was selected for reconstruction because it consisted of only 2 intrafusal muscle fibres and had a capsule which was situated nearer to one end than the other. The capsule was 0.6 mm long with a mid-capsular point 6.2 mm from the proximal end of the spindle (Fig. 8). The 2 intrafusal muscle fibres had equal length (8.7 mm) and, apart from the characteristic variation in diameter in the capsular region they maintained a more or less uniform diameter throughout their length. The diameter changes, at the capsular region, were much less irregular than those shown in the reconstruction of spindle B2. In the polar regions, the intrafusal muscle fibres were separated by extrafusal muscle fibres, but in the longer proximal pole they came together at intervals (Fig. 9), and at both ends they were inserted in the epimysium separately. Again the spindle capsule was in close relation to a band of epimysium.

FIGURE 8. Reconstruction of spindle D2. The limits of the capsule are indicated on each intrafusal fibre by white lines. The corrected lengths and diameters of the fibres are to scale; the relative positions of the intrafusal fibres are diagrammatic. The lengths of the fibres from the proximal end of the spindle are shown on the vertical scale. The levels from which the transverse sections shown in Fig. 9 were taken are marked a - j.

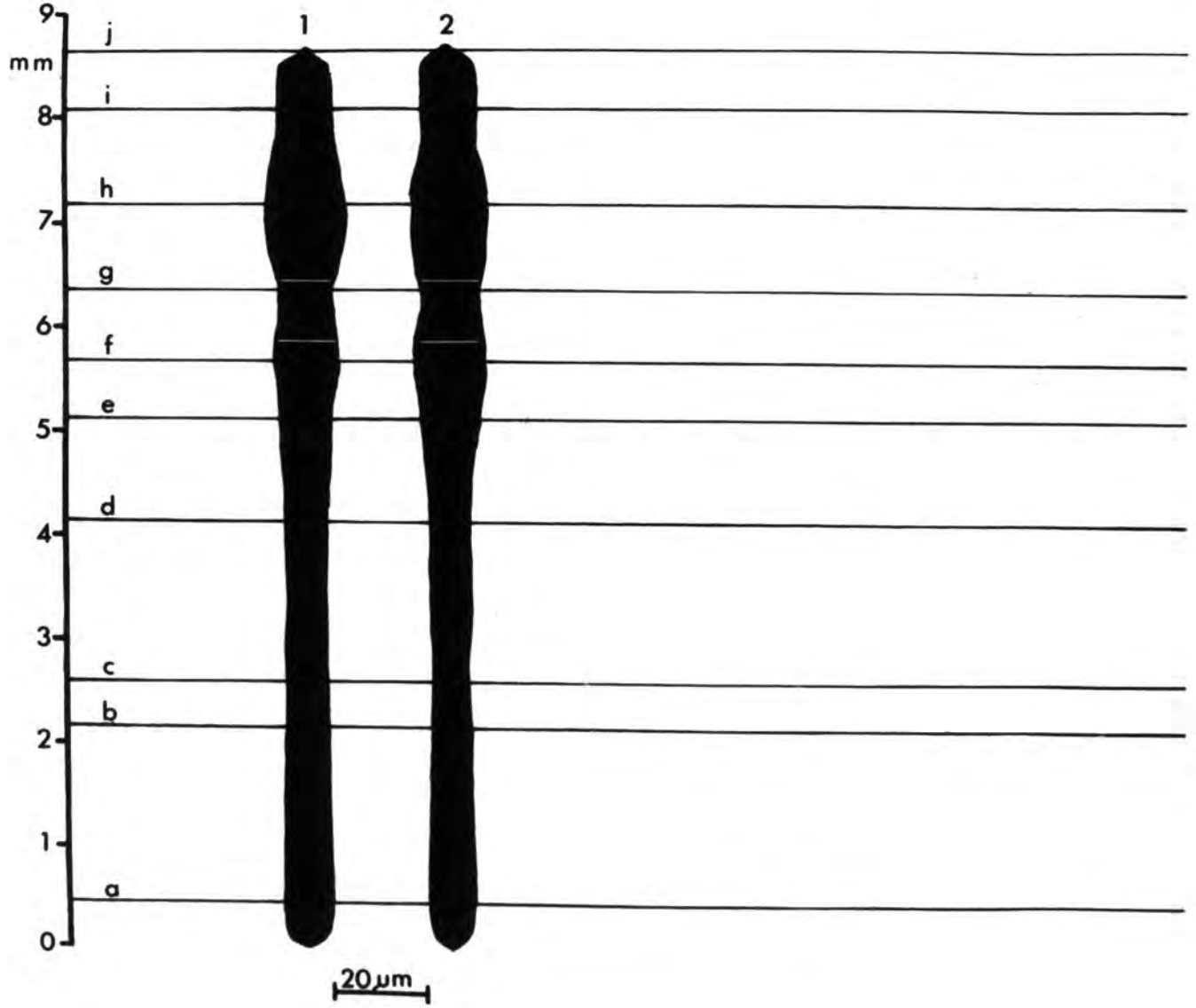
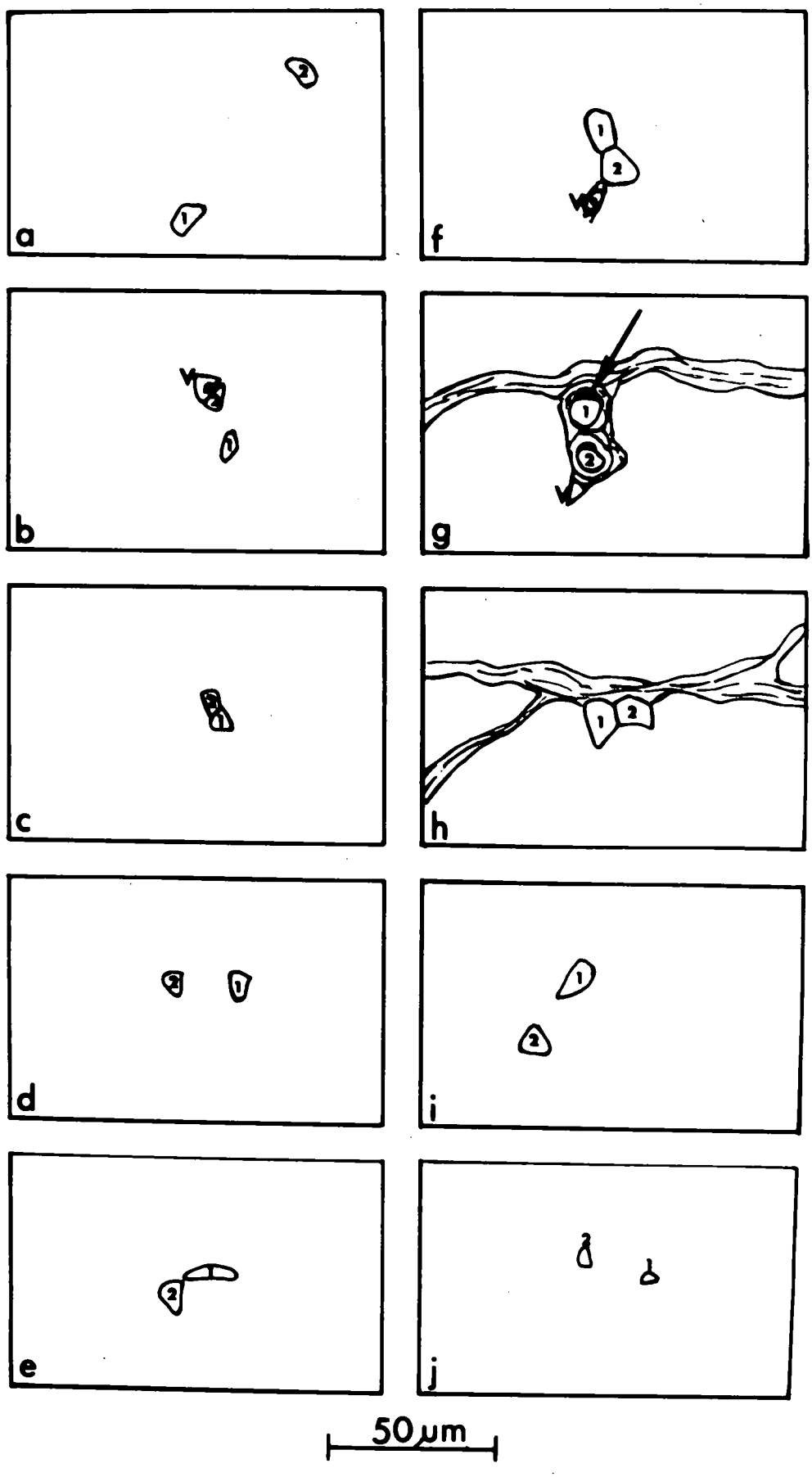
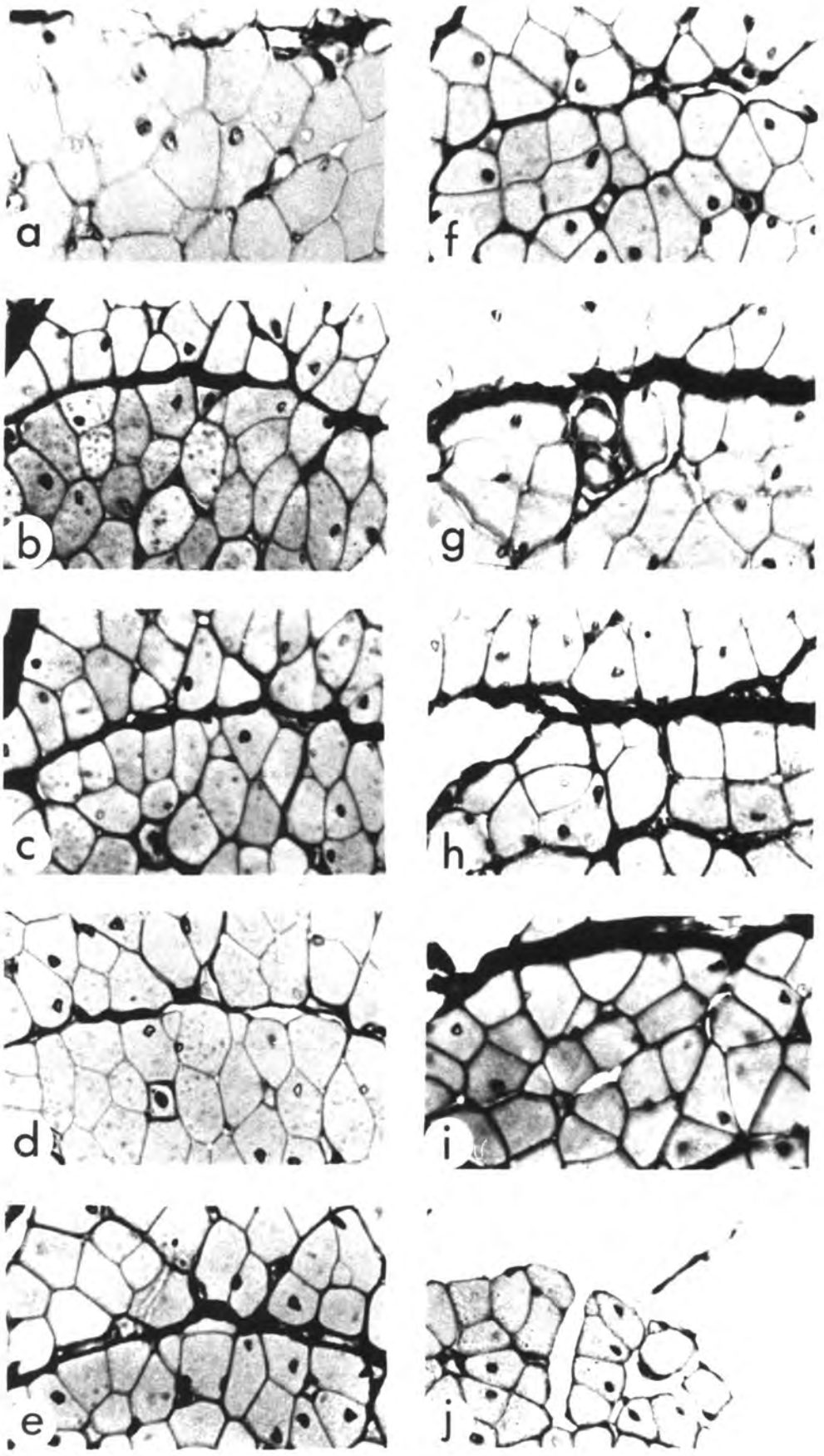

9
mm
8
7
6
5
4
3
2
1
0
j
i
h
g
f
e
d
c
b
a
1
2
20µm

FIGURE 9. Transverse sections of spindle D2. A key identifying the individual intrafusal fibres is given on the facing page. Note the changes in diameter and relative positions of the intrafusal fibres and the band of perimysium in relation to the capsule. Blood vessels (v) and satellite cell nuclei are present (arrow). H & VG.


a
b
c
d
e
f
g
h
i
j
50µm


a
f
b
g
c
h
d
i
e
j
50 µm

Spindle C4 was one of the 2 'tandem' spindles found among the 27 spindles examined in serial transverse sections. It was 8.3 mm long and consisted of 17 intrafusal muscle fibres (Figs. 10, 11). At the proximal end there were 14 intrafusal muscle fibres which all passed through the proximal capsule which was 0.7 mm long and its mid point was 1.1 mm from the proximal end of the spindle. Of these 14, only fibres 1 and 2 did not pass through the distal capsule which was 1.0 mm in length and its mid point was 5.9 mm from the proximal end of the spindle. Fibre 2 ran throughout all the intercapsular part of the spindle and ended just before the distal capsule. Three more intrafusal muscle fibres arose at different points in the intercapsular region of the spindle and passed with the other 12 fibres through the distal capsule.

All the intrafusal muscle fibres maintained a fairly uniform diameter in the intercapsular region, except fibre 14 which showed an increase in diameter. All the fibres showed the characteristic changes in diameter in both capsular regions, except fibre 4 which failed to show the intracapsular decrease in diameter when passing through the proximal capsule, instead it showed shallow indentations. The intrafusal muscle fibres were grouped together in both the capsular and the intercapsular regions, but in both polar regions they were separated into 2 groups which were inserted separately in the epimysium.

FIGURE 10. Reconstruction of the 'double tandem' spindle C4. The limits of the capsules are indicated by white lines. The corrected lengths and diameters of the fibres are to scale; the relative positions of the intrafusal fibres are diagrammatic. The lengths of the fibres from the proximal end of the spindle are shown on the vertical scale. The levels from which the transverse section shown in Fig. 9 were taken are marked a - j.

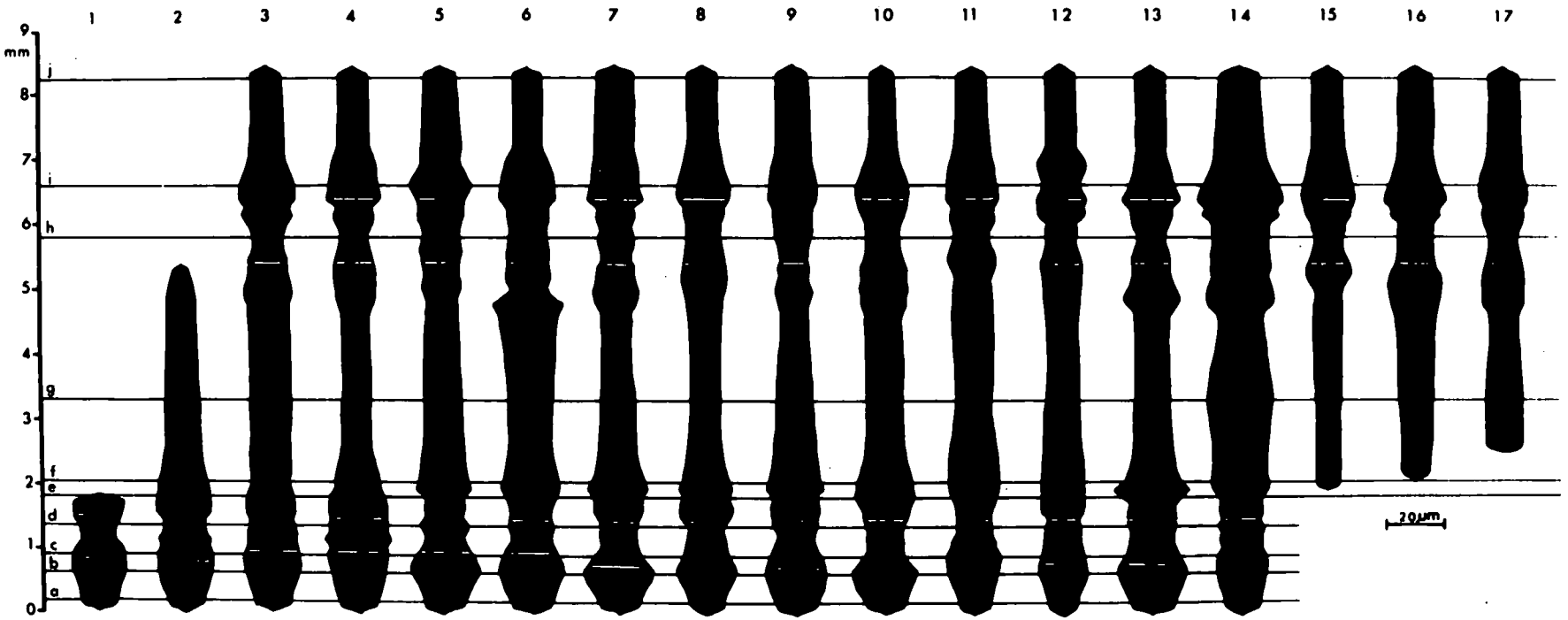
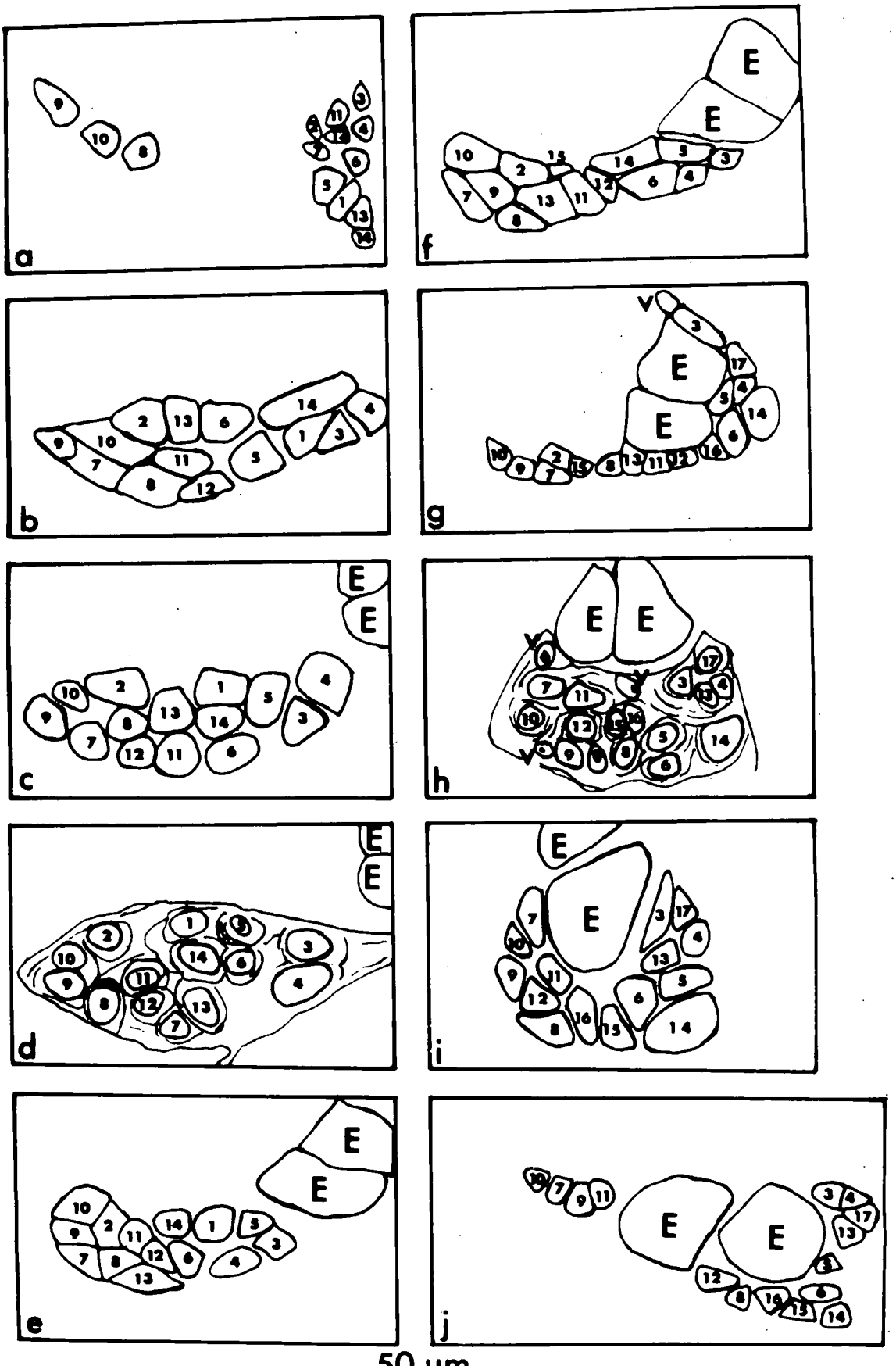
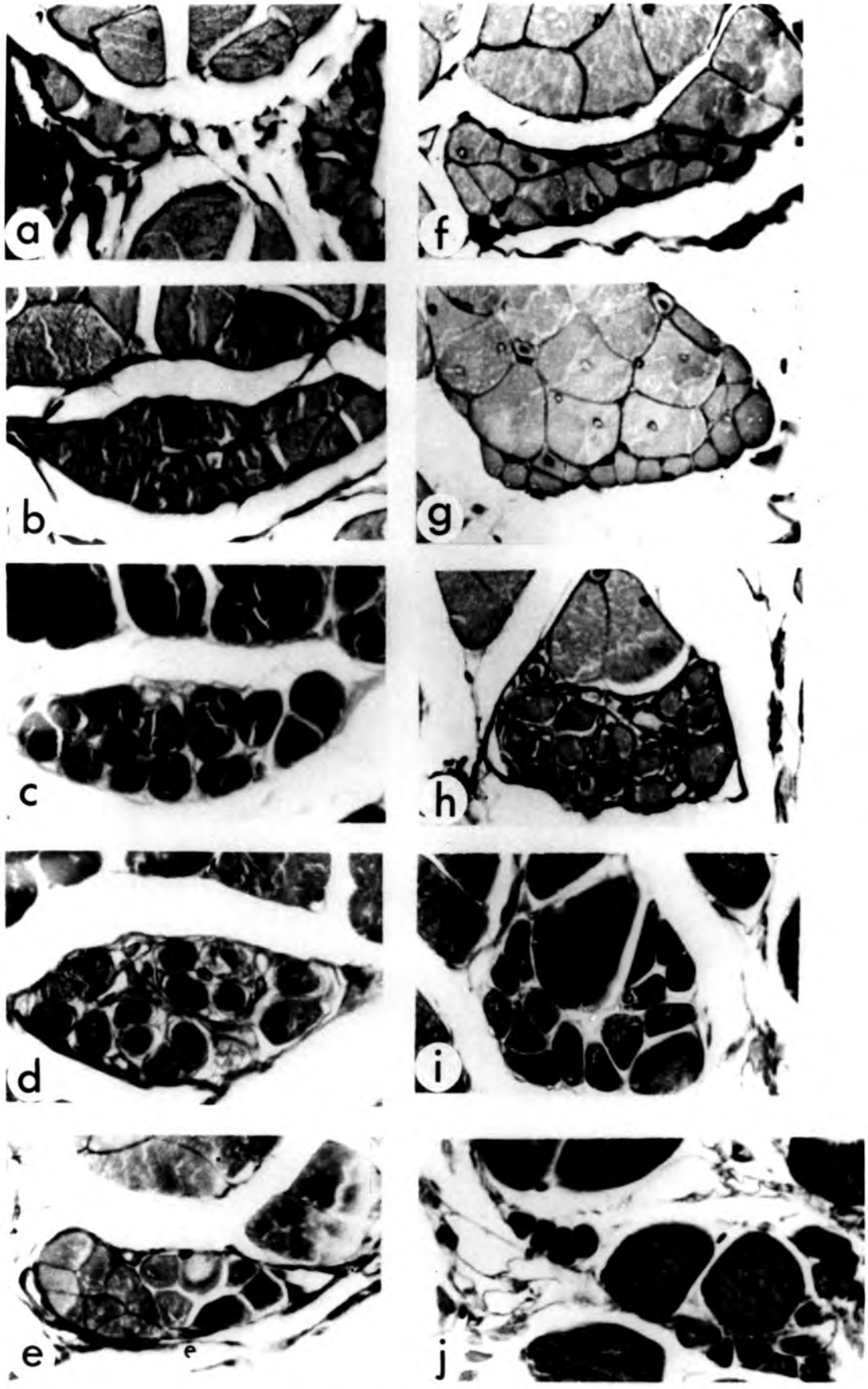

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
mm
9
8
7
6
5
4
3
2
1
0
j
i
h
g
f
e
d
c
b
a
20µm

FIGURE 11. Transverse sections of the 'double tandem' spindle C4. A key identifying the individual intrafusal fibres is given on the facing page. Note the changes in diameter, grouping and relative position of the fibres. Two extrafusal fibres (E) are closely associated with the spindle. Blood vessels (V) are present. Sections a, b, f, g andh are stained with H & VG, and c, d, e, i and j are stained with Masson's trichrome.


a
b
c
d
e
f
g
h
i
j
E
V
50 µm


a
f
b
g
c
h
d
i
e
e
j
50 µm

It could be seen (Fig. 11c) that the proximal capsule started to enclose some of the intrafusal fibres, and at a further point (Fig. 11d) all the fibres were enclosed. It should be noted that the grouping of the intrafusal muscle fibres within the capsules was not constant (Fig. 11 d-h), and therefore it could not be considered as 2 separate spindles lying parallel to each other. Two extrafusal muscle fibres accompanied the spindle for a considerable part of its length, but were not enclosed in the fibrous capsules.

4. Structure of the capsule

4.1 Light microscopy

The muscle spindles in this study were first identified in serial transverse sections. The connective tissue capsule in the equatorial region provided the only positive means of identifying them because of the spreading of the intrafusal muscle fibres in the extra-capsular region (see section 3.4), and the overlap of the diameter distribution of the intrafusal and extrafusal muscle fibres (see Fig. 4).

The length of the capsular region can be measured quite easily in the serial transverse sections although in some cases, the intrafusal fibres did not enter or leave the capsule simultaneously (see Figs, 6, 7, 10, 11). Table I shows the lengths of the capsules, the mean length was 1.05 mm

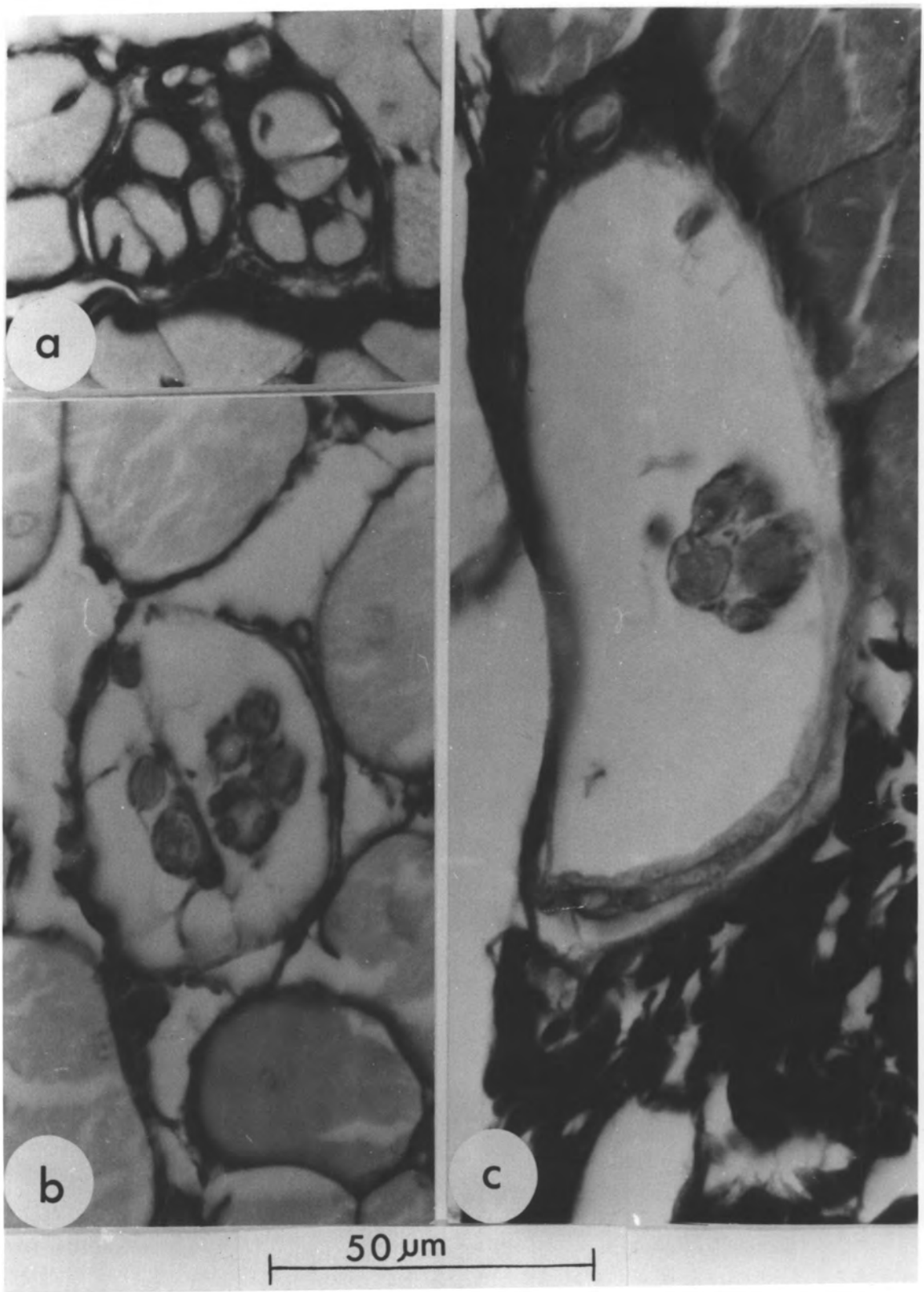
(range 0.5 to 1.9 mm). No relation was found between the length of a spindle and the length of its capsule. For example, spindle A2 was 10.3 mm long with a capsule 1.3 mm long, while spindle B5 was 10.5 mm long with a capsule of 0.5 mm. The capsule covered only a small part of the spindle length. When each capsule length was expressed as a percentage of its spindle length, the mean value for 25 capsules (tandem spindles excluded) was 11.6% (range 4.7% - 18.6%).

In transverse section, a striking feature of the capsular region of the tortoise spindle was the absence of a bulbous fluid-filled periaxial space, the connective tissue layers being closely applied to the intrafusal muscle fibres. In contrast, the frog and rat spindles from muscles prepared in the same way, clearly showed a periaxial fluid-filled space (Fig. 12).

Each capsule seen in serial sections, consisted of concentric connective tissue layers. The capsular cells were similar to the fibroblasts found elsewhere in the connective tissue elements of the muscle, and had flattened, thin, sometimes rounded deeply stained basophilic nuclei (Figs. 7, 11).

Each intrafusal muscle fibre had a sheath consisting of one or two layers of fibrous tissue, which separated it from the other intrafusal muscle fibres in the spindle. However, when two fibres were closely apposed, the connective

FIGURE 12. Transverse section of the capsular region of (a) tortoise, (b) frog and (c) rat spindles prepared in exactly the same way. The tortoise spindle lacks the fluid-filled space, conspicuous in the frog and rat spindles. In the tortoise spindle (a) each intrafusal muscle fibre has its own connective tissue sheath. Section a is stained with H & VG, and sections b and c are stained with Orcein and VG.


a
b
c
50 µm

tissue layers separating them disappeared and both were contained in the same connective tissue compartment (see Fig. 31; section 5.66). Several fibres (2-5) were held together as a group by a common sheath consisting of a double or triple layer of fibrous tissue. Finally the groups were bound together by another two to four layers of fibrous tissue. Each intrafusal muscle fibre was therefore separated from the outside of the spindle by five to eight layers of connective tissue. The pattern of grouping of the intrafusal muscle fibres often tended to change at different points within the capsule. For example, in the spindle shown in Figure 7e, intrafusal muscle fibres 1, 2 and 7 formed a group, but in another region of the same capsule, (Fig. 7f) the fibres 1 and 2 formed a group. In only 5 of the 27 spindles studied in serial sections, did the intrafusal muscle fibres maintain the same grouping throughout the length of the capsule.

The connective tissue capsule was thickest with the maximum number of lamellae and nuclei at the mid-capsular region. As it was traced towards the poles, the number of lamellae and nuclei gradually decreased. At the ends of the capsule, the connective tissue layers between the intrafusal muscle fibres usually disappeared just before the outermost layers of the capsule were broken and the intrafusal muscle fibres spread among the extrafusal muscle fibres (Figs. 7,

9, 11). Therefore, in a transverse section through the capsular region, the thickness of the capsule and the density of its nuclei gave a rough idea of the region of the capsule from which the section was taken. The endomysium of the intrafusal muscle fibres in the extracapsular region was not thicker than that of the extrafusal muscle fibres.

In transverse sections, the outer layers of the capsule sometimes formed a 'lateral extension' (Cooper & Daniel, 1963) which usually enclosed one, but rarely up to nine thick muscle fibres (Figs. 7, 13). These fibres were considered to be extrafusal because they were much larger than the intrafusal muscle fibres, and did not show the variations in diameter typical of the latter, and no sensory nerve endings were seen on them in teased preparations. Such lateral extensions of the capsule enclosing extrafusal muscle fibres have been described by Cooper and Daniel (1963) in the human spindle and Jones (1966a) in the spindles of Trichosurus valpecula.

Serially sectioned tortoise spindles stained for elastic fibres were carefully examined, but no elastic fibres were seen in the capsular and extracapsular regions. However, elastic fibres were found in the walls of the blood vessels (Fig. 14a). Elastic fibres were also found in frog and rat spindles (Fig. 14 b,c) where they were arranged longitudinally around the intrafusal muscle fibre.

FIGURE 13. Transverse section of the capsular region of a tortoise spindle. Some extrafusal muscle fibres (E) are enclosed within lateral extensions of the capsule. A branch of the muscle nerve (N) and blood vessels (V) are present. H & VG.

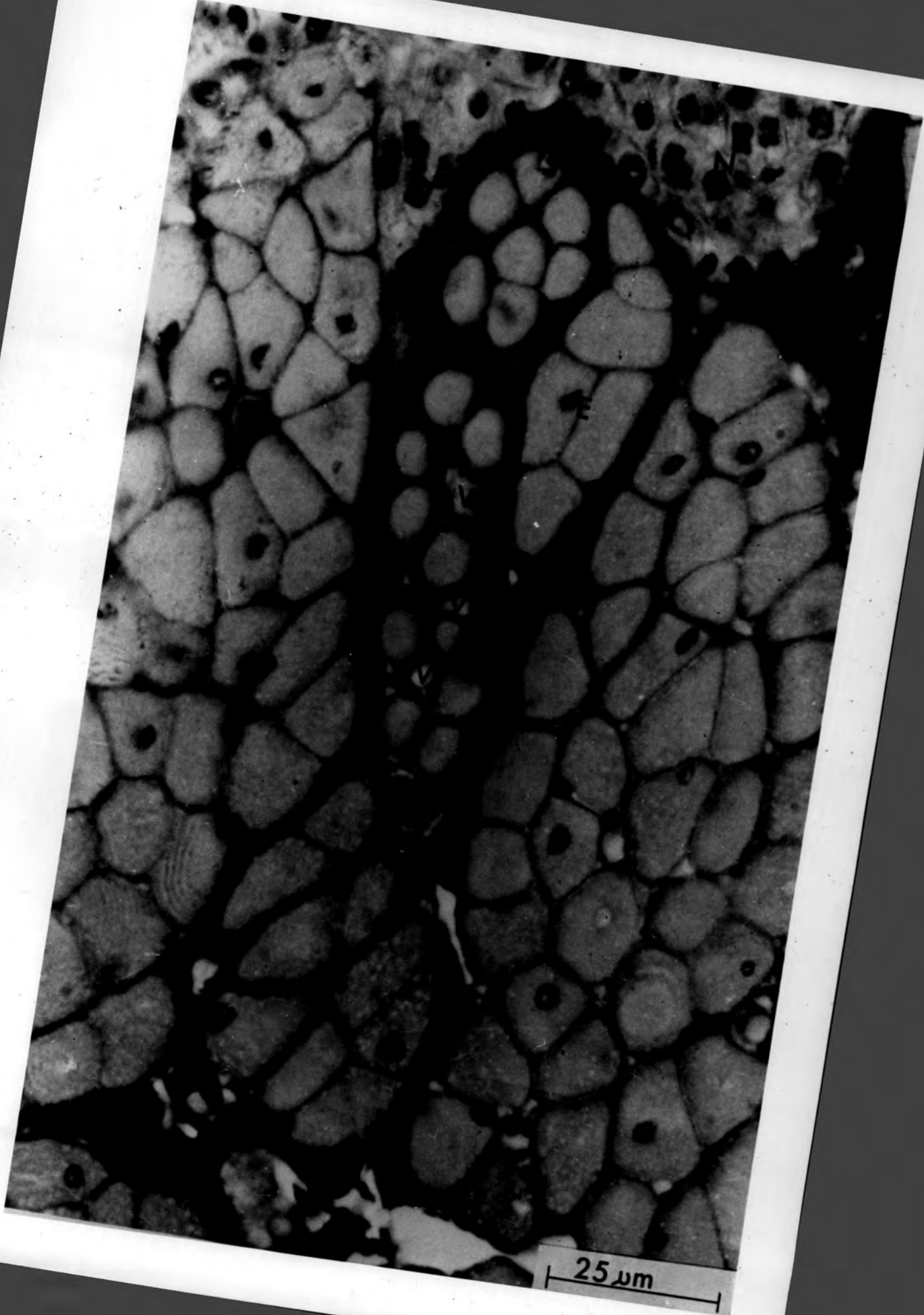

25 µm

FIGURE 14. Transverse section of the capsular region of (a) tortoise, (b) frog and (c) rat spindles stained for elastic fibres (arrows). Elastic fibres are not present in relation to the tortoise intrafusal fibres (IF), but present in an adjacent blood vessel (BV). Orcein & VG.


IF
IF
IF
IF
IF
IF
IF
V
a
b
c
20 µm

At least one blood vessel was seen in between the connective tissue layers of each spindle capsule, (e.g. Figs. 11h, 13). Sometimes it was difficult to distinguish a blood vessel with a nucleated red blood cell inside from an intrafusal muscle fibre unless it was traced through several sections.

4.2 Electron microscopy

Transverse and longitudinal sections from the capsular region of 12 spindles were examined under the EM. The results obtained confirmed the LM observations and provided more information concerning its fine structure.

Figure 15 shows an electron micrograph of a transverse section through the mid-capsular region of the spindle with 4 intrafusal muscle fibres, and figure 16 shows an electron micrograph of a longitudinal section through the same region in another spindle. In both, the intrafusal muscle fibres were seen to be directly surrounded by connective tissue layers with no fluid-filled space.

The capsule was not differentiated into outer and inner capsular sheaths, as is the case with amphibian (Robertson, 1956), mammalian (Landon, 1966), and other reptilian (Fukami & Hunt, 1970) spindles and only one type of capsular cell was detected. In fact it was not uncommon to see cytoplasmic processes from the same cell taking part in the formation of not only the outermost layers of the capsule, holding the

FIGURE 15. Electron micrograph montage of a transverse section of the mid-equatorial region of a spindle with 4 intrafusal muscle fibres (1-4). There is no fluid-filled space and each intrafusal fibre has its own connective tissue sheath, except fibres 3 and 4 which are in partial close apposition and have a common sheath. Processes from the same capsular cell (cc) participate in the formation of both the outer layers of the capsule and those around the individual intrafusal fibres (arrows). The intrafusal mitochondria (Mi) are of uniform size, and smaller than those of the extrafusal fibres (E). The arrangement of the myofibrils is identical in the intrafusal and extrafusal fibres. The intrafusal fibre nuclei (Nu) are flattened and peripherally situated. Some sensory endings (SE) are completely enclosed within sarcolemmal lips (*).

A	- sensory axon	Mi	- mitochondrion
BV	- blood vessel	Nu	- nucleus
C	- collagen	SC	- satellite cell
cc	- capsular cells	Sch	- Schwann cell
E	- extrafusal muscle fibre	SE	- sensory ending


C
BV
SE
SE
CC
Sch
Mi
3
SE
SE
BV
5 µm

FIGURE 16. Electron micrograph of a longitudinal section of the mid-capsular region of a spindle. There is no fluid-filled space. Part of a peripherally placed muscle nucleus (Nu) is present. The processes of the capsular cells (CC) are parallel to the long axes of the intrafusal fibres. All the fibres show the same pattern of striation. The upper fibre has slightly fewer glycogen granules (G) than the other two. A structure (*) resembling an enlarged sensory ending with densely packed mitochondria (Mi) is present and shown in Fig. 43b at a high magnification.

CC - capsular cell

G - glycogen

M - M line

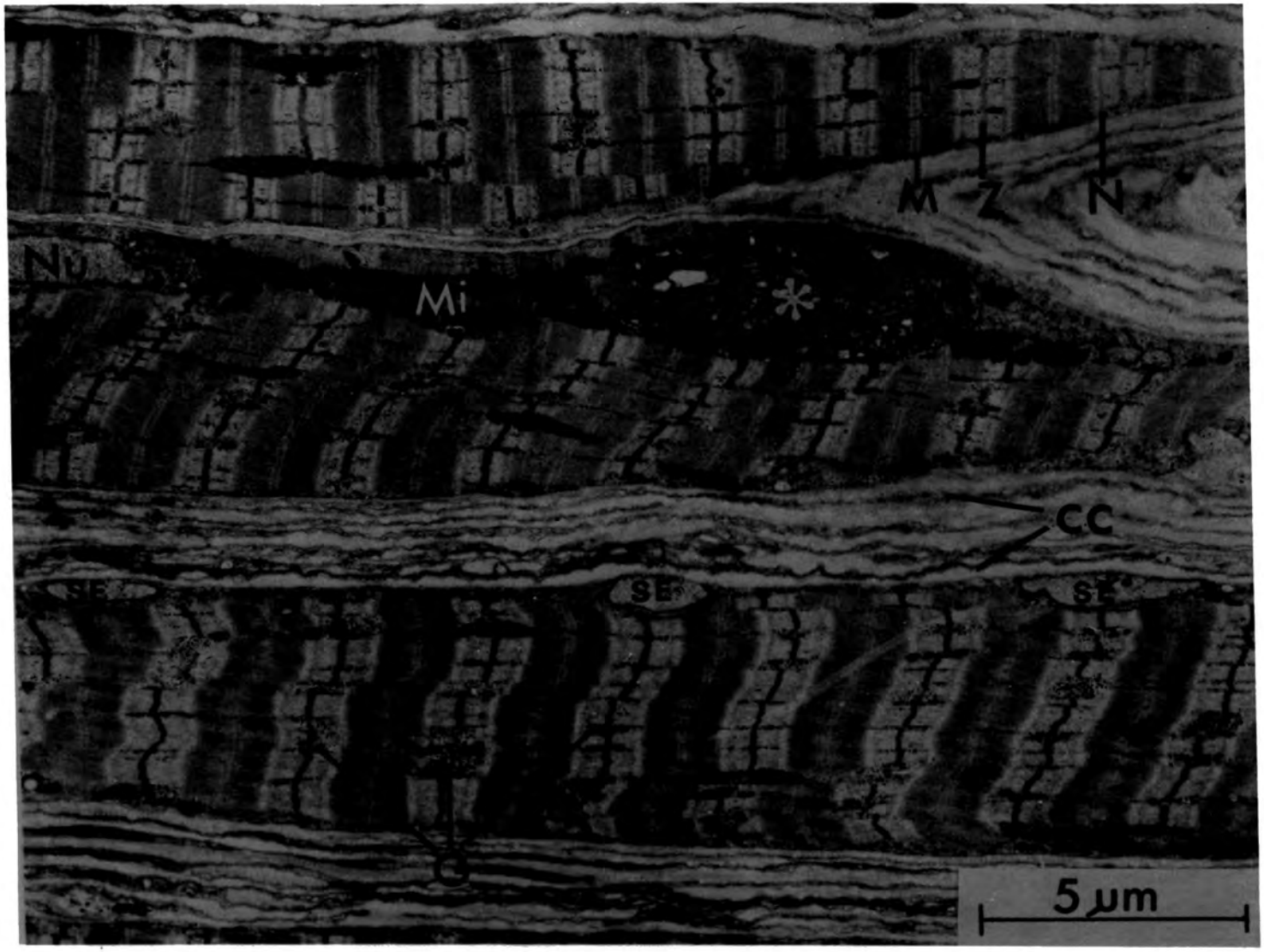
Mi - mitochondrion

N - N line

Nu - intrafusal fibre nucleus

SE - sensory ending

Z - Z disc


M
Z
N
Nu
Mi
CC
SE
SE
SE
5 µm

intrafusal muscle fibres together, but also the layers surrounding the individual intrafusal muscle fibres (Fig. 15).

The capsular cells had flattened, elongated, deeply stained nuclei with occasionally one or two nucleoli. At their centres, the nuclei were 1 - 2.8 μm thick and 4 - 11 μm long and were surrounded by a thin layer of cytoplasm 20 - 500 mμ. The capsular cell bodies were extensive and appeared in transverse sections as concentric, thin, but highly irregular, processes 0.2-0.9 mμ thick. These processes took origin from the cytoplasmic layer surrounding the nucleus and diverged around and between the intrafusal muscle fibres (Fig. 15). In longitudinal section these processes ran parallel to the intrafusal muscle fibres (Figure 16).

Other organelles were infrequent in these cells, they consisted of a few small mitochondria (0.16 - 0.21 μm), ribosomes, granular endoplasmic reticulum, and occasional glycogen granules (Fig. 17a). A prominent feature of the capsular cells was the presence of numerous flask-shaped pinocytotic vesicles with their necks opening to the exterior of the cell (Fig. 17b). Where the edges of two different cells overlapped, a tight junction was seen between their membranes (Fig. 17c). The capsular cells had a basement membrane which was sometimes interrupted (Fig. 18a).

FIGURE 17. High power electron micrographs of capsular cells showing the cell organelles and cytoplasmic inclusions. In (b) the nucleus is surrounded by a thin layer of cytoplasm. In (c) two capsular cell processes overlap and their plasma membranes fuse to form a tight junction (arrow).

BM - basement membrane

C - collagen

Ca - caveoli

CC - capsular cell process

ER - rough endoplasmic reticulum

G - glycogen granules

IF - intrafusal muscle fibre

Mi - mitochondrion

Nu - capsular cell nucleus

PV - pinocytotic vesicle

SP - sarcoplasm

R - ribosomes

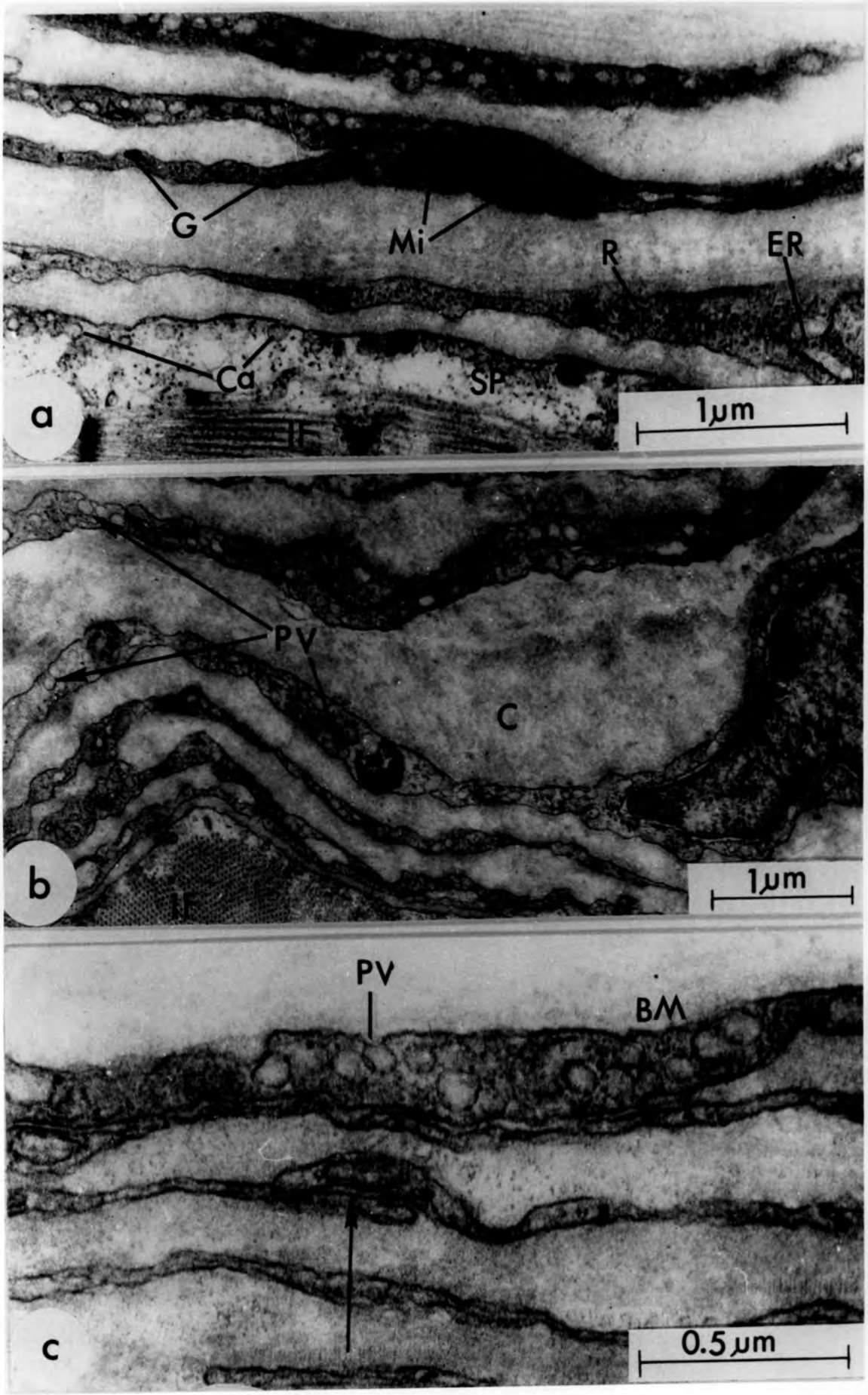

G
Mi
R
ER
Ca
SP
a
1µm
PV
C
b
1µm
PV
BM
c
0.5µm

FIGURE 18. Electron micrographs of transverse sections heavily stained with uranyl acetate to demonstrate circularly and longitudinally arranged collagen fibres (C) filling almost all the space between the capsular cell processes (CC) and the intrafusal fibres (IF). The basement membrane of the capsular cell processes is interrupted in a (*)

BM - basement membrane

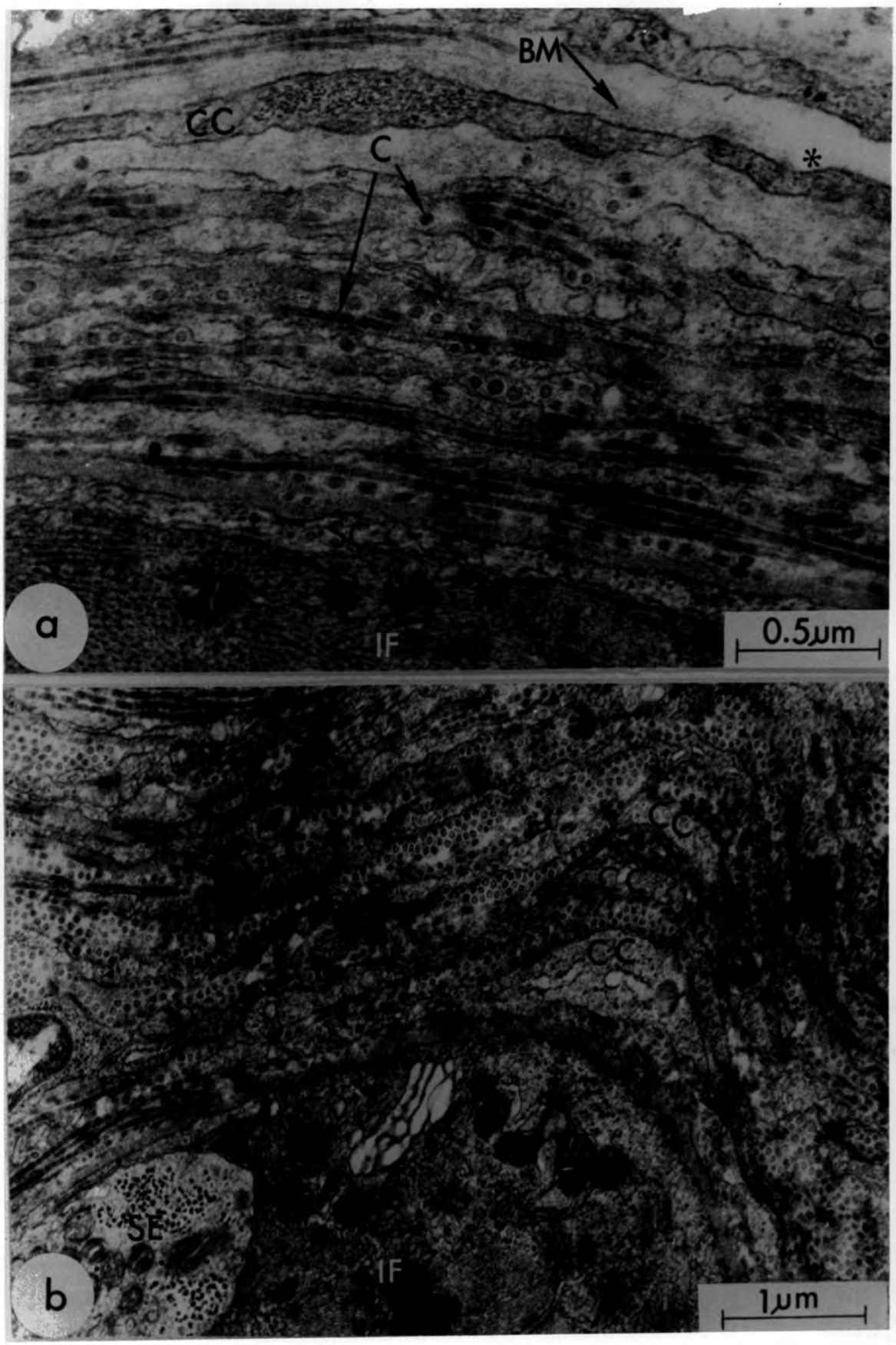
C - collagen fibres

CC - capsular cell processes

IF - intrafusal muscle fibre

SC - satellite cell process

SE - sensory ending


BM
CC
C
*
a
IF
0.5µm
CC
CC
SE
b
IF
1µm

The narrow intercellular spaces (13-960 mμ) were almost completely filled with collagen fibres (30-60 mμ in diameter) arranged in both longitudinal and circular planes, and amorphous basement membrane material (Fig. 18a,b). Myelinated and non-myelinated nerve fibres and capillaries were also seen in the intercapsular spaces, but no elastic fibres were observed.

5. Intrafusal muscle fibres

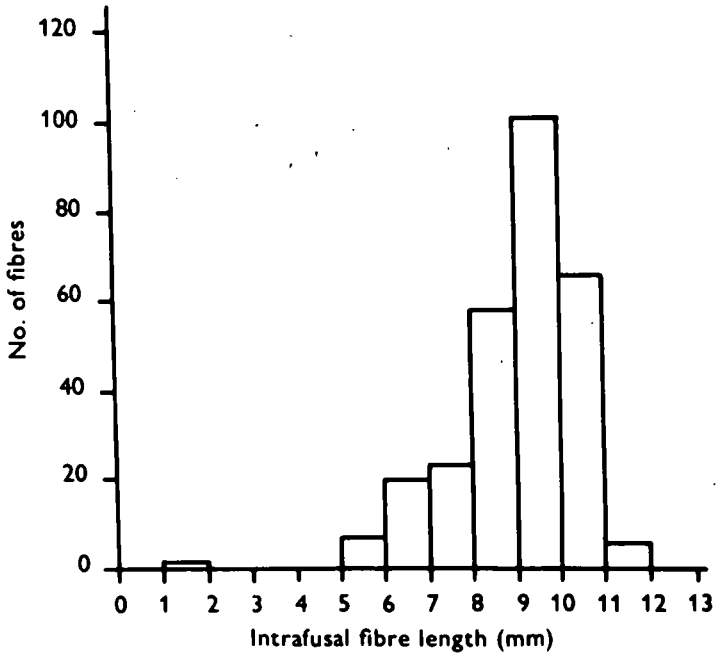
The LM was used to examine intrafusal muscle fibres in transverse sections and teased whole spindle preparations and the EM was used to examine both transverse and longitudinal sections of intrafusal muscle fibres to see whether they could be categorized into more than one type on the basis of length, diameter, nuclear structure and distribution, myofibrillar structure and histochemical activity.

5.1 Length

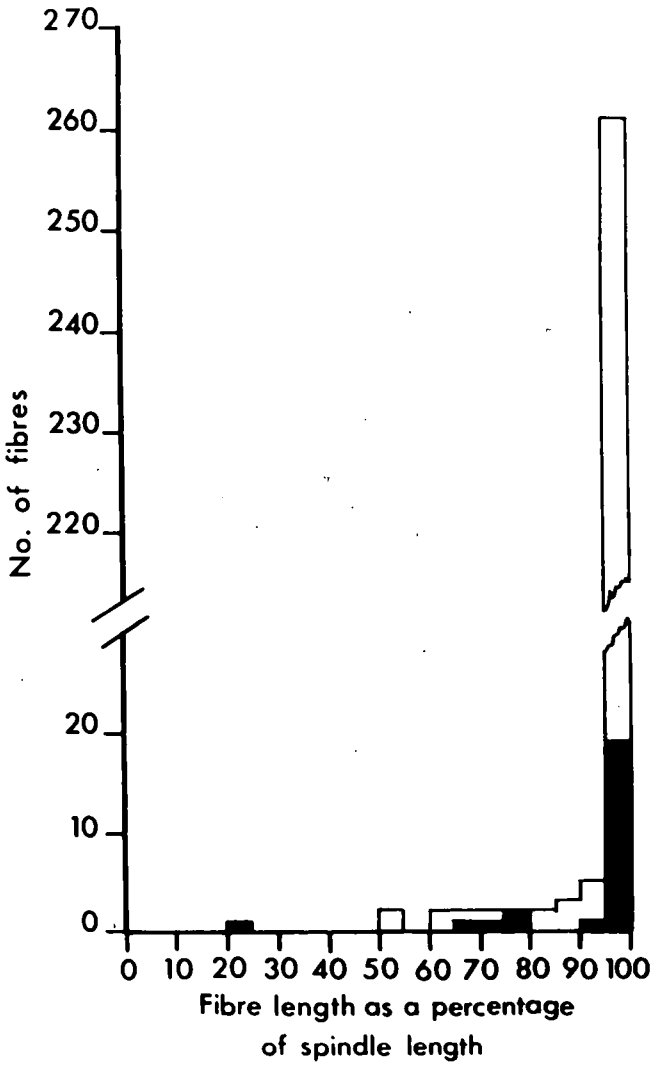
Figure 19a shows a histogram of the lengths of the 282 intrafusal muscle fibres found in 27 spindles examined in serial transverse sections. The distribution was found to be unimodal with a mean length of 9.1 mm and a standard deviation of 1.39 mm. However, the picture might have been distorted due to the fact that the spindles were not of uniform length, so the length of each intrafusal muscle fibre

FIGURE 19a. Distribution of the lengths, after correction for shrinkage, of 282 intrafusal fibres measured in serial sections. The mean length is 9.1 mm (S.D. 1.39 mm).

FIGURE 19b. Distribution of the lengths of the 282 intrafusal fibres measured in serial sections, expressed as a percentage of spindle length. The fibres of 'tandem spindles' are indicated by the shaded parts of the columns.


No. of fibres
120
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13
Intrafusal fibre length (mm)

a


No. of fibres
270
260
250
240
230
220
20
10
0
0 10 20 30 40 50 60 70 80 90 100
Fibre length as a percentage
of spindle length

b

was expressed as a percentage of the total spindle length. The frequency distribution of percentage length (Figure 19b) showed that 94% of the intrafusal muscle fibres extended for more than 95% of the total spindle length.

It was of interest that the shortest intrafusal muscle fibre (20% of total spindle length) was seen in one of the tandem spindles (spindle C4, Fig. 10). In fact, of the 18 fibres which were less than 95% of the total spindle length, 6, including the shortest fibre, were found in 'tandem' spindles (Fig. 19b).

In teased preparations all the intrafusal muscle fibres in a spindle were practically of the same length.

5.2 Diameter

The reconstructions of spindles shown in Figures 6, 8 and 10 illustrate the characteristic and consistent variation in the diameters of the intrafusal muscle fibres. Generally they maintained a uniform diameter in the polar regions, and enlarged as they approached both poles of the capsule to attain a maximum just prior to their entry into the capsule. The fibre diameter then decreased to reach a minimum in the mid-capsular region. In some fibres the changes in diameter in the capsular region were not smooth but rather irregular with occasionally a smaller, third peak in the capsular region (see Fig. 6).

In the capsular region the decrease in intrafusal muscle fibre diameter was always roughly proportional to the thickness of the capsule around the fibre, i.e. as the connective tissue increased towards the mid-capsular region, so the diameter of the fibres decreased.

The pattern of variation in diameter along the intrafusal muscle fibre length was confirmed when teased preparations were examined, although no detailed measurements were carried out on them.

Jones (1966a) has described a similar change in the diameter of the intrafusal muscle fibres of the Trichosurus valpecula spindles and a similar decrease has been demonstrated in the intracapsular diameter of the intrafusal muscle fibres of man (Cooper & Daniel, 1956), cat (Boyd, 1962) and frog (Barker & Cope, 1962).

Due to this variation in the diameters of the intrafusal muscle fibres, the mid-polar region was selected for intrafusal muscle fibre measurements (Methods, section 5.13(b)). The mean diameter value for each of the 282 intrafusal muscle fibres was plotted in a histogram shown in Figure 4, together with the mean diameters of 300 extrafusal muscle fibres. The distribution of the diameters of the intrafusal muscle fibres is unimodal with a mean value of 10.6 mm and a standard deviation of 2.30 mm. It can be seen that this distribution slightly overlaps that of the extrafusal muscle fibres.

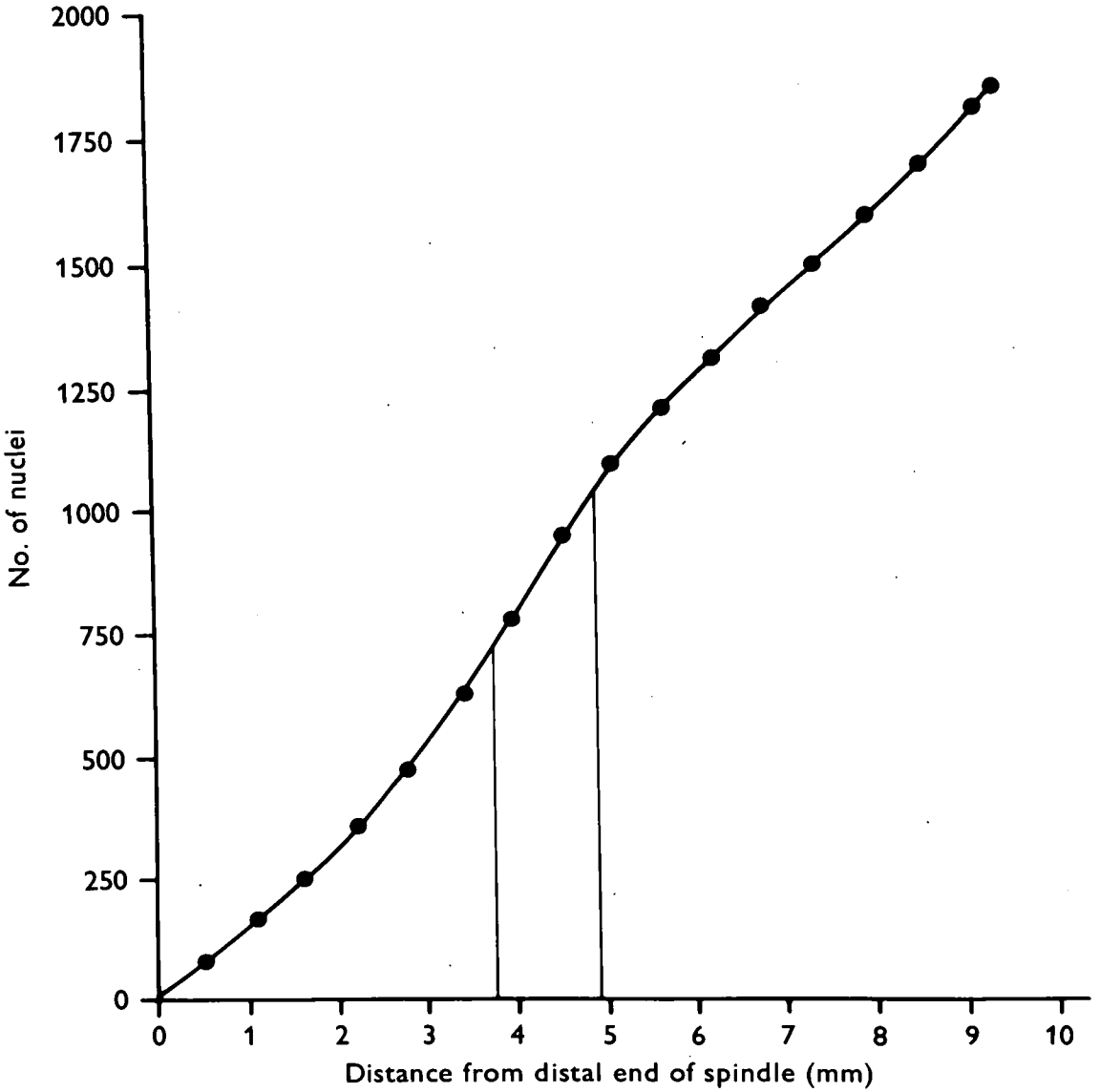
5.3 Nuclear distribution and structure

The distribution of nuclei within each of the above-mentioned 282 intrafusal muscle fibres was obtained by noting the number and position of the nuclei, taking care not to count the same nucleus more than once. Figure 20 shows the combined cumulative distribution for the eleven intrafusal muscle fibres of spindle D9, in which a detailed quantitative assessment was made. It can be seen from the figure that the nuclei were fairly evenly distributed along the length of the muscle fibres, with a slightly higher density in and around the capsule. This distribution was typical of all the intrafusal muscle fibres studied.

In transverse sections of an intrafusal muscle fibre, nuclei, if present, usually occurred singly, but occasionally two and rarely three nuclei were seen in the same section. No aggregations of nuclei resembling the nuclear-bags of mammalian spindles (Barker, 1948) were seen in any of the 282 intrafusal muscle fibres examined.

No myotube was observed in any of the intrafusal muscle fibres. The nuclei in the capsular region showed no apparent structural difference from those in the polar regions or those found in the extrafusal muscle fibres. In most cases they were peripherally placed both in the capsular and extracapsular regions.

FIGURE 20. The combined cumulative distribution of the nuclei of the 11 intrafusal fibres of spindle D9 examined in serial sections, relative to the length of the spindle. There is a slight increase in nuclear density in the capsular region, denoted by the two vertical bars.


2000
1750
1500
1250
1000
750
500
250
0
No. of nuclei
0
1
2
3
4
5
6
7
8
9
10
Distance from distal end of spindle (mm)

These observations were confirmed in teased whole spindle preparations and the material examined under the EM.

5.4 Myofibrillar density

Transverse sections stained with Masson's trichrome technique were examined to see if there was any variation in the staining properties in different intrafusal muscle fibres and at various points along a single fibre. Each fibre took the stain evenly throughout its entire length and the myofibrils were continuous and at no point were they interrupted or replaced by elastic or reticular tissue. The intrafusal muscle fibres took up the stain to the same extent as the surrounding extrafusal muscle fibres. All the intrafusal muscle fibres, as well as the extrafusal muscle fibres, had uniformly distributed, tightly packed myofibrils, and the sarcoplasm was very much reduced and could hardly be seen (Figs. 7 and 10).

5.5 Histochemistry

A histochemical study was carried out to determine whether there was more than one type of intrafusal muscle fibre. A total of 22 spindles (204 intrafusal muscle fibres) was examined in the present study.

Sudan black B, which has been reported as showing muscle fibre types (Lännergren & Smith, 1966) stained all the muscle fibres, intrafusal and extrafusal, uniformly (Fig. 21).

Staining for the mitochondrial oxidative enzyme succinic dehydrogenase stained both intrafusal and extrafusal muscle fibres uniformly. However, in the capsular region, the formazan granules, representing the mitochondria, were smaller than in the extracapsular regions, where they were almost of the same size as those found in the extrafusal muscle fibres (Fig. 22). This observation concerning the variation in the size of the mitochondria was subsequently confirmed with the EM (section 5.64)

Staining for phosphorylase and ATPase activity showed two distinct types of extrafusal muscle fibres, one with high activity (dark), the other with low activity (pale) and occasionally a third type with intermediate activity was seen, although the difference was not so marked with the ATPase. The activity of these enzymes along the length of a given extrafusal muscle fibre appeared to be fairly uniform and no relation between the enzymatic activity of the fibres and their diameters was seen. (Fig. 24). It could be seen that in the capsular region all the intrafusal muscle fibres, with few exceptions, showed low activity (pale) but gradually the activity of both enzymes increased towards the

FIGURE 21. Transverse section of the capsular region of a spindle stained with Sudan Black B. All the muscle fibres, intrafusal and extrafusal, are stained uniformly.

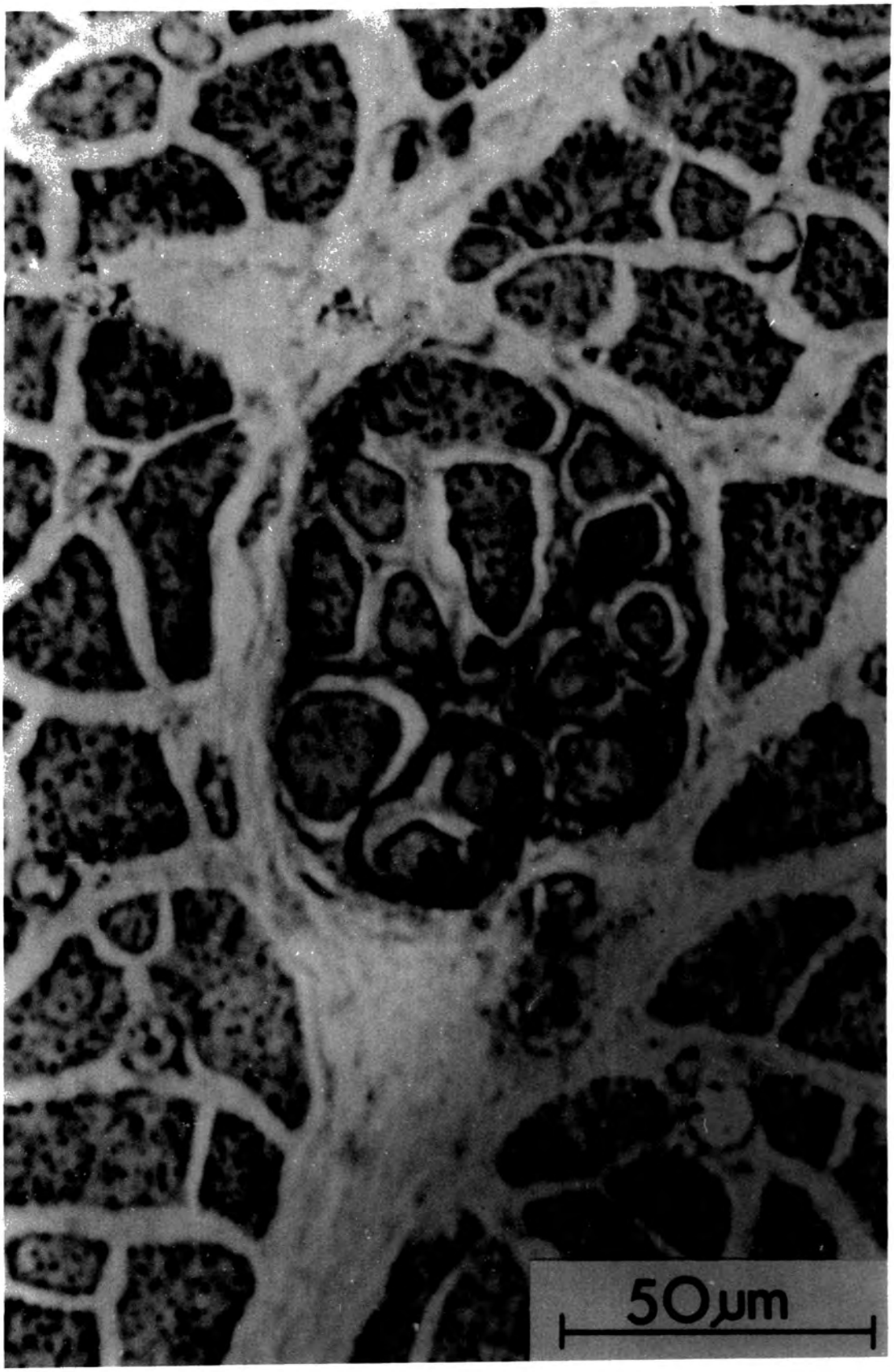

50µm

FIGURE 22. Transverse sections of (a) the capsular region and (b) the extracapsular region of a spindle, stained for the mitochondrial oxidative enzyme succinic dehydrogenase. In the capsular region (a) the mitochondria of the intrafusal fibres (1 - 3), represented by formazan granules, are smaller than those of the extrafusal fibres (E). In the extracapsular region (b) the mitochondria are large and of the same size as those of the extrafusal fibres. The activity of the extrafusal and intrafusal fibres is identical and uniform along their lengths.

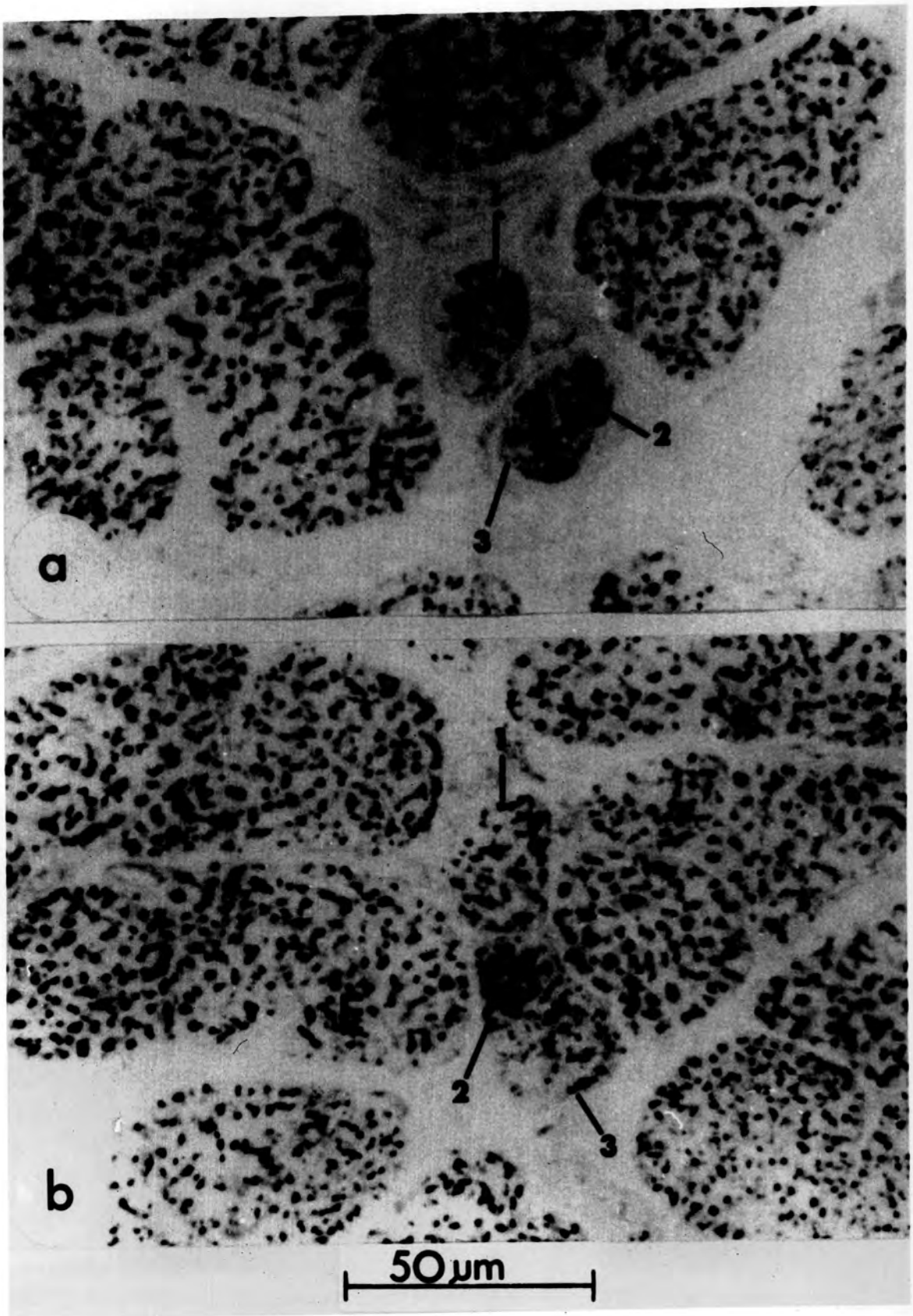
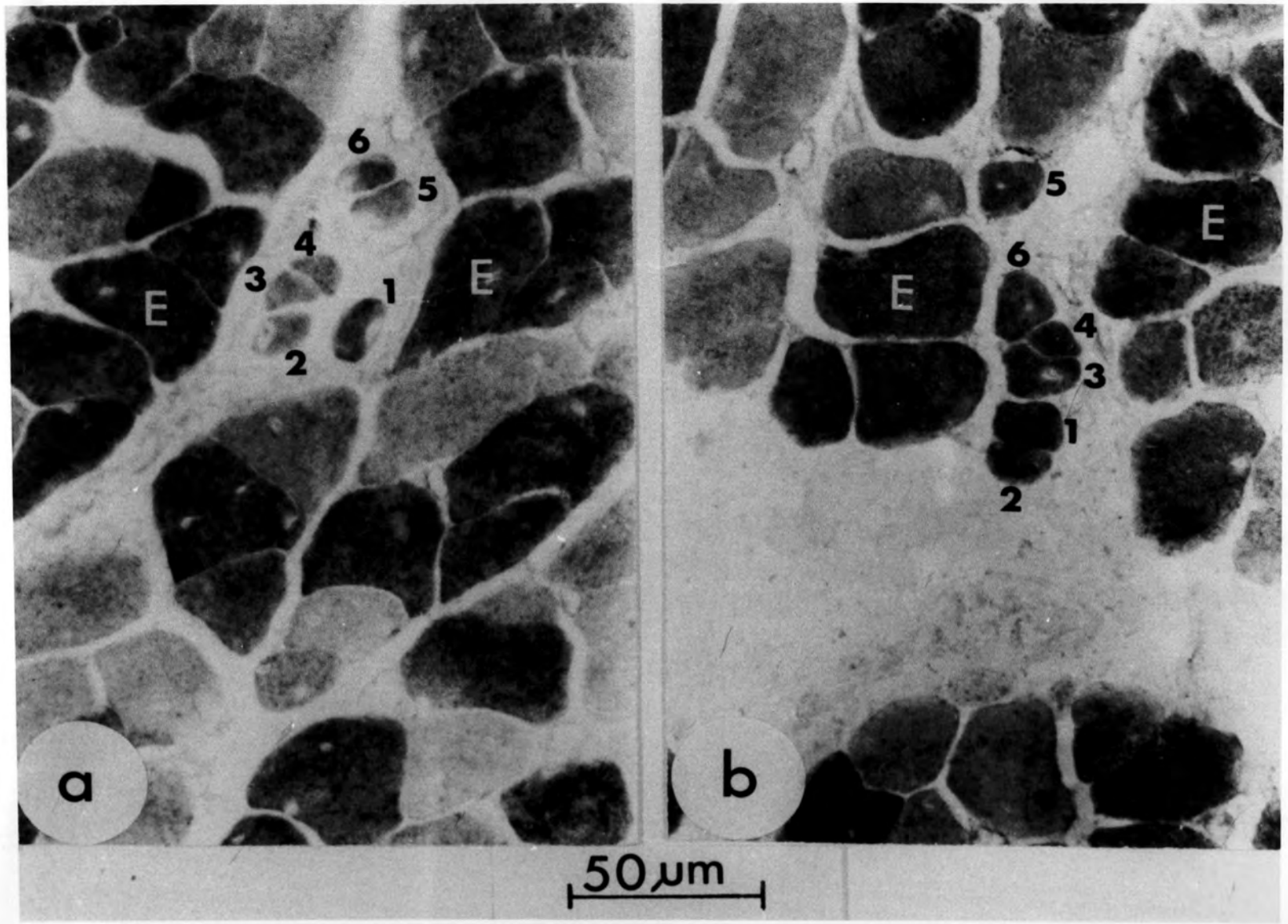

a
2
3
b
2
3
50µm

FIGURE 23. Transverse sections of (a) the capsular region and (b) the extracapsular region of a spindle stained for phosphorylase activity. All the intrafusal fibres (1 - 6) and two extrafusal fibres (E) have been identified to facilitate comparison. In the capsular region (a) all the intrafusal fibres have low activity, whereas in the extracapsular region (b) they are as dark as the extrafusal fibres (E) of high activity.


6
5
4
3
E
1
E
2
a
5
E
6
E
4
3
1
2
b
50 µm

polar regions where all the fibres attained high activity (Fig. 23). This change in enzymatic activity was not simultaneous in all the fibres but the number of fibres with high activity gradually increased as the intrafusal muscle fibres were traced from the mid-capsular region towards the polar regions (Fig. 24).

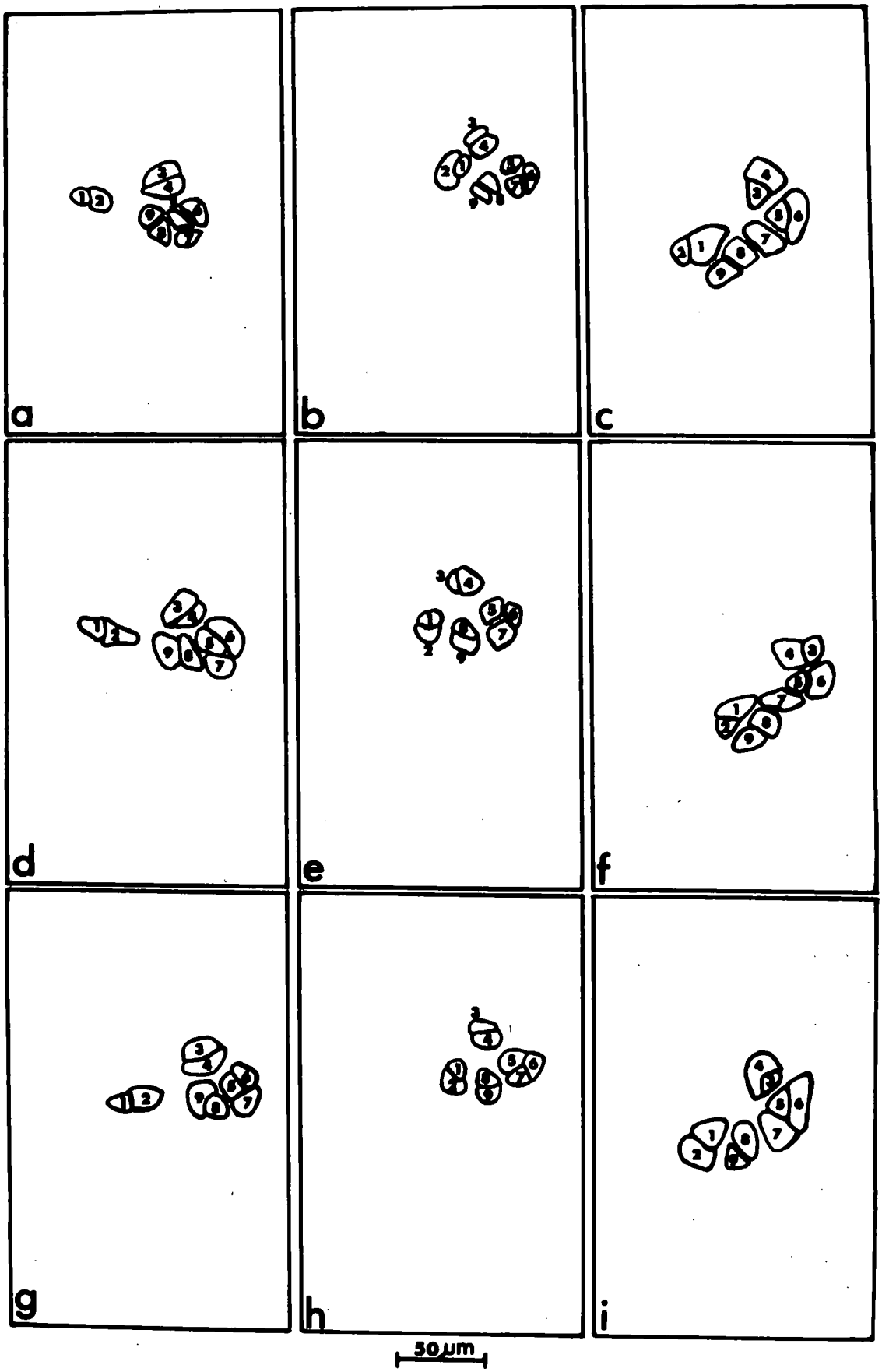
5.6 Fine Structure

Under the EM the intrafusal muscle fibres in the capsular region were easily distinguished from the extrafusal muscle fibres by their connective tissue layers, associated sensory nerve endings and their smaller size. All the intrafusal muscle fibres examined (101) had the same myofibrillar arrangement and structure and were indistinguishable in these respects from the extrafusal muscle fibres.

5.61 Sarcomeres

In longitudinal sections (Fig. 25) the sarcomere length of both intrafusal and extrafusal muscle fibres was about the same. The mean length of 16 sarcomeres of 4 intrafusal muscle fibres was 2.60 μm and the corresponding value for 16 sarcomeres of 4 extrafusal muscle fibres was 2.56 μm. The measurements were taken from fibres in the same section to minimize the difference which might arise from unequal shrinking or differences in plane of cutting.

FIGURE 24. Transverse sections of a spindle stained for phosphorylase (a - c), ATPase (d - f), and succinic dehydrogenase (g - i) activities. The sections in each column are from the same level of the spindle. A key identifying the intrafusal muscle fibres is provided on the facing page to facilitate comparisons. In the mid-capsular region (centre column) most of the intrafusal fibres have low phosphorylase and ATPase activity (pale fibres). Towards the poles (first and third columns), the activity of these enzymes increases. Three types of extrafusal muscle fibre can be distinguished by their phosphorylase and ATPase activities. The succinic dehydrogenase activity remains constant along the length of the intrafusal muscle fibres and is the same as the single type of extrafusal muscle fibre.


a
b
c
d
e
f
g
h
i
50 µm

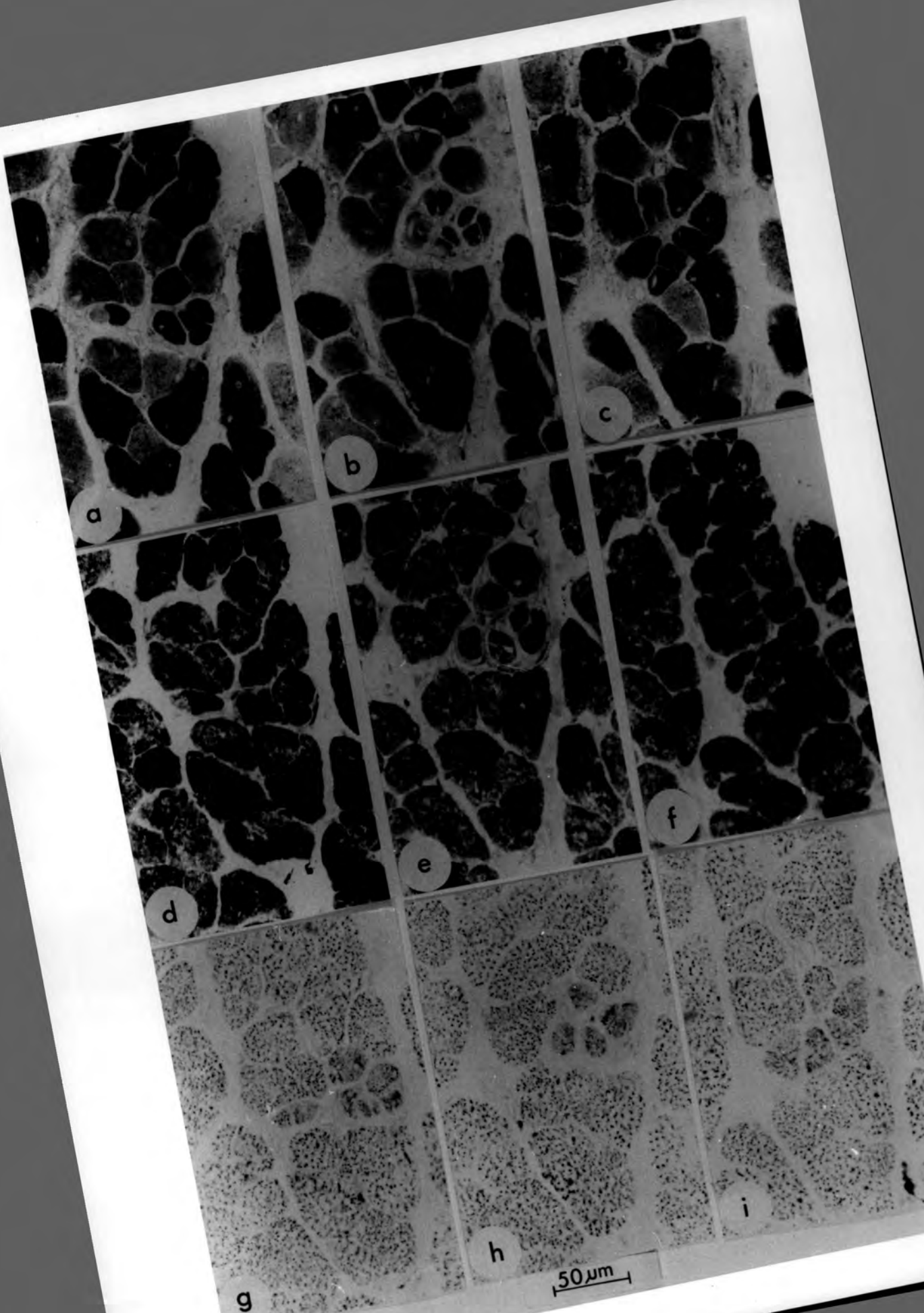

a
b
c
d
e
f
g
h
i
50 µm

FIGURE 25. Low power electron micrograph of a longitudinal section of an intrafusal fibre (IF) with a closely applied capsular sheath (CSh), and extrafusal fibres (E). With the exception of the presence of the capsule and the smaller diameter, the intrafusal and extrafusal muscle fibres are similar. In both, the Z discs are of the same thickness and run straight across the fibres, the nuclei (Nu) are flattened and peripherally placed, and the mitochondria are longitudinally arranged.

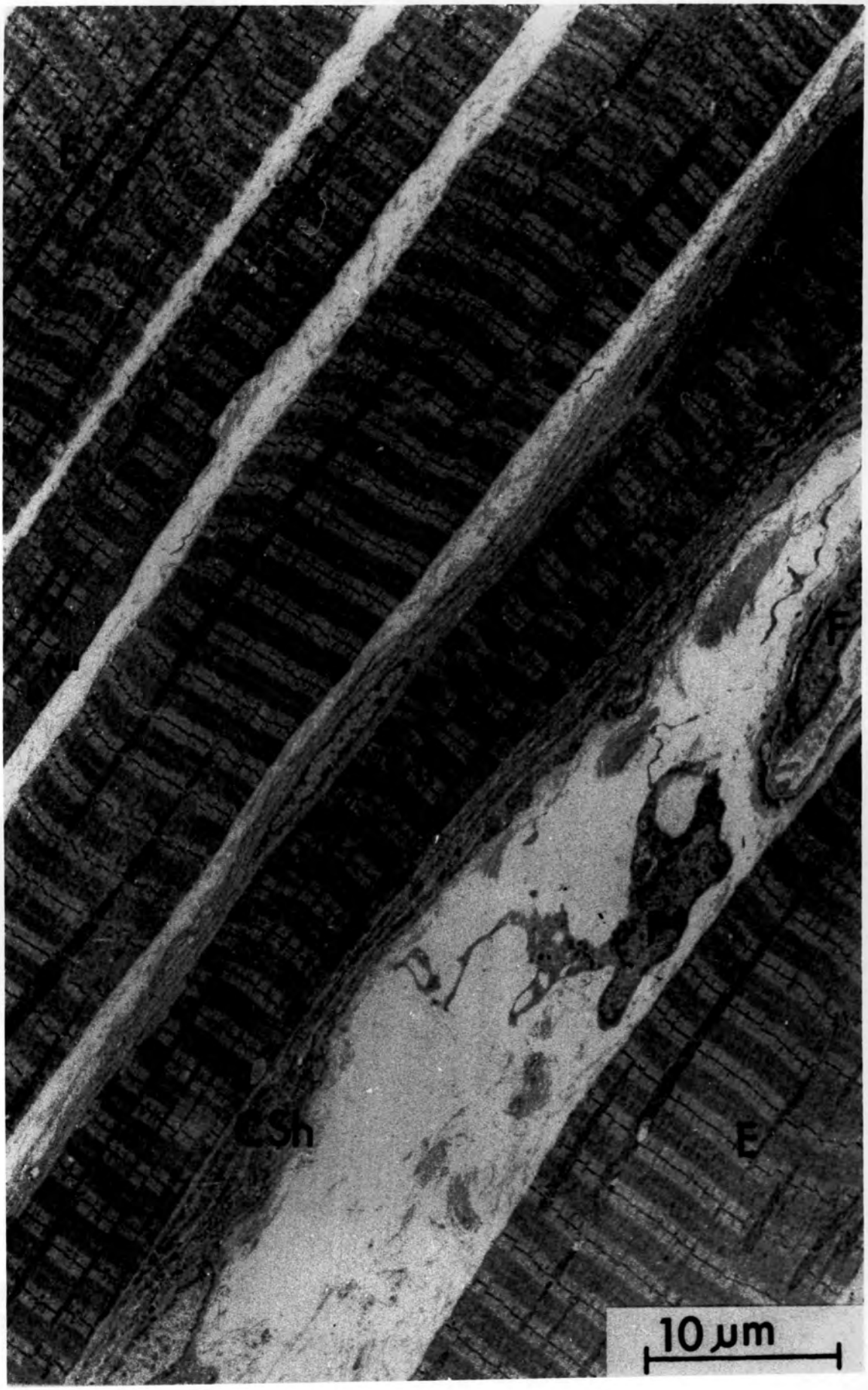
CSh - capsular sheath

E - extrafusal muscle fibre

F - fibroblast

IF - intrafusal muscle fibre

Nu - muscle nucleus


F
Sh
E
10 µm

The sarcomeres of both intrafusal and extrafusal muscle fibres showed well marked H bands with prominent M lines in the A bands and dark N lines in the I bands. The sarcomeres were clearly delineated by Z discs which were of more or less the same thickness and ran a more or less straight course across the myofibrils.(Fig. 26).

5.62 Myofibrils and myofilaments

The myofibrils in a transverse section passing through an A band, were ill-defined and fused together as a single mass of myofilaments among which the longitudinal tubules of the sarcoplasmic reticulum were seen (see section 5.63). In a transverse section through an I band, the myofibrils were more clearly delineated due to the presence of the sarcoplasmic reticulum, transverse tubules, triads (see section 5.63) and glycogen particles(Fig. 27).

In transverse section the A and I filaments showed the well-known hexagonal arrangement (Huxley & Hanson, 1960) and the distance between A filaments was 26-30 mμ in both intrafusal and extrafusal muscle fibres.

In longitudinal section the A filaments were 1.6 μm long, with a slight thickening and increased electron density at the level of the M lines (Fig. 26). Transverse sections at the level of the M line revealed that the A filaments were interconnected by fine bridges in this region (Fig. 28a).

FIGURE 26. Higher power electron micrograph of a longitudinal section of an intrafusal muscle fibre. The mitochondria tend to be near the prominent straight Z discs. The A filaments are enlarged at the M line (M). The longitudinal tubules (L) of the sarcoplasmic reticulum form networks at the A and I bands, and their electron-dense terminal sacs form triads with the transverse tubules (T) of the T-system. Some parts of the T-system are arranged longitudinally (arrows).

L - longitudinal tubule

M - M line

N - N line

T - transverse tubule

Z - Z disc


1 µm
I
I
N
N
T
Z


L
A
I
T
Nu
SE
G
A
2 µm

The I filaments were regularly arranged at their insertion into the Z discs, and showed a slight thickening for about 30-40 mμ before ending in the Z discs in regular square arrays (Fig. 28b, c).

5.63 Sarcoplasmic reticulum, transverse tubules and triads

The sarcoplasmic reticulum (Figs. 26, 27, 29) consisted of longitudinally oriented electron dense tubules 30-70 mμ in diameter, running parallel to and in between the myofibrils. In longitudinal section these tubules formed a network surrounding the myofibrils at the I band region and sometimes at the A band region. The tubules enlarged to form terminal sacs in the I bands, but these sacs did not surround the whole myofibril. These sacs as well as the tubules were electron dense due to the presence of fine granules which were more marked in the former.

The smaller tubules, 16-64 mμ in diameter, of the transverse system (T-system of Andersson - Cedergren, 1959) were seen to run not only transversely around the myofibrils, and across the intrafusal muscle fibre forming a network at the I band, but also longitudinally across the Z discs and A bands (Figs. 26, 29a b). Therefore they have the three dimensional arrangement described by Page (1968) for the

FIGURE 28a. Electron micrograph of a slightly oblique transverse section of an intrafusal muscle fibre. At the M line region (M) the A filaments are slightly enlarged, more electron dense, and connected by fine bridges (arrows) giving a dark appearance. The light H band (H) lacks these cross bridges. The I band is dark due to the presence of the I filaments (I), but the A filaments are slightly smaller and lighter in this region as compared with the M line region.

FIGURE 28b. Electron micrograph of a slightly oblique transverse section of an intrafusal muscle fibre. In the I band (I) the I filaments are irregularly arranged but as they approach their insertion into the Z disc they become thicker and form regular square arrays (*).

FIGURE 28c. Electron micrograph of a longitudinal section of an intrafusal muscle fibre. The I filaments (I) thicken as they insert onto the Z disc (Z).

H - H band

I - I filaments

M - M line

Z - Z disc

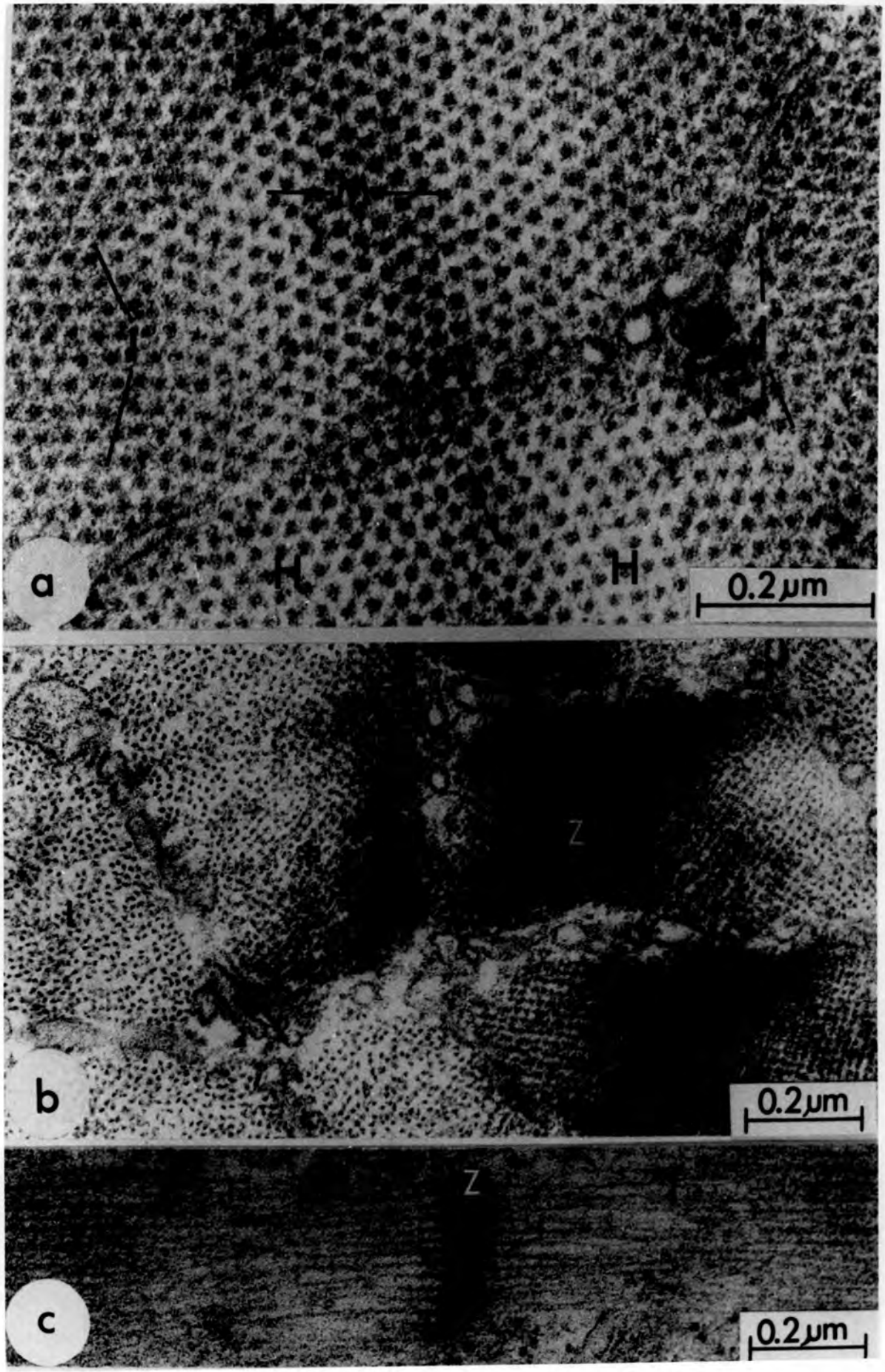

a
H
H
0.2 µm
b
Z
0.2 µm
c
Z
0.2 µm

FIGURE 29. High power electron micrographs showing further details of the arrangement of the sarcoplasmic reticulum and the T-system. In (a) a longitudinal tubule (L) forms a terminal sac (TS) adjacent to a Z disc (Z). Part of the 'transverse' tubules (T) run longitudinally across the A band and the Z disc. In (b) the contribution of the terminal sac (TS) of the longitudinal tubules and the transverse tubules (T) to the triads (Tr) is clearly shown at the I band on both sides of the Z disc (Z). In transverse section (c) 3 electron-dense terminal sacs abut onto an electron-transparent longitudinally orientated t-tubule (arrows). Transverse tubules (T) can also be seen running around the myofibrils and across the muscle fibre.

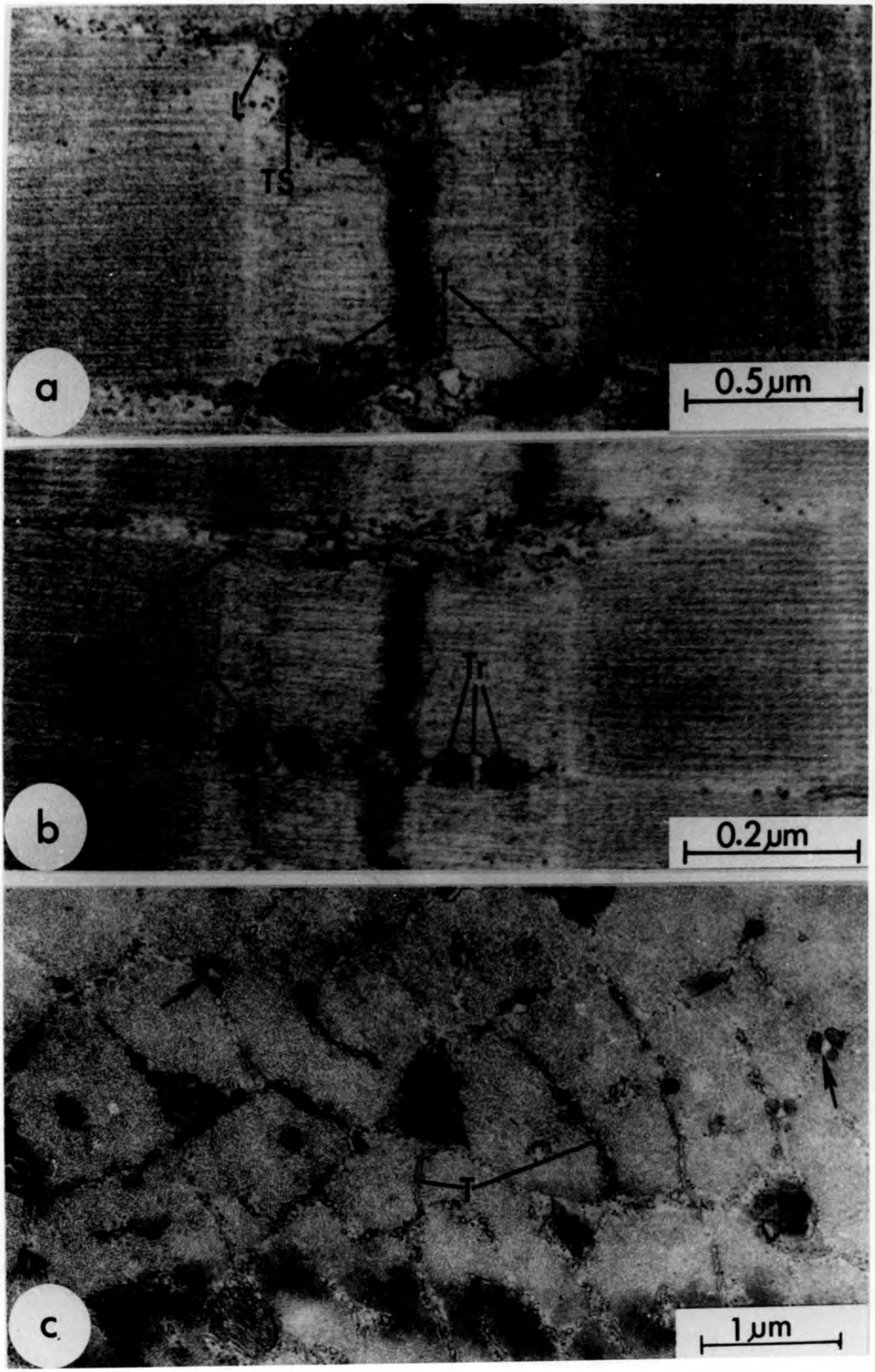
L - longitudinal tubule

T - transverse tubule

Tr - triad

TS - terminal sac of longitudinal tubule

Z - Z disc


TS
a
0.5μm
b
0.2μm
c
1μm

extrafusal muscle fibres of the tortoise. These 'transverse' tubules appeared less electron dense than those of the sarcoplasmic reticulum and no granules were seen inside them.

In a longitudinal section, a transverse tubule was usually flanked by only two terminal sacs of the sarcoplasmic reticulum, which were in close apposition to the transverse tubule forming a triad (Figs. 26, 29a, b). Triads when present were only seen in the I band on both sides of the Z discs in both intrafusal and extrafusal muscle fibres. In transverse sections passing through the I band, the centrally placed transverse tubule was sometimes flanked by more than two terminal sacs (Fig. 29c), and as many as four terminal sacs were seen flanking a single transverse tubule. This arrangement confirms the three-dimensional structure of the 'T - system', because at this point the 'transverse' tubules must be running in the same longitudinal plane as the sarcoplasmic reticulum.

The results of the study of fine structure so far presented confirm the observations of Page (1968) on the fine structure of the extrafusal muscle fibres of the tortoise and show them to be true for the intrafusal muscle fibres. In addition, further details of the structure and arrangement of the myofibrils and myofilaments have been described in the present study.

5.64 Mitochondria

In longitudinal sections these ellipsoid organelles were seen lying in between the myofibrils, with their long axes parallel to that of the muscle fibre. In the same muscle fibre, they might be placed singly, in which case one end was usually near to a Z disc, or in a row stretching for several sarcomeres (see Fig. 25, 26). The extrafusal muscle fibres did not differ in this respect.

In transverse sections at the mid-capsular region, the mitochondria, in all the intrafusal muscle fibres constituting a spindle, were of the same size (see Fig. 15). The mean diameter of 245 mitochondria in this region where sensory nerve endings were present, was 0.25 μm (range 0.12-0.5 μm). In those regions of intrafusal muscle fibres nearer to the pole of the capsule where there were no sensory nerve endings (Fig. 30), the value for 101 mitochondria was 0.41 μm (0.15-0.8 μm). The mean diameter of 52 mitochondria in the intrafusal muscle fibres just outside the capsular region was found to be 0.47 μm (0.22-1.0 μm). For the extrafusal muscle fibres the value for 56 mitochondria was 0.51 μm (0.25-1.15 μm). Thus there was an inverse relation between the sensory nerve endings on the intrafusal muscle fibres and the size of the mitochondria they contained. This result supports the observation made on transverse sections stained for the succinic dehydrogenase activity (see section 5.5).

FIGURE 30. Electron micrograph of a transverse section of the capsular region of a spindle. Besides the structural features already shown, it can be seen that the mitochondria of the only two fibres without sensory endings (*) are the same size as those of the extrafusal muscle fibres (E), whereas the mitochondria of those fibres with sensory endings (SE) are considerably smaller. All the fibres have the same arrangement of myofibrils regardless of the size of mitochondria.


10 µm

5.65 Other Cell components

A layer of sarcoplasm of variable thickness (60-560 mμ) separated the plasma membrane from the myofibrils. In this layer innumerable flask-shaped caveolae (Rayns, Simpson & Bertaud, 1968) 50-90 mμ in diameter were present with their necks opening to the exterior of the cell (Fig. 18a). On the outer perimeter, each intrafusal muscle fibre was seen to be surrounded by a basement membrane which also enclosed, in those regions where they were present, sensory nerve endings and satellite cells.

The nuclei in the equatorial region were seen to be flattened, peripherally placed and with no apparent structural difference from those of the extrafusal muscle fibres (Figs. 25, 27, 30).

Vesicles and branching tubules belonging to the Golgi apparatus were also seen in the juxtanuclear regions of the intrafusal muscle fibres.

Aggregations of glycogen granules were seen most frequently in the I band region, where the triads were located (see Fig. 16). Although some fibres contained more glycogen granules than others, they were otherwise identical. This variation in amount of glycogen most probably corresponded to the variation in phosphorylase activity along the intrafusal muscle fibres (see section 5.5).

5.66 Close apposition of intrafusal fibres

In each intracapsular region examined in serial transverse sections under LM in this study, several instances were observed where the intrafusal muscle fibres seemed to fuse together. The connective tissue between two muscle fibres disappeared, so that they were contained in one connective tissue compartment, and the apposed surfaces of the two muscle fibres became straight. It was extremely difficult to distinguish the two muscle fibres for about 20–40 μm. They then became separated again by connective tissue layers (Fig. 31). This was often repeated either between the same two intrafusal muscle fibres or between different fibres.

Figure 32a, b show electron micrographs taken at different magnifications of the region of close apposition of two intrafusal muscle fibres. Although at low magnification the two fibres looked as if they were fused together at higher magnification no real fusion or cytoplasmic continuity was seen. In this region not only the connective tissue layers were absent, but also the basement membrane of the muscle fibres was seen to be reflected from one fibre to the other instead of lying between them. Thus the distinct, continuous and strictly parallel plasma membranes of both intrafusal muscle fibres were separated by a gap of 19.5 mμ over most of the region. At intervals the membranes

FIGURE 31. Transverse sections selected from the capsular region of spindle B1, stained with Masson's trichrome. All the intrafusal fibres (a - g) and the associated extrafusal fibre (E) have been labelled to facilitate comparison between sections. Fibres f and g gradually come into close apposition (a, b) until they can be differentiated only with difficulty (c) and then separate again (d).

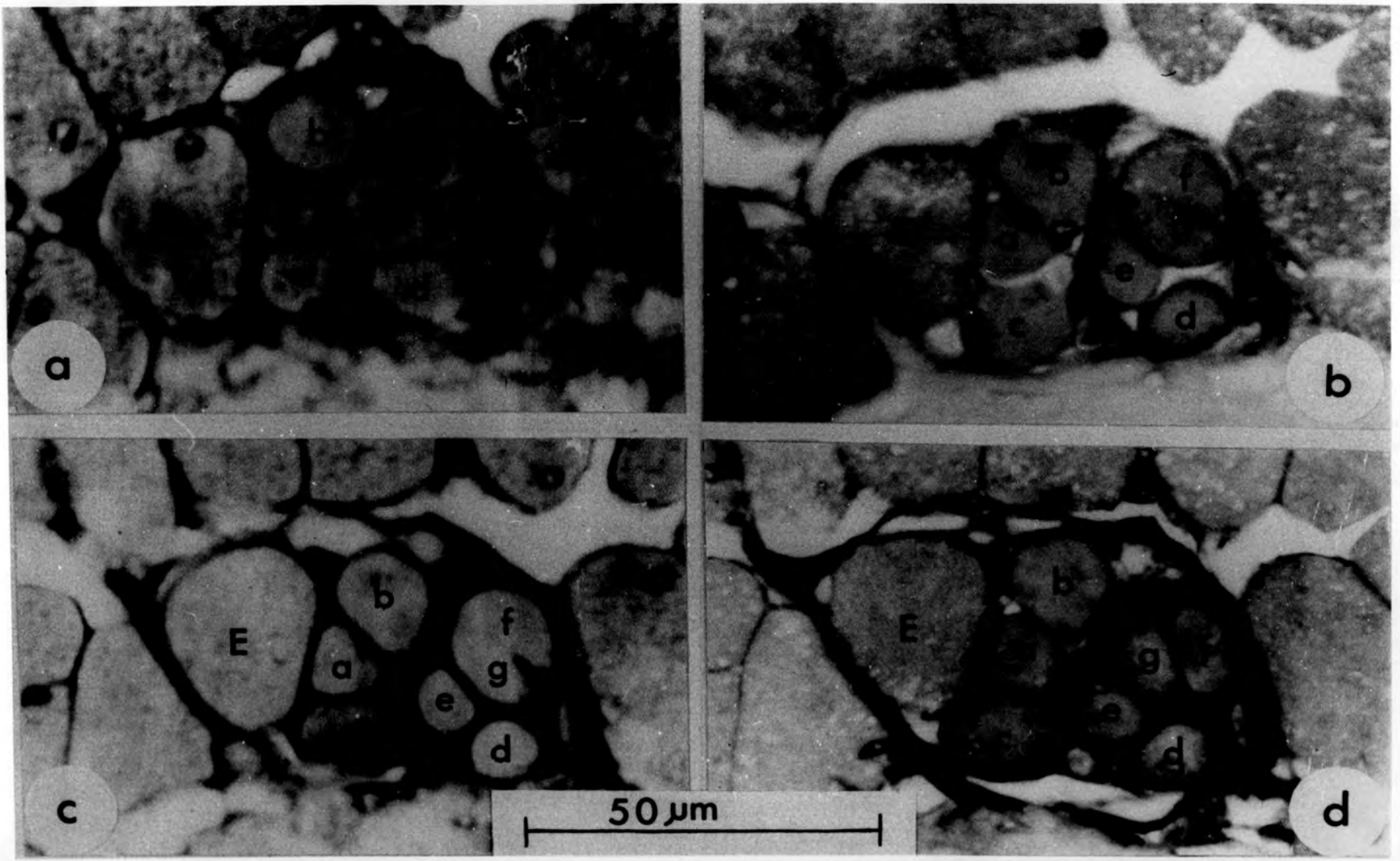
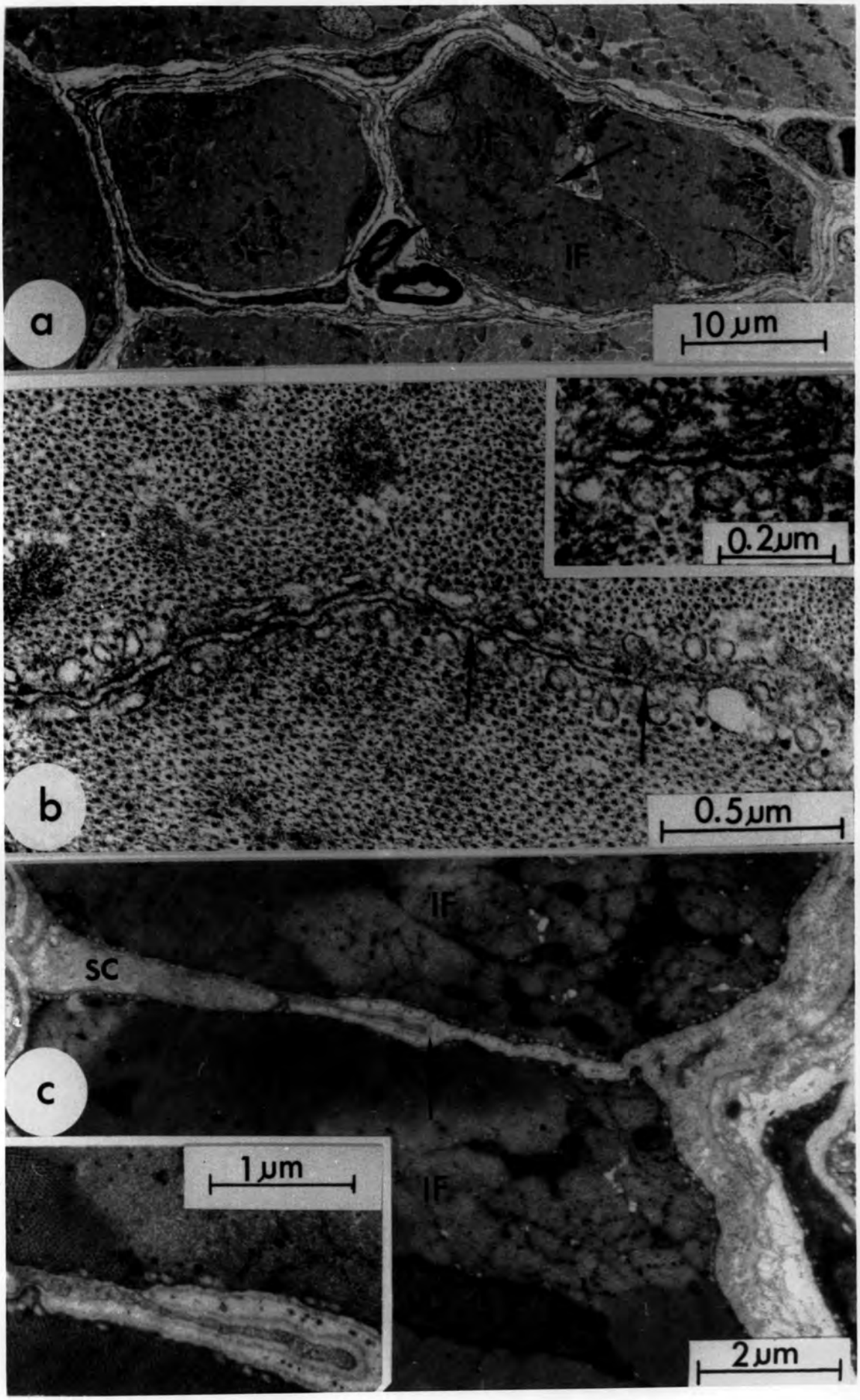

a
b
e
d
b
E
a
f
g
e
d
c
50 µm
E
b
g
d
d

FIGURE 32a. Electron micrograph of a transverse section of 2 intrafusal muscle fibres (IF) in close apposition. Their common border is indicated by the arrows.

FIGURE 32b. Higher power electron micrograph of part of the common border between the two fibres in Fig. 32a. There is no cytoplasmic continuity between the two fibres. The inset shows the region between the arrows at a still greater magnification. Periodic patches of electron-dense material are associated with filamentous material crossing the gap between the membranes.

FIGURE 32c. Electron micrograph of two intrafusal muscle fibres (IF) in an early stage of apposition. A satellite cell process (SC) still separates the fibres. The inset shows the interposing satellite cell process at a higher magnification. It is enclosed in the muscle fibre basement membrane as it is reflected from one fibre to the other (arrow). Osmium fixation.


a
IF
10 µm
b
0.2µm
0.5µm
c
IF
SC
1µm
IF
2µm

appeared darker, with an accumulation of electron dense material in the adjacent sarcoplasm. This electron dense material was seen to bridge the intercellular gap between the two fibres.

Always at the region of close apposition between two intrafusal muscle fibres, a satellite cell (see section 5.67) binding the two fibres together was seen and was enclosed in the muscle fibre basement membrane which was reflected from one fibre to the other (Fig. 32c).

This absence of cellular continuity between two closely apposed intrafusal muscle fibres and the absence of fusion between their plasma membranes is in agreement with what has been reported in the intrafusal muscle fibres of the cat spindle (Corvaja, Marinozzi & Pompeiano, 1967).

5.67 Satellite Cells

In the capsular region, under the LM, flattened deeply basophilic nuclei were seen in close association with the intrafusal muscle fibres. They could easily be mistaken for peripherally placed muscle fibre nuclei. However, they were differentiated by being larger, more elongated and more deeply stained. They fit tightly around a considerable part of the circumference of the intrafusal muscle fibres (Figs. 7e, f, 9g).

Under the EM these nuclei proved to be those of the satellite cells (Mauro, 1961) which were particularly evident in the sensory region, where they were associated with every intrafusal muscle fibre. They gradually decreased in number, as the sensory nerve endings decreased towards the poles of the capsule. Figure 33 shows a satellite cell nucleus which contained a deeply stained nucleolus, and was surrounded by a thin layer of cytoplasm. The thin extensive bodies of these cells appeared as thin processes (40-320 mμ thick) around the intrafusal muscle fibres. These processes more or less enclosed the sensory nerve endings (Fig. 33b) when present and took part in binding the intrafusal muscle fibres when they were in close apposition. These cells were so close to the intrafusal muscle fibres that their plasma membranes were separated by only 19.5 mμ, and were enclosed within the basement membrane of the muscle fibres. Sometimes processes from one satellite cell covered two adjacent intrafusal muscle fibres. Figure 33c shows such a case and it should be noted that the basement membrane is reflected from one muscle fibre to the other along the satellite cell process.

Satellite cells were relatively devoid of organelles, apart from the nuclei, a few small mitochondria (0.15-0.2 μm), granular endoplasmic reticulum, occasional ribosomes and glycogen granules (Fig. 33d).

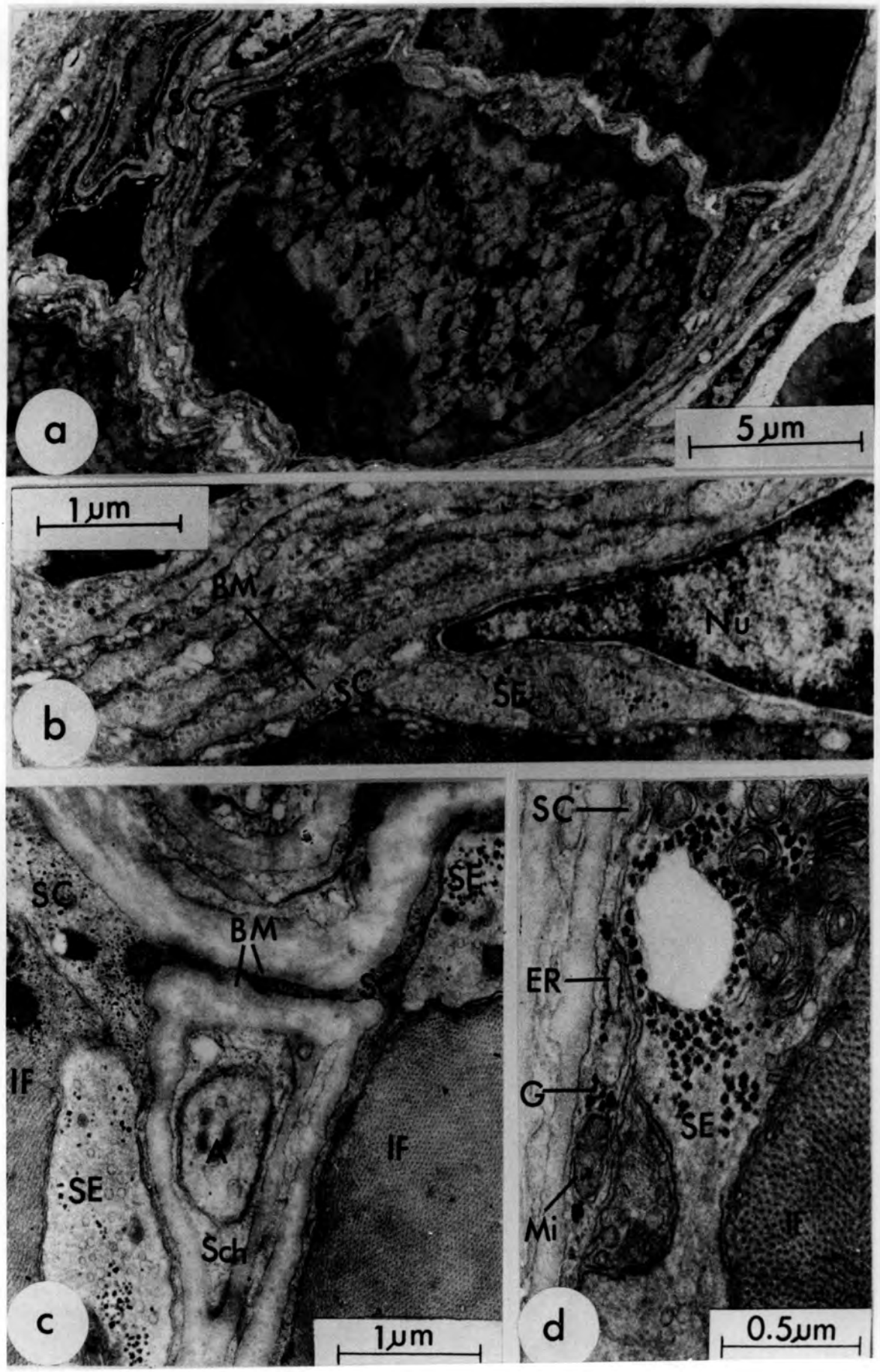
FIGURE 33a. Electron micrograph of a transverse section of an intrafusal muscle fibre (IF). The satellite cell nucleus (SC) contains an electron-dense nucleolus and is enveloped by a thin layer of cytoplasm, which extends to form long processes around the fibre.

FIGURE 33b. Electron micrograph showing the relationship of the satellite cell processes (SC) to the sensory endings (SE). Both are enclosed within the basement membrane (BM) of the muscle fibre.

FIGURE 33c. Electron micrograph showing a satellite cell process (SC) passing between two adjacent intrafusal muscle fibres (IF). The basement membrane (BM) is reflected from one fibre to the other, along the satellite cell process.

FIGURE 33d. Electron micrograph of the organelles and inclusions of a satellite cell process (SC).

A	sensory axon	IF	intrafusal muscle fibre
BM	basement membrane of intrafusal muscle fibre	Mi	mitochondrion
		Nu	satellite cell nucleus
ER	granular endoplasmic reticulum	SE	sensory ending
		SC	satellite cell
G	glycogen granules	Sch	Schwann cell process


a
5 µm
1 µm
BM
SC
SE
Nu
b
SC
SE
BM
IF
SE
A
Sch
IF
c
1 µm
SC
ER
G
SE
Mi
IF
d
0.5µm

The satellite cell processes could be distinguished from those of the capsular cells by their being enclosed in the basement membrane of the intrafusal muscle fibres and by being devoid of the pinocytotic vesicles which were a striking feature of the capsular cells.

5.7 Branching

Two cases were observed in the serially sectioned material, where an intrafusal muscle fibre appeared to branch. One of the fibres of spindle D4 was single for most of its length, including the intracapsular region, then it branched as it approached the distal end of the capsule. The two arms of the branched part were both 2.9 mm long. A 'double tandem' spindle (D6) also had a fibre which remained single until the point of emergence from the proximal capsule, where it branched. Both arms of the branched fibre passed through the distal capsule and were 4.6 mm long.

The separation of the two arms of the branched fibres was very ill-defined at first and became gradually more distinct, although the branches remained in close relation over their entire length.

Since only these two examples of the branching of intrafusal muscle fibres were found in the LM examination of 282 intrafusal muscle fibres, it was not surprising that, in

spite of the most careful examination, no such examples were found in the EM preparations.

6. Sensory innervation

6.1 Light microscopy

Eighty-two teased whole spindle preparations from EDBI muscle were examined for their sensory innervation, of these 61 were stained with methylene blue, and 21 with silver.

Each teased spindle was served by a single, heavily myelinated, sensory nerve fibre which always divided into two or three main branches before it entered the spindle capsule. In all except one case, in which the sensory nerve fibre entered the sensory region parallel to the intrafusal muscle fibres (Fig. 34a), the sensory nerve or its main branches entered the capsule at right angles to the long axis of the spindle (Fig. 34b). Within the spindle capsule, these main branches passed in opposite directions, parallel to the intrafusal muscle fibres and started to divide dichotomously and the branches went in opposite directions, parallel to the intrafusal muscle fibres. This 'T' shaped branching was repeated several times before the axon lost its myelin sheath. The non-myelinated fine axon terminals meandered over the surface of the intrafusal muscle fibres, and gave fine endings which in methylene blue preparations appeared as varicose threads (Fig. 34c).

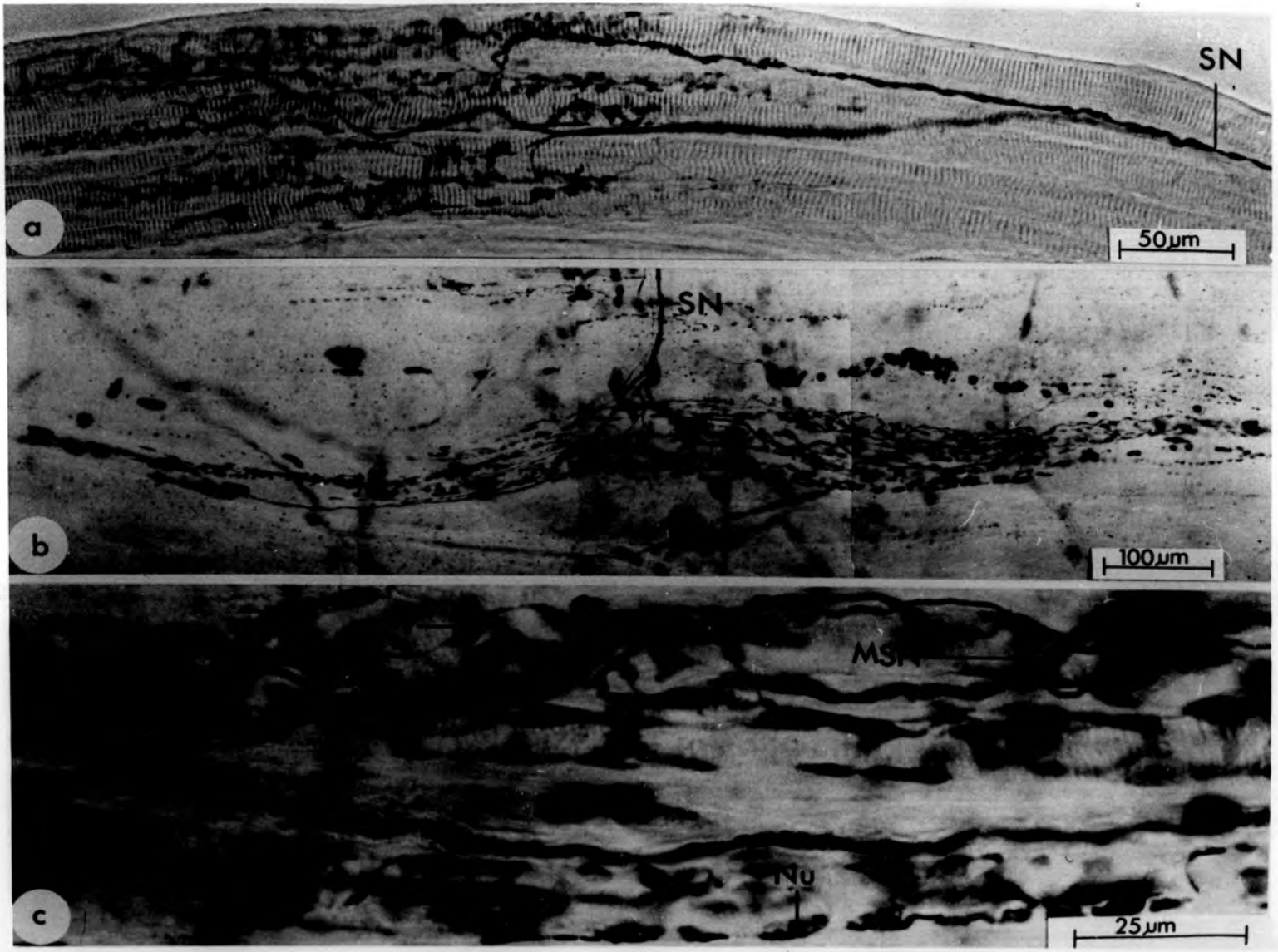
FIGURE 34. Teased methylene blue preparations to show the details of sensory innervation. Typically the sensory nerve enters the spindle at right angles to the long axis of the intrafusal muscle fibres as in (b). In a single case shown in (a) the sensory nerve entered the spindle in the long axis of the intrafusal muscle fibres. The sensory nerve (SN) branches dichotomously with the myelinated branches (MSN) passing in opposite directions. The non-myelinated axon terminals meander over the intrafusal muscle fibres. The endings are fine bearing varicose synaptic contacts (v).

Nu - intrafusal fibre nucleus

MSN - myelinated branch of sensory nerve

SN - sensory nerve

V - varicose synaptic contact


SN
a
50µm
SN
b
100µm
MSN
Nu
c
25µm

Only one type of sensory nerve ending was seen and these never formed spirals or rings around the intrafusal muscle fibres, although the myelinated main branches of the sensory nerve fibre sometimes followed a spiral course, encircling one or more intrafusal muscle fibres (Figs. 34b, 35b).

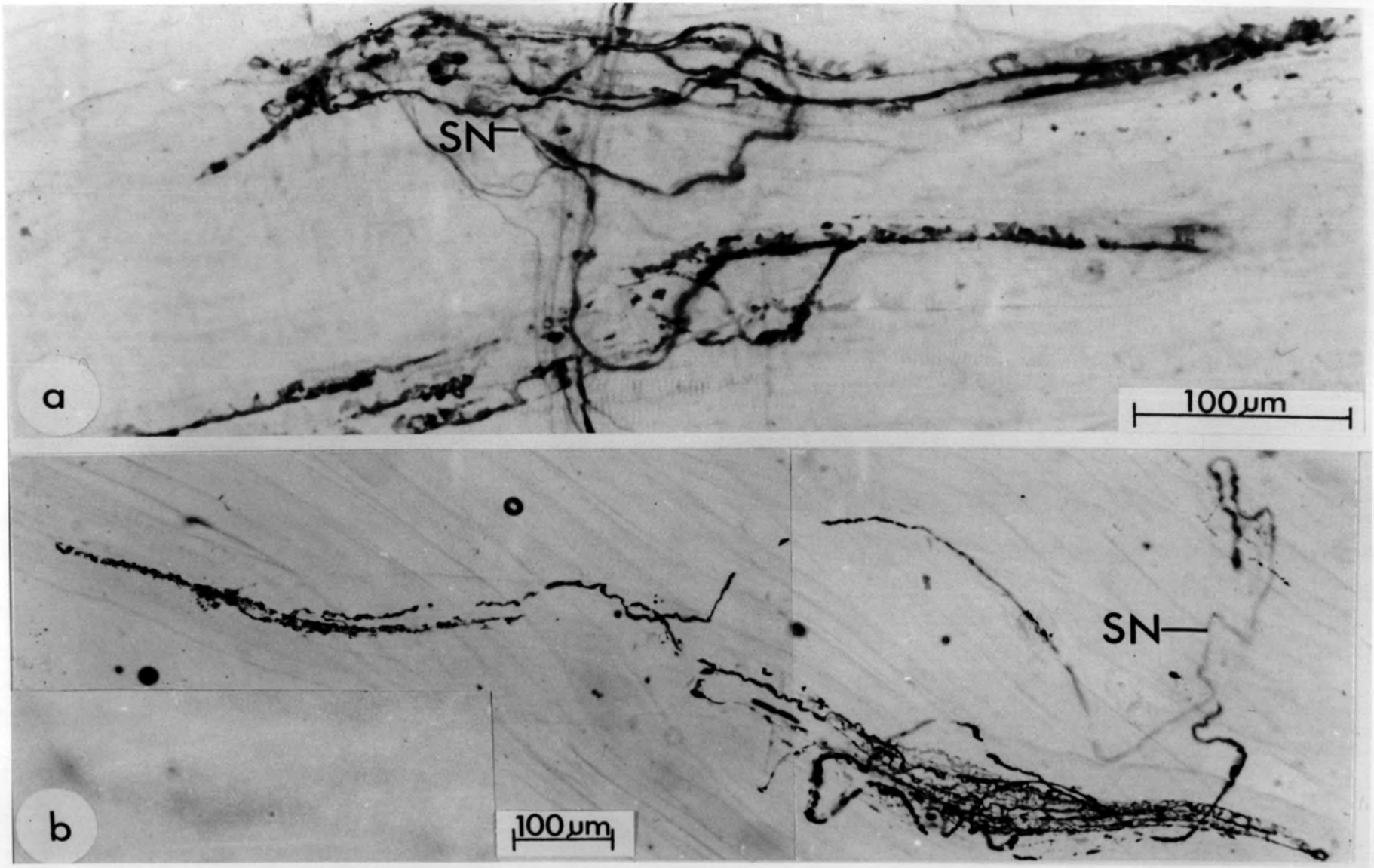
In no case was a spindle served by more than one sensory nerve fibre. On the contrary, of the 82 teased whole spindles, 6 cases were seen in which the same sensory nerve fibre divided to supply two spindles separated by varying numbers of extrafusal muscle fibres (Fig. 35a). A single case was seen where the same sensory nerve innervated three spindles (Fig. 45a).

Only one 'double tandem' spindle was seen, in which both sensory regions (0.3 mm apart) were served by the same sensory nerve fibre, which divided into two main branches, one supplying each sensory region (Fig. 35b). In the proximal sensory region all the intrafusal muscle fibres received sensory endings, while in the distal sensory region only two intrafusal muscle fibres did so.

Each intrafusal muscle fibre had a definite region of sensory innervation which was of variable length (0.28-0.75 mm). However in a given spindle the lengths of the sensory regions were more or less the same for every intrafusal muscle fibre although they did not necessarily lie alongside one another. This is shown to the left of Fig. 34b where only one intrafusal

FIGURE 35a. Teased methylene blue preparation of two spindles supplied by branches of a single sensory nerve fibre (SN).

FIGURE 35b. Teased methylene blue preparation of a 'double tandem' spindle. Both sensory regions are supplied by branches of the same sensory nerve (SN). In places, the myelinated portions of the sensory nerve pursues a spiral course around the intrafusal muscle fibres.


SN
a
100 µm
SN
b
100 µm

muscle fibre has a sensory ending while the other intrafusal fibres have their sensory regions further to the right.

The transverse striations of the intrafusal muscle fibres were continuous in the region of the sensory nerve endings (e.g. Fig. 34a).

Figure 36 shows the distribution of 50 sensory nerve fibre diameters measured at a point 1 mm from spindle entry in methylene blue preparations. The distribution is unimodal with a mean diameter of 5.2 μm and a standard deviation of 1.08 μm.

6.2 Electron microscopy

Under the EM, mitochondria, neurofilaments, neurotubules, microvesicles and glycogen granules normally present in other vertebrate nerve fibres (e.g. Fawcett, 1967) were seen in both myelinated and non-myelinated parts of sensory nerve fibres (Figs. 37, 38a). In addition, structureless vacuoles of different diameters ranging from 0.1 to 0.8 μm were also seen frequently. Around these vacuoles large amounts of glycogen granules were arranged, and on only one occasion a vacuole was seen surrounded by a limiting membrane (Fig. 33d). Such vacuoles were also frequently seen in the sensory endings (e.g. Figs. 39b, 41b), and are believed to be identical to the bleached fat droplets described by Fawcett & McNutt (1969) in cardiac muscle.

FIGURE 36. Distribution of the mean diameters of 50 sensory nerve fibres measured in teased methylene blue preparations. The mean is 5.2 μm (S.D. 1.08 μm).

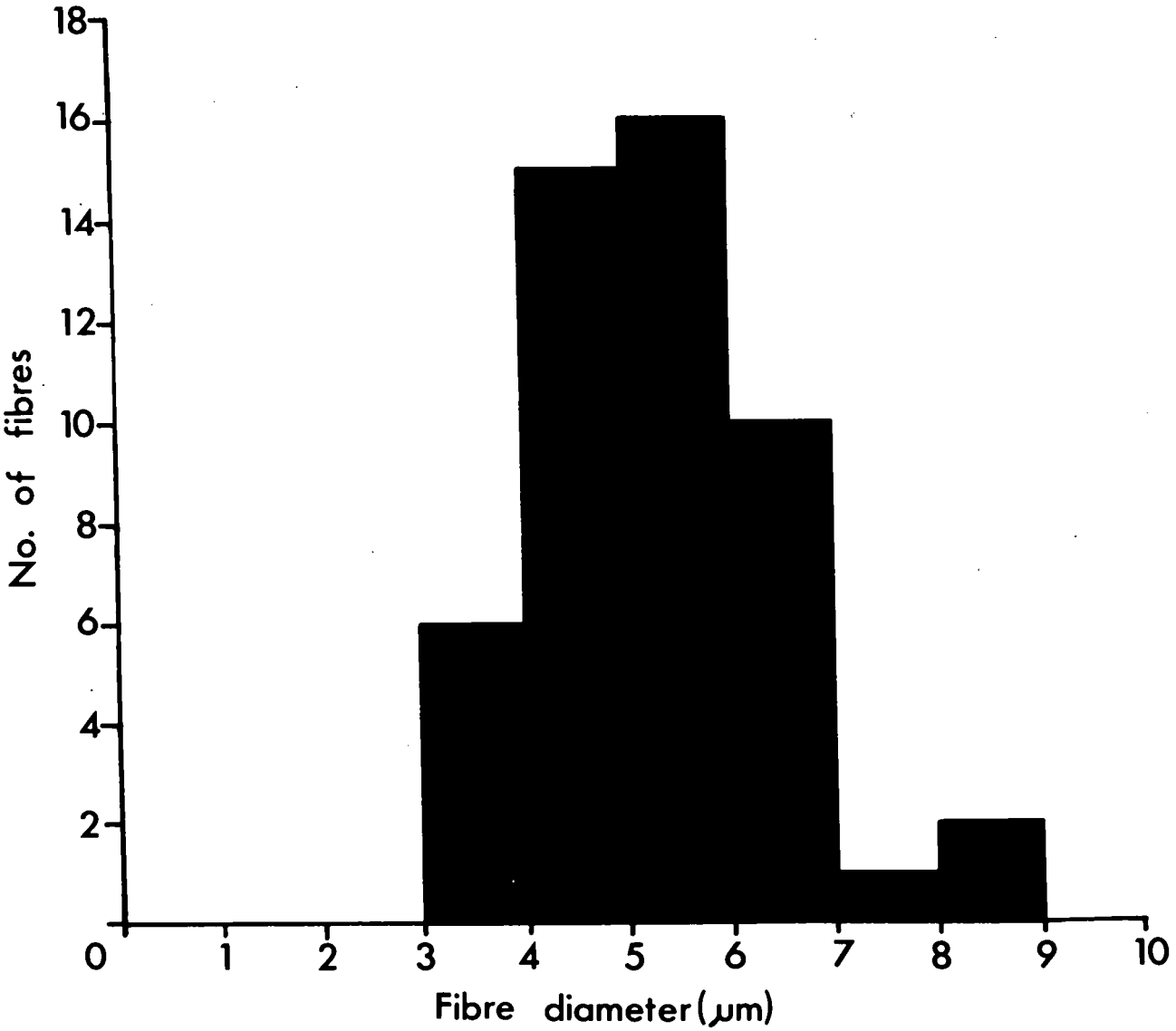

18
16
14
12
10
8
6
4
2
No. of fibres
0
1
2
3
4
5
6
7
8
9
10
Fibre diameter (μm)

FIGURE 37. Electron micrograph of a myelinated branch of a sensory nerve.

A - axoplasm

Mi - mitochondrion

nf - neurofilaments

nt - neurotubules

V - vesicle

VD - vesicle containing dense body

* - ? leached fat droplet surrounded by glycogen granules


V
A
n
VD
nf
1 µm

The slender 0.1-0.2 μm terminal axons on the other hand (Fig. 38b, c) were comparatively devoid of organelles and contained neurofilaments only. As these terminal axons enlarged to form sensory endings, microvesicles started to reappear but no mitochondria were seen in that region of the ending adjacent to the terminal axon.

The sensory nerve endings were irregularly arranged on the circumference of the transversely sectioned intrafusal muscle fibres (Fig. 39a). In longitudinal section the region of contact of a single sensory ending might cover as many as four sarcomeres, generally the sensory terminals were ellipsoid in shape and had their long axes parallel to those of the intrafusal muscle fibres (Fig. 39b). They usually lay in more or less deep, trough-like, depressions in the surface of the intrafusal muscle fibre. The sarcolemma was folded on both sides of the ending to form a lip which extended over the outer surface of the ending for a variable distance and at times completely surrounded the ending (Fig. 39a). Satellite cell processes generally took part in the formation of the lips of the trough in which the sensory ending lay (Fig. 40a, b). In some cases a sensory terminal sat proud on the surface of the muscle fibre, in which case the lip of the trough was formed almost entirely by satellite cell processes which sometimes completely enclosed the ending (Fig. 41a, b).

FIGURE 38a. Electron micrograph to show the fine structure of a non-myelinated region of a sensory nerve fibre.

FIGURE 38b, c. Electron micrograph of a terminal axon (TA) as it enlarges to form a sensory ending (SE). The terminal axon contains only neurofilaments (nf) and at this region, the sensory ending lacks mitochondria.

A - axoplasm

ER? - ? rough endoplasmic reticulum

G - glycogen granules

IF - intrafusal muscle fibre

Mi - mitochondrion

MV - multivesicular body

nf - neurofilament

Sch - Schwann cell process

SE - sensory ending

TA - terminal axon

V - vesicle

VD - vesicle containing dense body

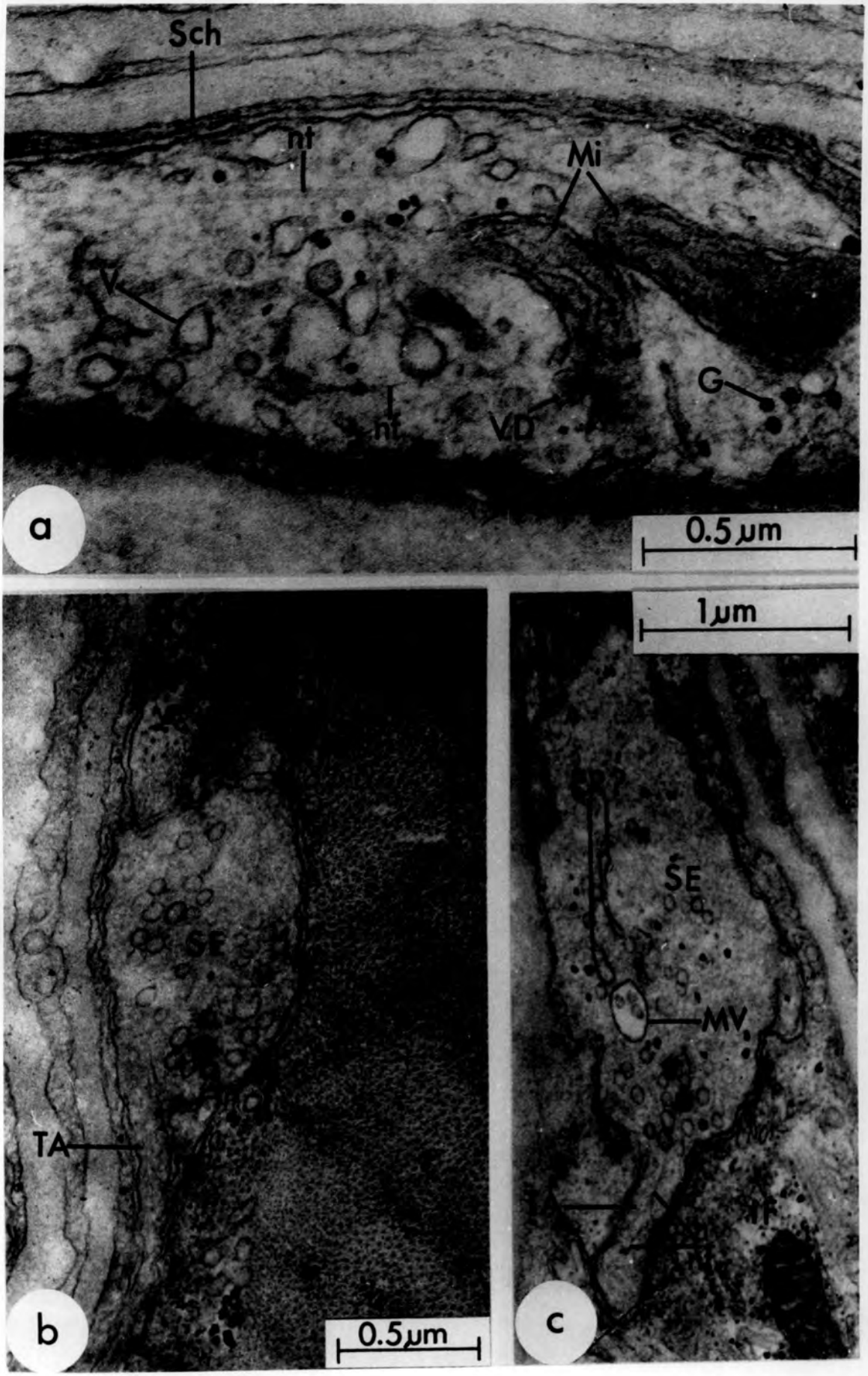

Sch
nt
Mi
V
G
nt
VD
a
0.5 µm
1µm
SE
TA
b
0.5µm
SE
MV
c

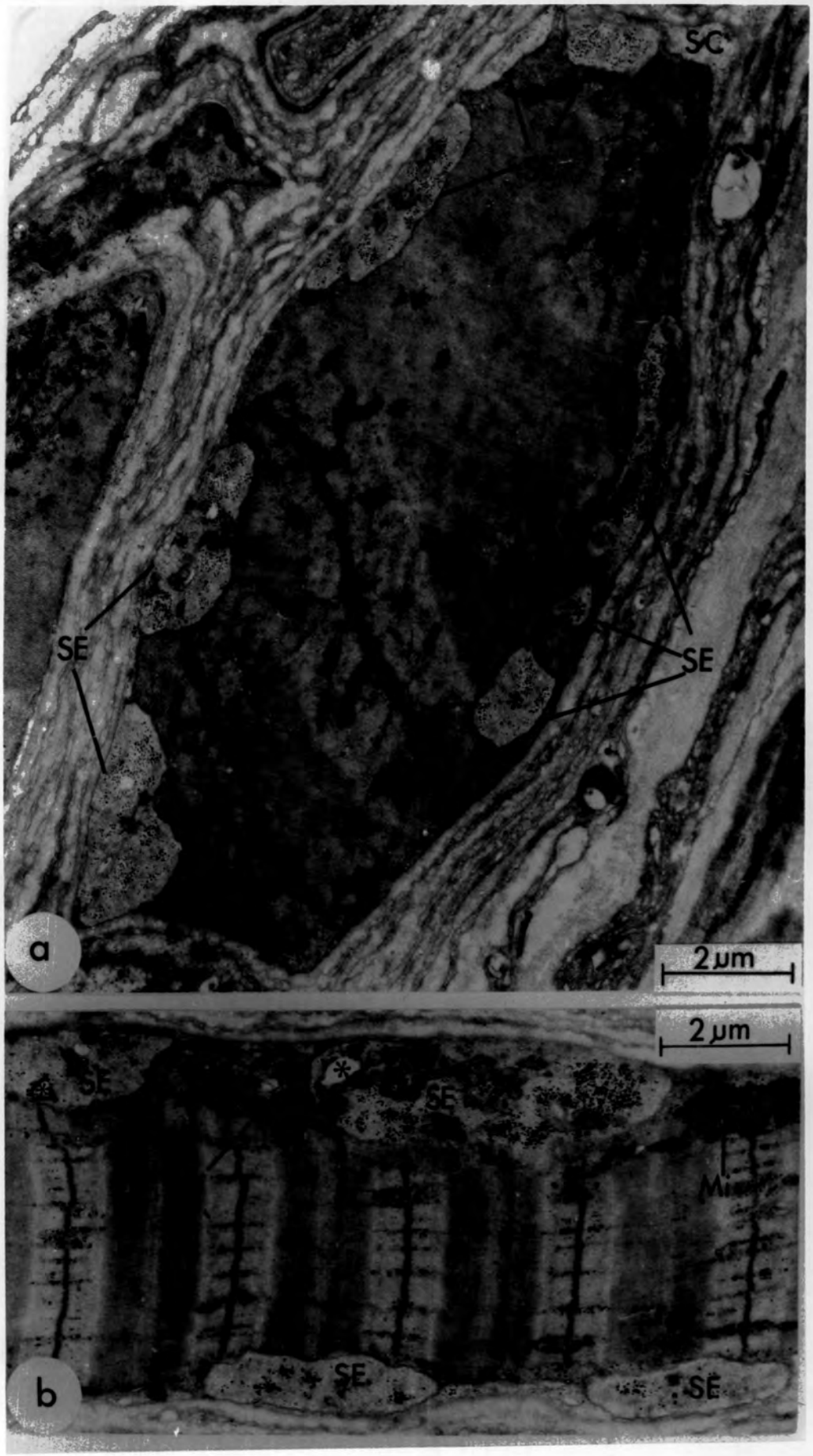
FIGURE 39a. Electron micrograph of a transverse section of an intrafusal fibre showing the irregular arrangement of the sensory nerve endings (SE), in contrast to the well-known spiral arrangement of other species. Some of the endings (*) are completely enclosed by the sarcolemma.

FIGURE 39b. Electron micrograph of a longitudinal section of an intrafusal muscle fibre. The sensory endings (SE) are elongated in the long axis of the intrafusal muscle fibres. A leached fat droplet (*) is present in one sensory ending.

Mi - mitochondrion

SC - satellite cell

SE - sensory ending


SC
SE
SE
a
2 µm
2 µm
SE
SE
Mi
SE
SE
b

In most transverse and longitudinal sections, the sensory endings were seen to contain variable numbers of mitochondria (0.12-0.24 μm in diameter) which were smaller and less elongated than those of the muscle fibre. In most cases the mitochondria tended to aggregate in the centre of the endings, but occasionally mitochondria were completely absent (Fig. 41a). Other organelles including clear vesicles and vesicles containing a dense body were seen in variable numbers, the former were by far the more common and had a diameter range of 35-80 mμ, the latter were less frequent, with a diameter range of 53-120 mμ; the dense body inside was 40-55 mμ in diameter. Multivesicular bodies (0.24-0.3 μm) were also seen (see Figs. 38c, 40b) containing from 3 to 6 vesicles (30-40 mμ). Tubular profiles probably belonging to the endoplasmic reticulum were also present (Fig. 38c). Glycogen granules were present in great numbers and were deeply stained in material post-fixed in Dalton's chrome osmium (see Fig. 40a, b).

All the above organelles tended to aggregate in the centre of the cytoplasm which usually appeared fluffy and more electron dense at the periphery of the terminal.

The sensory myoneural junction was generally smooth with no junctional folds. The plasma membrane of the sensory terminals was separated from that of the intrafusal muscle

FIGURE 40a. High power electron micrograph of a sensory ending (SE) and its organelles. At the left a satellite cell process (SC) forms the lip of the trough; on the right the lip is formed by the sarcolemma (S). The basement membrane (BM) of the muscle fibre is continuous over the ending and the satellite cell. Note that there is no gap separating the basement membrane from the underlying structures (cf Fig. 40b).

FIGURE 40b. Electron micrograph of a sensory ending (SE). At the left a satellite cell (SC) gives processes above and below the ending; on the right the lip is formed by the sarcolemma (S). The basement membrane (BM) of the muscle fibre is continuous over the ending and the satellite cell. The basement membrane is separated from the muscle fibre and the sensory ending by a gap, and the glycogen granules are less deeply stained than in Fig. 40a. (Osmium fixation).

BM - basement membrane of the muscle fibre
G - glycogen granules
Mi - mitochondrion
MV - multivesicular body
SC - satellite cell
SE - sensory ending
V - vesicle
VD - vesicle containing a dense body

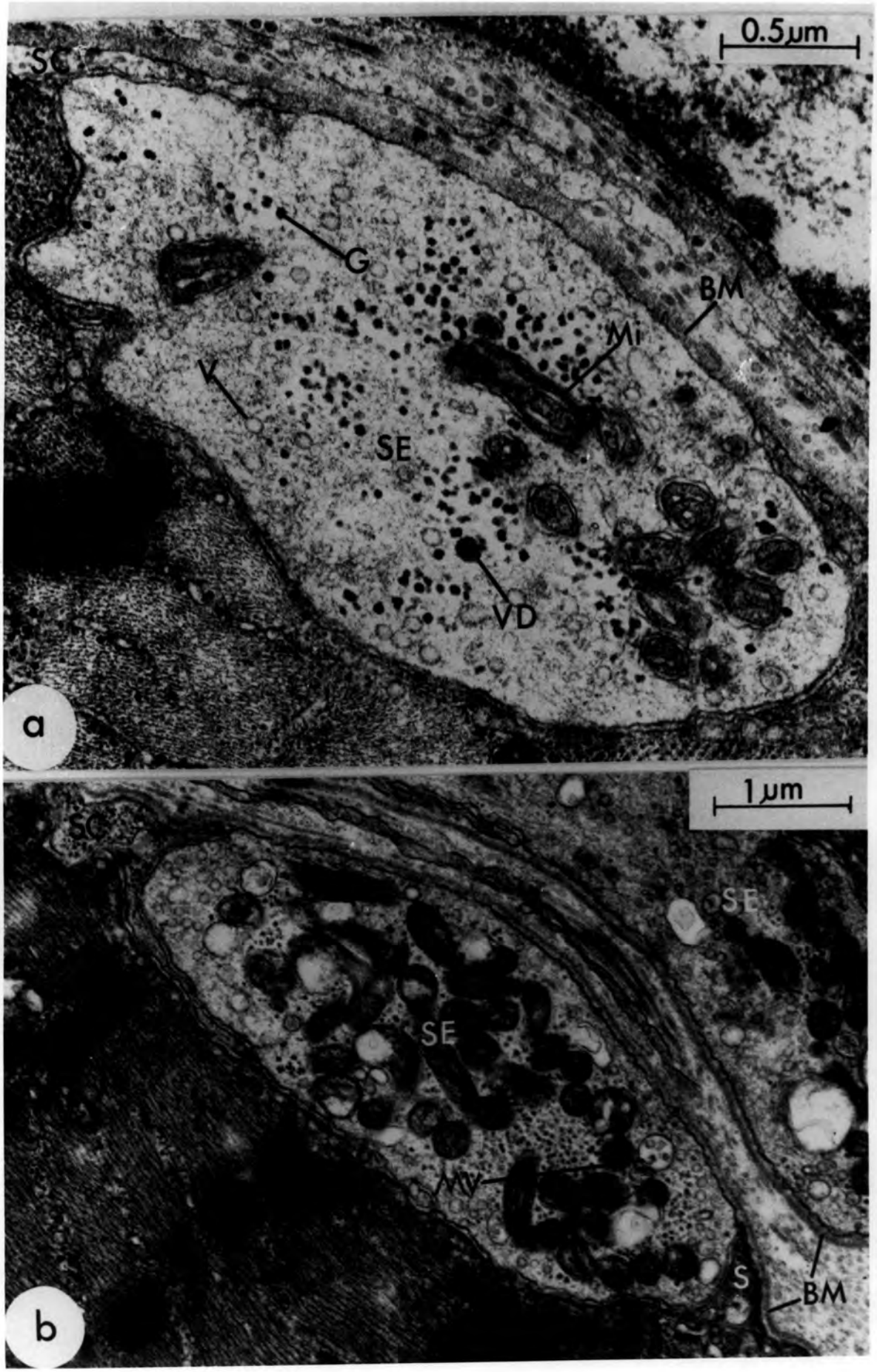
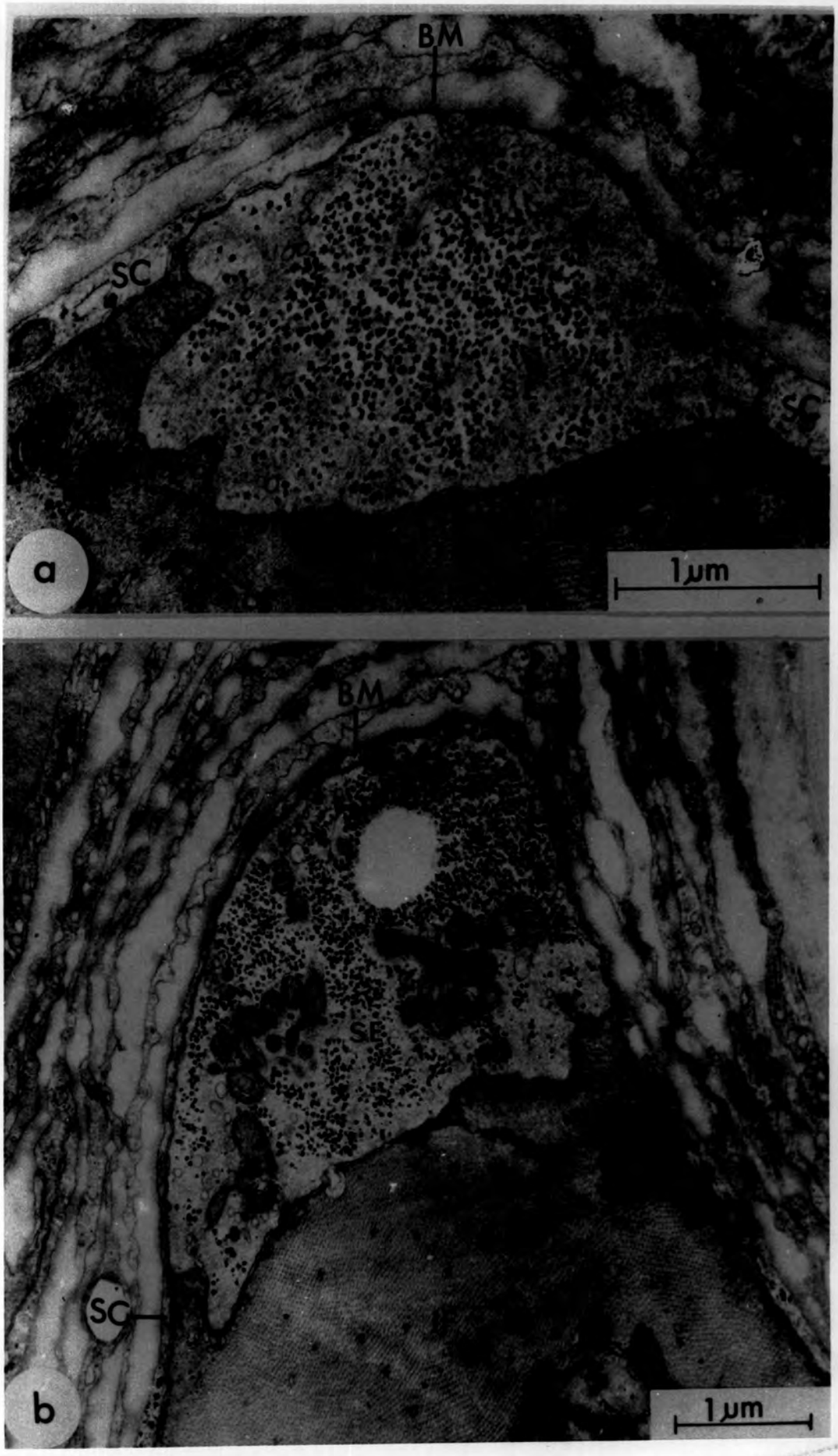

0.5µm
SC
G
BM
Mi
V
SE
VD
a
1µm
SC
SE
SE
MV
S
BM
b

FIGURE 41. Electron micrographs of sensory endings. The endings (SE) do not lie in troughs but 'sit proud' on the intrafusal fibres (IF). The lips at the edges of the ending are formed almost entirely by satellite cell processes (SC), which completely surround the ending in (b). The mitochondria are completely absent from the ending in (a).

BM - basement membrane of the muscle fibre

IF - intrafusal muscle fibre

SC - satellite cell process


BM
SC
SC
a
1µm
BM
SE
SC
b
1µm

fibre by a gap of about 19.5 μm. At intervals the membranes were more electron dense with accumulation of electron dense amorphous and filamentous material bridging between them (Fig. 42a). A gap of the same width was seen between the plasma membranes of the satellite cell processes and those of the intrafusal muscle fibres and the sensory ending (Fig. 42b).

Instances were seen where the membranes of the muscle fibre and the sensory ending were ill defined and appeared to be discontinuous. In many cases it was suspected that these irregularities could be caused by the plane of sectioning, but regions were seen in which it was reasonable to suspect that cytoplasmic continuity did exist. In Fig. 42c, for example, the membrane of the sensory terminal is fused with that of the intrafusal fibre so that the two membranes have the appearance of a single membrane that was folded double on either side of the gap. In the region of the gaps, cytoplasmic material of the same intensity of staining, could be seen connecting the intrafusal muscle fibres and the sensory terminal. Such continuity was not commonly seen.

In cases where sensory terminals were seen in the clefts between pairs of intrafusal fibres in close apposition, a satellite cell process was present between one of the intrafusal fibres and the sensory ending to restrict, but not to obliterate, the common border between the two cells (Fig. 43a).

FIGURE 42a. Electron micrograph of the relation of the plasma membranes of the sensory ending (SE) and the intrafusal muscle fibre (IF). At intervals there are accumulations of electron dense material (arrows) with bridges of filamentous material.

FIGURE 42b. Electron micrograph of the relation of the plasma membranes of the sensory ending (SE), intrafusal muscle fibre (IF) and satellite cell processes (SC). The gaps separating the plasma membrane of these structures are of more or less same magnitude.

FIGURE 42c. Electron micrograph showing an apparent discontinuity (arrow) in the plasma membranes of the intrafusal muscle fibre (IF) and the sensory ending (SE). The inset shows the region of the discontinuity at a higher magnification. The two plasma membranes appear to be fused.

BM - basement membrane of the muscle fibre

IF - intrafusal muscle fibre

SC - satellite cell process

SE - sensory ending

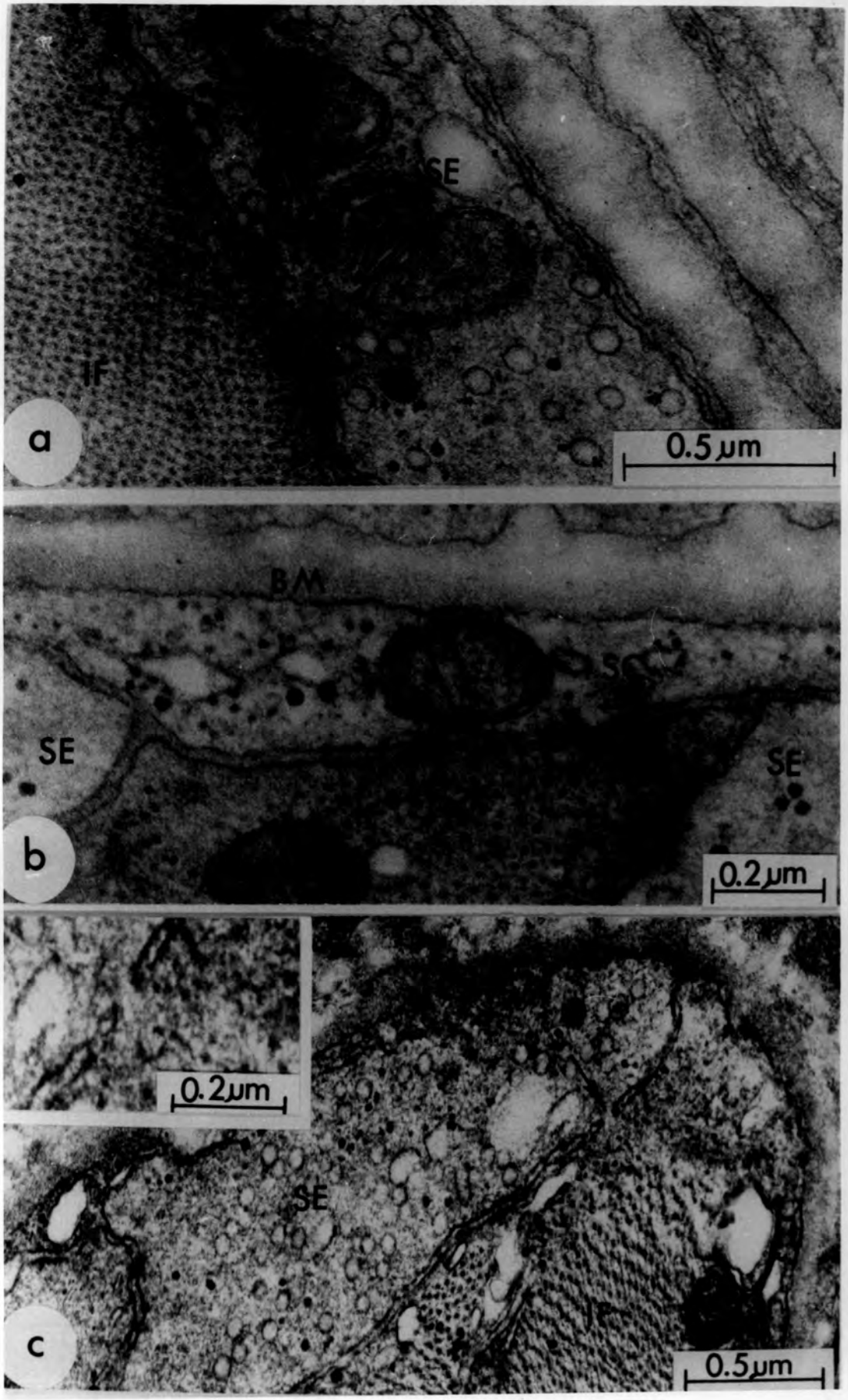
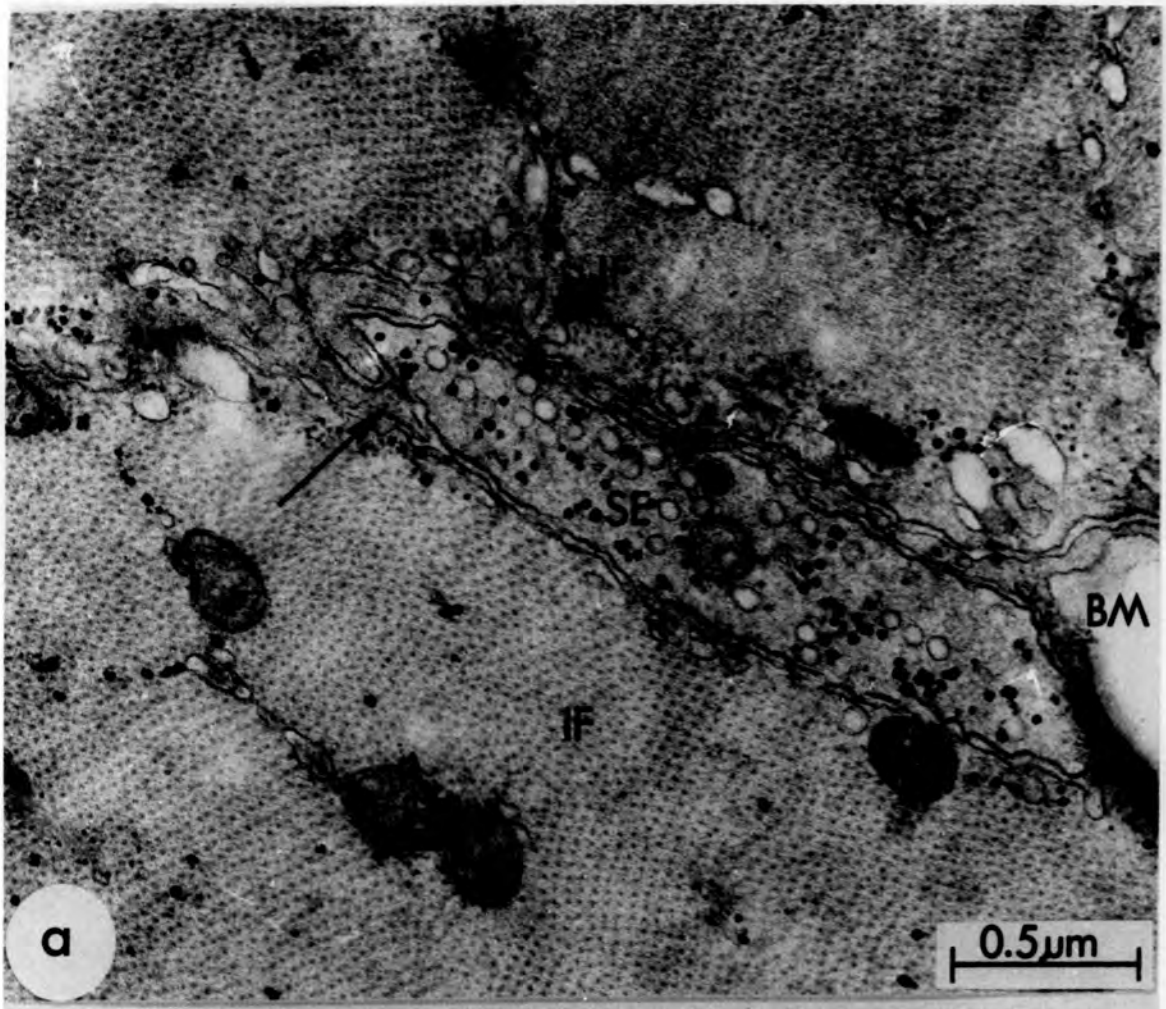
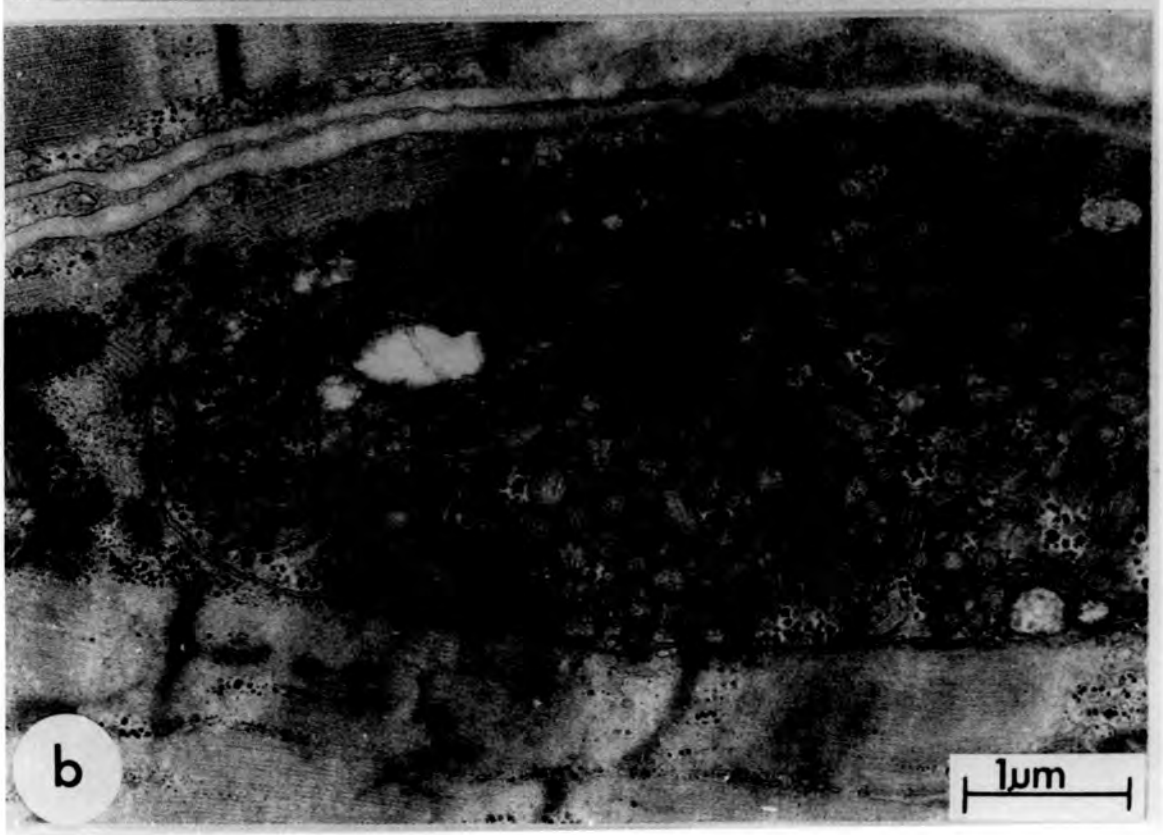

SE
IF
a
0.5 µm
BM
SE
SE
b
0.2µm
0.2µm
SE
c
0.5µm

FIGURE 43a. Electron micrograph of two intrafusal muscle fibres (IF) in close apposition. A sensory terminal (SE) lies between them but is separated from the upper fibre by a satellite cell process, except at the arrow. The basement membrane (BM) is reflected from one fibre to the other and over the satellite cell.

FIGURE 43b. Higher power electron micrograph of the structure shown in Fig. 16. It resembles a sensory ending packed with mitochondria, arranged in transverse and longitudinal planes.


SE
BM
IF
a
0.5µm


b
1µm

On one occasion a structure rather larger than a sensory terminal (Figs. 16, 43b) was found. It was tightly packed with mitochondria which were of the same size as those found in sensory endings, but they were not randomly oriented. One group lay in a plane parallel to the axis of the muscle fibre; the other group lay at right angles to this plane. The limiting membrane of this structure was separated from the sarcolemma by a gap similar to that seen between other sensory endings and the sarcolemma.

Katz (1961) described 'microladders' in the frog intrafusal muscle fibres in relation to the sensory endings. Similar structures were also seen by Gruner (1961) in man, by Landon (1966) in rat and by Corvaja et al. (1969) in cat. Such structures were not seen in the present study in relation to the tortoise sensory endings.

7. Motor innervation

As indicated elsewhere (Methods, section 6) the morphological similarity between fusimotor and skeletomotor endings was one of the criteria used to establish whether a given ending on an intrafusal fibre was sensory or motor. Therefore studies upon the motor innervation of both the extrafusal and intrafusal muscle fibres were carried out.

7.1 Skeletomotor innervation

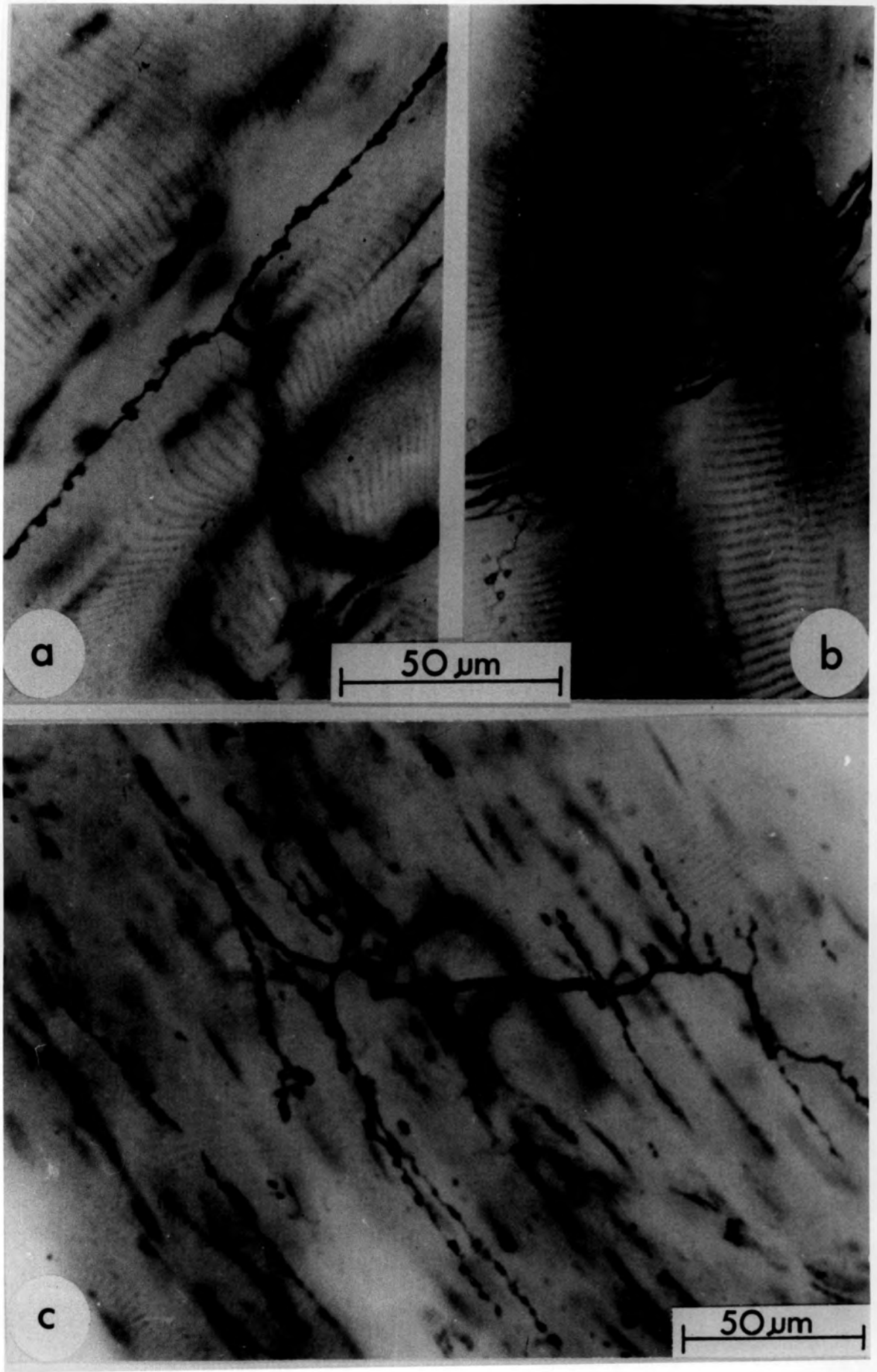
In silver preparations, two main types of motor nerve endings, plates and grapes, were found. In each case a parent axon divided to supply endings (all of the same type) to several muscle fibres (Fig. 44b, c).

Plate endings were found in all muscles stained by the modified de Castro method as well as in those muscles stained by the methylene blue method in which the motor endings were visible. The terminal axon approached the muscle fibre at right angles to its long axis and, at the point of contact, the terminal axon divided into two branches which passed in opposite directions along the surface of the muscle fibre and parallel to its long axis. The two arms of the 'T' shaped plate thus formed were, in most cases, of more or less equal length and had rounded synaptic contacts extending over one or two sarcomeres. These synaptic contacts were regularly arranged along both arms of the 'T' junction and separated from each other by about two sarcomeres. The endings were seen lying flush with the surface of the muscle fibres and no nucleated sole-plates or Doyere's eminences were observed. Occasionally muscle fibres supplied with two plate endings were seen (see Fig. 46c). Plate endings were supplied by thick myelinated axons 3 to 5 μm in diameter (25 axons measured).

FIGURE 44a. Teased silver preparation of an extrafusal plate ending. The ending is T-shaped with more or less regularly-spaced rounded synaptic contacts.

FIGURE 44b. Teased silver preparation of an extrafusal grape ending. The synaptic contacts are borne on separate non-myelinated terminal axons. A single axon branches to supply endings to several muscle fibres.

FIGURE 44c. Teased silver preparation of an extrafusal motor axon which branches to supply plate endings to several muscle fibres.


a
50 µm
b
c
50 µm

Grape endings were found only in muscles stained with the modified de Castro method. Their non-appearance in methylene blue stained material was probably due to either their different staining properties or the capriciousness of the method. Grape endings were not linearly arranged along the long axis of the muscle fibres, but tended to lie irregularly across them. In Figure 44b it could be seen that either each synaptic contact had its own short unmyelinated terminal axon which was a branch of the parent axon, or the fine terminal axon meandered across the surface of the muscle fibre giving irregularly scattered synaptic contacts. The synaptic contacts have more or less the same dimensions as those of the plate endings. The nerve fibres giving the grape endings were 1 to 3.5 μm in diameter (25 axons measured).

Distinction between plate and grape endings could be made in the majority of cases, but sometimes the appearance was intermediate, with several terminal branches shorter than those of plate endings and each branch made more synaptic contacts than those found on grape ending.

7.2 Fusimotor innervation

The motor innervation of tortoise muscle spindles was studied in 36 teased whole spindle preparations, 15 of which were stained by the methylene blue method and 21 by the silver method.

The fusimotor endings were always seen to lie only in the extracapsular region of the muscle spindles (Fig. 45a). In three of the spindles examined, the motor nerve fibre entered the capsule, with the sensory nerve fibre, instead of the more usual separate entry at the extracapsular region near the region of synaptic contact. However, even in these three cases, the motor nerve did not give any endings in the capsular region, but pursued a sinusoidal course to the extracapsular region (Fig. 45b).

Both grape and plate endings were observed on the intrafusal muscle fibres. They were similar to the extrafusal endings except that the arms of the 'T' junction of the plate endings tended to be smaller, and that the grape endings had fewer synaptic contacts. In every case where the nerve was unbroken they could be seen to be collaterals of the skeletomotor nerve fibres (Fig. 46).

In methylene blue preparations, only plate endings were seen, in the silver stained preparations both the plate and grape endings were stained. Table II shows the details of the motor innervation of the 21 spindles stained with silver. From that table it could be seen that each spindle had one or two areas of motor innervation on each pole, except for one spindle (spindle 5) which had two zones on one pole and none on the other. However this might have been due to faulty impregnation at that pole since it was very lightly stained, and therefore this spindle is not

FIGURE 45a. Teased silver preparation of three muscle spindles supplied by a single sensory nerve (arrow). Motor nerves (MN) enter the spindle in the extracapsular region.

FIGURE 45b. Teased silver preparation of a spindle showing a motor nerve (MN) entering the capsular region with the sensory nerve (arrow). The motor nerve passes out of the capsule before giving endings.

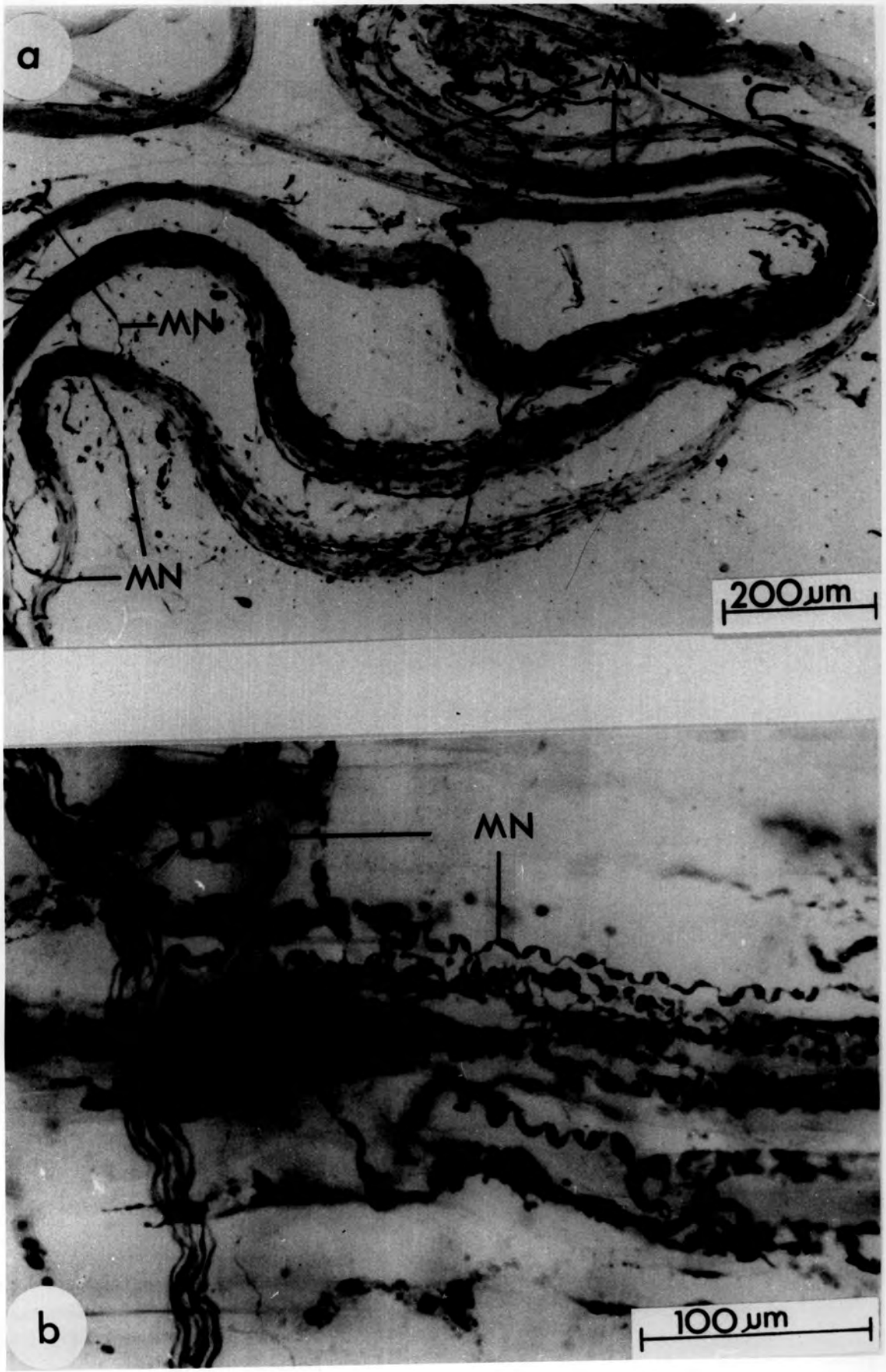

a
MN
MN
MN
200 µm
MN
b
100 µm

FIGURE 46a. Teased silver preparation of an intrafusal plate ending. The ending is smaller than the extrafusal plates (cf Fig. 44a, c).

FIGURE 46b. Teased silver preparation of an intrafusal grape ending. There are fewer synaptic contacts than in extrafusal grapes (cf. Fig. 44b). The axon goes out of the field to supply grape endings to extrafusal muscle fibres.

FIGURE 46c. Teased silver preparation of a motor nerve supplying two plate endings to an extrafusal muscle fibre (E). A collateral branch supplies a plate ending to one of the three small intrafusal muscle fibres (arrows).

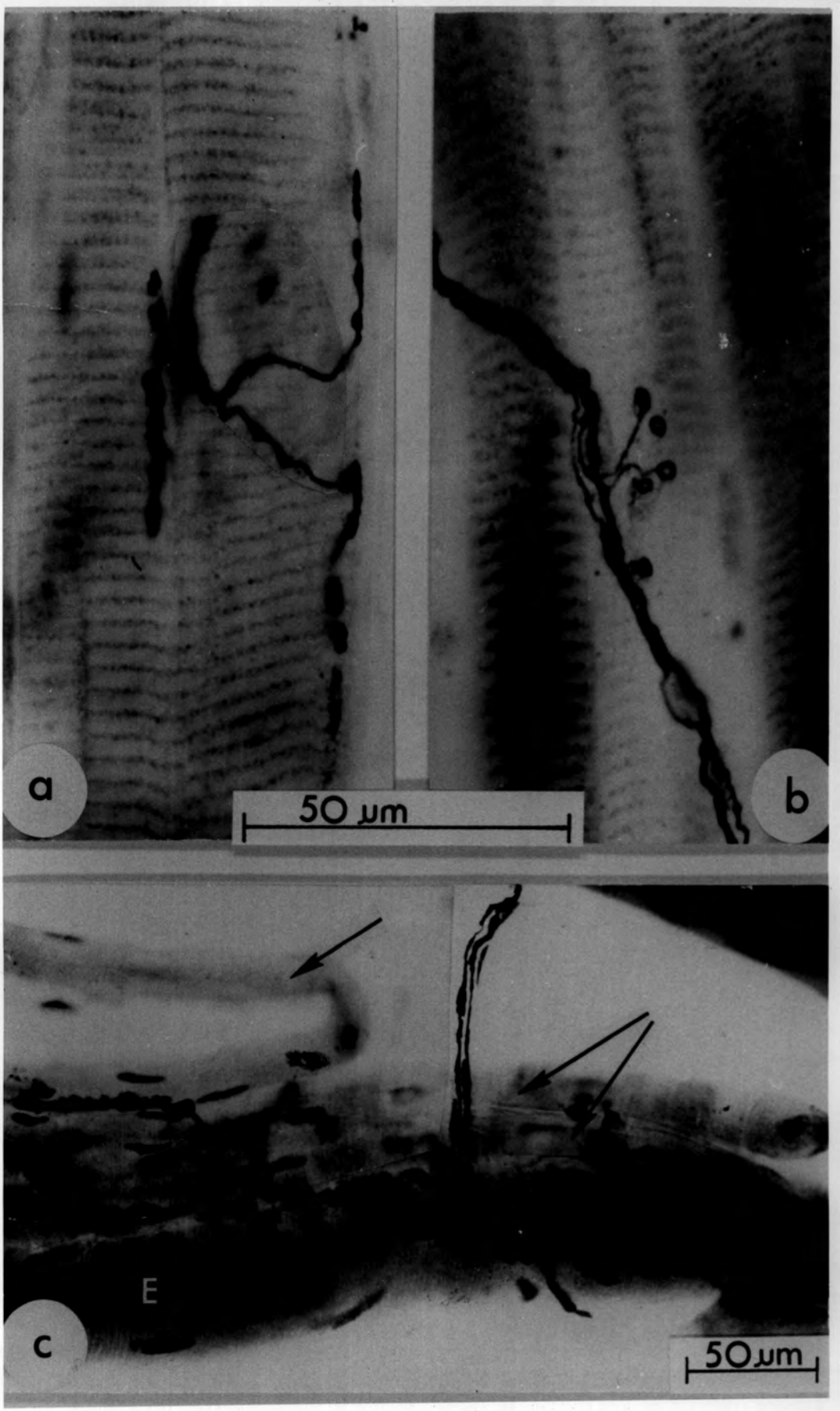

a
50 µm
b
E
c
50µm

TABLE II

The number of zones of each type of motor innervation (plate only, grape only or mixed) on each pole of 21 'single' spindles stained with silver

Thirteen spindles have both plate and grape endings; seven have plate endings only, excepting spindle 5 (see Text).

Spindle Number	Number of motor zones					
	Pole 1			Pole 2		
	Plate	Grape	Mixed	Plate	Grape	Mixed
1	-	2	2	-	-	2
2	1	-	-	-	-	1
3	-	-	1	1	-	-
4	2	-	-	-	-	1
5	2	-	-	?	?	?
6	-	-	1	2	-	-
7	1	-	-	1	-	-
8	1	-	-	1	-	-
9	1	-	-	2	1	-
10	1	-	-	-	-	1
11	-	-	1	2	-	-
12	1	-	-	1	-	-
13	1	-	-	1	-	-
14	1	-	-	1	-	-
15	1	-	-	1	-	-
16	-	-	1	-	-	1
17	2	-	-	-	-	1
18	2	-	-	1	-	-
19	1	-	-	-	-	2
20	-	-	1	1	-	1
21	1	-	-	-	-	1

included in the following remarks.

Each zone of innervation might contain plate endings only, grape endings only or both (mixed). Seven spindles had plate endings only while the rest had both types of endings. The number of plate endings per spindle always exceeded the number of intrafusal muscle fibres so that some of the fibres must be multiply innervated. However it was not possible to see whether both types of endings occured on the same intrafusal muscle fibre.

8. General observations on spindles in other muscles

This section gives general remarks on muscle spindles of another hind limb muscle, the sartorius, a fore limb muscle, biceps brachii, and four retractor capitis muscles. The observations were made on fresh frozen sections taken at 0.25 mm intervals and stained with H & VG. No attempt was made to carry out a detailed study on serial sections of these muscles since the aim was to see whether the main features of the muscle spindles in the EDBI muscle were common to those of the other muscles.

The capsular count varied considerably from muscle to muscle, thus in the sartorius muscle it was 44, in the biceps brachii, 15 and in the 4 retractor capitis muscles values of 0, 0, 2 and 3 were recorded. The high capsular count in the sartorius was partly due to the relatively high proportion of tandem spindles (25%).

The number of intrafusal muscle fibres in the capsule was variable; the mean values were : 9.2 (range 3-17) for sartorius spindles; 8.6 (5-12) for biceps brachii spindles; 2.6 (2-3) for the 5 retractor capitis spindles. Therefore not only were spindles rare in retractor capitis muscles, but also they contained fewer intrafusal muscle fibres.

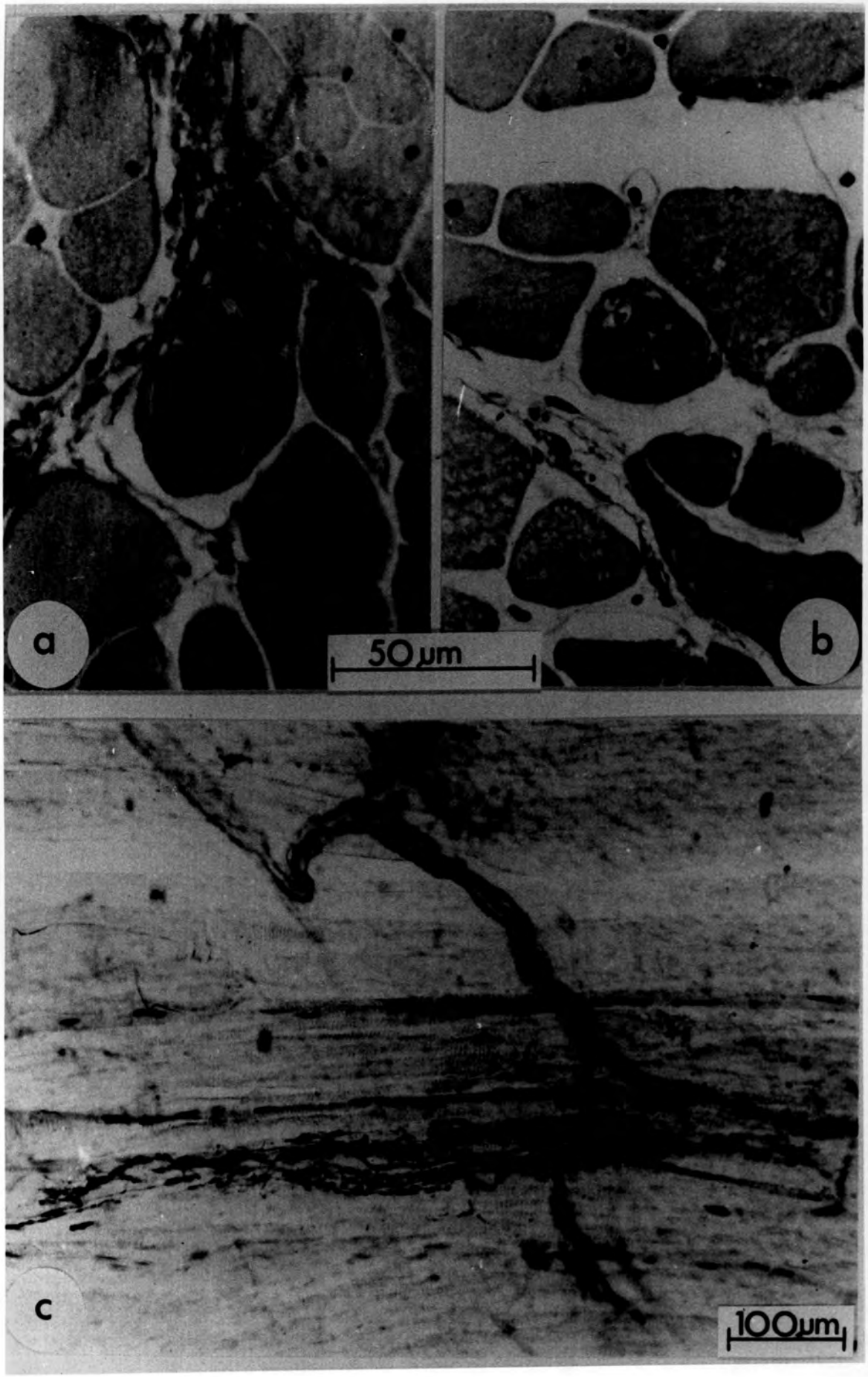
By LM the capsular structure of the spindles in the different muscles was similar to that found in the EDBI muscle. No fluid-filled space was seen and each intrafusal muscle fibre had its own connective tissue capsule (Fig. 47a, b).

No nuclear bags were seen and the myofibrils were continuous throughout the capsular region. Although no detailed measurements were made, the variation in diameter of individual intrafusal muscle fibres was similar to that described in EDBI muscles (section 5.2).

Twelve teased whole spindle preparations from biceps brachii, stained with methylene blue had only one sensory nerve fibre each and the mode of branching and the configuration of the sensory terminals were like that of spindles of the EDBI muscle (Fig. 47c). Since the methylene blue preparations did not consistently demonstrate the fusimotor innervation, no attempt was made to study it, but the 'T'-shaped plates, were seen on the extrafusal muscle fibres of the biceps brachii and sartorius muscles.

FIGURE 47a, b. Transverse sections of the capsular region of spindles from (a) biceps brachii and (b) retractor capitis. As seen in the spindles of EDBI (Fig. 12), the intrafusal muscle fibres each have individual connective tissue sheaths and there is no fluid-filled space.

FIGURE 47c. Teased silver preparation of the sensory region of a spindle from biceps brachii. The branching of the sensory nerve fibre and the form of the endings is identical to that seen in the spindles of EDBI (Figs. 34, 35).


a
50µm
b
c
100µm

IV DISCUSSION

Number of spindles per muscle

The four muscles studied in serial sections showed a wide variation in their spindle content (4 to 10), which clearly is not related either to individual variation or to the side of the animal from which the muscles were taken (see Table I). All the animals were adult and of approximately the same weight, and although there is no information on the effect of age on the number of spindles in chelonians, Cuajunco (1927) showed that in the pig all the spindles are formed before the 160 mm length of the embryo is reached. Cuajunco (1940) also showed that the number of spindles did not increase after the 15th week of foetal life of the human embryo. Even though the spindle content of the muscles might be a function of muscle size, number of motor units or some other parameter, the unexpected discrepancy between the two muscles (C and D, Table I) taken from the same animal cannot be explained. In this respect the EDBI muscle of the tortoise differs from mammalian muscle since Barker (1962) and Chin, Cope and Pang (1962) have shown that in a pair of muscles taken from the same animal (cat) the capsular content is so closely equivalent that the capsular content of one muscle can be worked out if

the content of the other is known. Also, they showed that the capsular content in a muscle varies in different individuals but the lower the value, the narrower is the range.

Although it was not one of the aims of this study to do a detailed quantitative evaluation of the number of spindles, and despite the lack of sufficient data on which to base a firm statistical conclusion, it can be said, that there is no such close relationship in the EDBI muscle of the tortoise (see Table I). The data from the four muscles tend to indicate that the number of spindles varied by a relatively greater amount than that shown by Barker (1962) and Chin et al. (1962) in the cat.

Number of intrafusal muscle fibres per spindle

The number of intrafusal muscle fibres per spindle varies in different species and indeed in different muscles of the same animal as shown in Table III. It can be seen that, with the exception of reptiles, all species so far studied possess multifibrillar spindles. Although it is agreed that the snake and lizard muscle spindles are monofibrillar the earlier reports on the tortoise spindle were conflicting. Thus Giacomini (1898) stated that both monofibrillar and multifibrillar spindles are present while Huber and de Witt (1897) were unable to demonstrate the presence of monofibrillar spindles. In the present study

TABLE III

The number of Intrafusal muscle fibres per spindle in different muscles of various species

Species	Muscle	Number of intrafusal fibres	Reference
Man	Superior oblique Inferior oblique First lumbrical	4 - 14) 2 - 11) 2 - 13)	Cooper and Daniel, 1963.
Opossum	Lumbricals	4 - 20	Jones, 1966a.
Cat	Tenuissimus Soleus Interosseous	4 - 8) 5 - 9) 5 - 13)	Boyd, 1962.
Chicken	Anterior and posterior latissimus dorsi	1 - 8	Barker, 1968.
	gastrocnemius	2 - 10	de Anda & Rebollo, 1967.
Snakes	In all muscles studied so far	1	Cipollone, 1897.
Lizards	In all muscles studied so far	1 (rarely 2)	Szepsenwol, 1960.
Tortoise	?	1 - 5	Giacomini, 1898
	Vasti	2 - 8	Huber & de Witt, 1897.
Alligator	?	2 - 5	Hines, 1930.
Frog	Extensor digitorum longus IV	3 - 12	Gray, 1957.

however, no monofibrillar spindles were seen in all the sectioned and teased preparations (over 200 spindles in all), and only three spindles with two intrafusal muscle fibres were seen, two of which were in retractor capitis muscles. The mean number of intrafusal muscle fibres was 10.4, within the range of 2-17, for the spindles found in serially sectioned EDBI muscles. When the spindles found in transverse sections of other muscles were taken into consideration the mean number of intrafusal muscle fibres per spindle was 9.0 within the range of 2-19.

The higher value of the present study as compared with that of Huber and de Witt might be attributed to either the difference in species used and/or the different muscles studied. Indeed, the spindles seen in retractor capitis muscles (Results, section 8), although not subjected to detailed study, show a lower number of intrafusal muscle fibres (2-3 per spindle) as compared with those of the EDBI muscle (2-19).

Even though the number of muscle spindles per muscle varies widely as does the number of intrafusal muscle fibres per spindle, there is no compensatory increase in the number of intrafusal muscle fibres per spindle in those muscles containing fewer spindles.

Spindle length

The spindle length differs in different species, in different muscles of the same animal and in different spindles of the same muscle. In human extraocular muscles, Cooper & Daniel (1949) found spindles ranging from 50 μm to over 1 mm which did not run the whole length of the muscle.

According to the data of Boyd (1962), the lengths of spindles in cat tenuissimus muscles were in the range 3 to 12 mm, while in the much shorter interosseus muscle they were in the range 0.8 - 4.5 mm long. From the results of Jones (1966a), who made detailed studies on the four lumbrical muscles in the limb of Trichosurus valpecula, it is noted that the second lumbrical which is the longest of the four had the highest mean spindle length. So, in these mammals at least, although the spindles do not run the whole length of the muscle and are very variable in length, there appears to be a direct relation between the length of the muscle and the length of the spindles it contains.

In reptiles, Szepsenwol (1960) stated that some spindles of the lizard, Anolis crestatellus, had the same length as the muscle while others were situated in the distal half only. Proske (1969) observed that in the lizard Tiliqua nigrolutea, the muscle spindles extended for three-quarters of the length of the muscle. Therefore in these animals it can be said that longer muscles contain longer spindles.

In the frog, Rana temporaria, Gray (1957) stated that all the 'spindle systems' ran the whole length of the extensor digitorum longus IV (EDL IV) and were 17 mm long. Barker (1962) found that in R. regulosa and R. guentheria, the length of 'spindle systems' in the EDL IV ranged between 17.13 and 26.52 mm, and the length of 'single spindles' were from 4.5-18.45 mm, while the spindle length in pectoral cutaneous muscle ranged from 6.72 to 7.84 mm. Barker concluded that the long spindles of the EDL IV were characteristic of that muscle.

In the present study, the spindles in the EDBI muscle of the tortoise have a length of 55% to 79% of the total length of the muscle, which is a similar situation to that observed by Proske (1969) in the lizard. However, it should be remembered that the lateral side of the EDBI muscle is slightly shorter than the medial side, and therefore the absolute length of the muscle is greater than the actual length of the majority of extrafusal muscle fibres (see Fig. 3). Thus a spindle near the shorter lateral side of the muscle might be almost as long as the surrounding extrafusal muscle fibres, which in turn is related to the overall length of the muscle, so that the longest spindles are found in the longest muscle, and the shortest spindle in the shortest one (see Table I). However, a final conclusion on this point

must depend upon the result of further studies of other muscles of different lengths.

The Capsule

Huber and de Witt (1897) described the spindles in the tortoise Chrysemys picta and Emys meleagris as having the same general structure as those of other vertebrates. They described the capsule as follows: 'The intrafusal muscle fibres, from two to eight in number, are surrounded by an axial sheath, periaxial lymph space and capsule, each intrafusal muscle fibre being further surrounded by its own connective tissue sheath, which is partly fused to the axial sheath or connected with it by bands or septa of fibrous tissue.' (pp. 210-211). In contrast, the present investigation has clearly shown that in EDBI muscle as well as in other muscles of the limbs and the neck, the spindle capsules lack both the axial sheath and the periaxial fluid-filled space.

In order to determine whether the absence of the lymph space was an artifact, control muscles from frog and rat, whose spindles are known to have a periaxial fluid-filled space were simultaneously processed in the same way as tortoise muscles. Transverse sections of these muscles clearly showed the fluid-filled space. Further, Cooper & Daniel (1949), stated that paraffin embedding exaggerates the

fluid-filled space and therefore considered it to be an artifact at least in part.

In their figures, Huber and de Witt (1897) did not show the lymph space they described in the text, and the space separating the innermost layer of the connective tissue capsule from the intrafusal fibres is of more or less the same magnitude as the spaces between the layers constituting the fibrous capsule. It therefore seems likely that the fluid-filled space they described was most probably due to severe shrinkage which created a false space around each intrafusal muscle fibre, as well as in between the layers of the connective tissue capsule.

Another possible explanation is that both C. picta and E. meleagris, have a different capsular structure from that of T. graeca, but it would be remarkable if such closely related animals exhibited such a fundamental difference in the structure of their spindles.

The observation of Huber & de Witt that each intrafusal muscle fibre has its own connective tissue sheath was confirmed in this study. However, these authors did not remark that in this respect the tortoise spindle differs from all the previously described muscle spindles.

The EM study described in the Results section 4.2, showed beyond doubt that there is no fluid-filled space between

the capsule and the intrafusal muscle fibre and that the narrow spaces between the innermost layer of the capsule and the intrafusal fibres are not greater than those in between the layers of the capsule, and like the latter are almost completely filled with collagen fibres. Thus, in its fine structure, the capsule of the tortoise spindle differs from those of the other species so far studied which have a prominent fluid-filled space (Robertson, 1956, 1960 - frog; Merrillees, 1960 - rat; Corvaja et al, 1969 - cat; Fukami & Hunt, 1970 - snake;). The absence of a fluid-filled space is not the only aspect in which the tortoise capsule differs from those already investigated in other vertebrates. In the capsules of mammalian, reptilian and amphibian spindles, the cells of the multi-layered 'outer capsular sheath' can be distinguished from those of the single-layered 'inner capsular sheath' by differences in their fine structure (see for example, Landon, 1966; Corvaja et al, 1969). In contrast, the capsule of the tortoise spindle is composed of only one sheath and all the capsular cells have the same fine structure and must therefore be considered as being of one type. They appear to resemble those of the 'outer capsular sheath' of other species and it must therefore be assumed that it is the inner capsular sheath which is missing in the tortoise.

Reptilian spindles can be differentiated into two types on the basis of the size of the capsule and its length relative to the length of the spindle (Barker, 1968), but no such differentiation was observed in tortoise spindles in the present study.

The tortoise muscle spindle by LM and EM is seen to lack the elastic fibres found in rat spindles, Merrillees (1960) and Landon (1966), in human spindles by Cooper & Daniel (1967), and in frog spindles during the present study.

Therefore it can be concluded that the capsule of the tortoise spindle is much simpler in construction than the mammalian, amphibian and other reptilian spindles, so far studied, in lacking a fluid-filled space and elastic fibres, and in having only one capsular sheath composed of a single cell type.

However, some similarities can be seen between the capsular structure of the tortoise spindle and those found in human extraocular muscles (Cooper & Daniel, 1949), where there is a small periaxial space in the equatorial zone which disappears at the poles where 'the capsule sends in many stout bands or septa which encircle and bind together the individual intrafusal muscle fibres, thus giving a very different picture from that seen in the equatorial zone.', and 'At the poles also the capsule invests the intrafusal fibres more closely, and no periaxial space is seen, even in paraffin sections.' (Page 7). This picture of the polar part of the capsule of the

human extraocular muscle spindles most resembles that seen in the capsular region of tortoise muscle spindles.

Intrafusal muscle fibres

In all the different vertebrates whose spindles have so far been examined in detail at least two types of intrafusal muscle fibres can be distinguished by differences in the general morphology such as length, diameter, nuclear distribution, fine structure and histochemical activity.

The work on the mammalian muscle spindles reveals at least two morphologically different types of intrafusal muscle fibres, namely the nuclear-chain and nuclear-bag fibres (Barker, 1948; Boyd, 1962; Cooper & Daniel, 1963; Jones, 1966a). The thick, long nuclear-bag fibre usually extends beyond the poles of the fibrous capsule, and has an equatorial aggregation of round vesicular nuclei, and loses the transverse striations at this region. Electron microscopic studies have shown that the nuclear-bag fibre has ill-defined H bands and lacks M lines (Landon, 1966; Corvaja, et al, 1969).

The thin short nuclear-chain fibre on the other hand, seldom extends beyond the poles of the capsule and has an equatorial region, containing only a single central row of vesicular nuclei. Unlike the nuclear-bag fibre, a nuclear-chain fibre shows a considerable diminution in its

intracapsular diameter. The studies of the fine structure show that a nuclear-chain fibre has well defined H bands and M lines, furthermore the mitochondria are larger than those found in a nuclear-bag fibre (Landon, 1966; Corvaja et al., 1969).

However the nuclear-chain and nuclear-bag fibres of the cat interosseus muscle spindles can only be recognized, using LM, by the arrangement of their equatorial nuclei and not by differences in length and diameter (Boyd, 1962).

Histochemical studies show at least three types of intrafusal muscle fibres. Intrafusal muscle fibres with high, low and intermediate succinic dehydrogenase activity are present in the spindles of various vertebrates (Ogata & Mori, 1962, 1964). The rat, rabbit and cat muscle spindles contain at least three types of intrafusal muscle fibres as recognized by differences in their succinic dehydrogenase, phosphorylase,and mitochondrial adenosine triphosphatase activities (Yellin, 1969; Barker & Stacey, personal communication).

In birds nuclear-bag and nuclear-chain fibres have been described together with an intermediate type (de Anda & Rebollo, 1967). Rebollo & de Anda (1967) stated that these three types of intrafusal muscle fibres can be distinguished by differences in their histochemical activity.

Among the reptiles, snake and lizard spindles although monofibrillar, show two types of intrafusal muscle

fibre according to differences in the structure of the equatorial region (Szepsenwol, 1960; Barker, 1968). The long thin capsulated intrafusal fibre shows transverse striations throughout its length including the sensory region which shows a slight increase in nuclear density, and therefore resembles the mammalian nuclear-chain fibre. The diameter of this type of fibre is fairly uniform along its entire length. In contrast, the short thick capsulated intrafusal muscle fibre shows an increase in diameter and interruption of the transverse striations at the equatorial region which is filled with an accumulation of nuclei, and therefore resembles the mammalian nuclear-bag fibre. A recent study of the fine structure of both types in snake (Fukami & Hunt, 1970) confirms these differences and shows that while myofibrils are present in the equatorial region of the long thin capsulated fibres, they are replaced by various organelles and nuclei in those of the thick short capsulated fibres. Apart from that, both fibres are identical in their fine structure, but differ from the extrafusal fibres in that they lack M lines, the Z discs are thicker and triads are rare or absent.

The frog spindle has two types of intrafusal muscle fibre, a short thin one with few nuclei, somewhat resembling mammalian nuclear-chain fibres, and a long thick fibre with more nuclei, but no nuclear-bags have been observed (Barker &

Cope, 1962). Page (1966) described the fine structure of two different types of intrafusal muscle fibre, one of which has the characteristics of the fast (twitch) extrafusal muscle fibre of the frog. The other type of intrafusal fibre has characters intermediate between those of the fast and slow (non-twitch) extrafusal muscle fibres which she described in an earlier study (Page, 1965). Barker, (1968) identified his large intrafusal muscle fibres with the 'twitch-like' fibres of Page and the small fibres with her structurally 'intermediate' fibres.

In the present study attention has been paid to the nuclear distribution, to exclude the possibility that there may be two types of intrafusal muscle fibre which are similar in length and diameter but different in their nuclear arrangement as is the case in the spindles of the cat interosseus muscles (Boyd, 1962). However only one type of intrafusal muscle fibre has been recognized in both sectioned and teased preparations, on the basis of length, diameter, nuclear distribution, fine structure and histochemical activity. Although there is a slight increase in the nuclear density at the capsular region, there is no nuclear aggregation like those present in the mammalian nuclear-bag fibres and the short thick capsulated spindles of snakes and lizards. The characteristic variations in the diameter of the tortoise

intrafusal muscle fibres resemble the variations described in the mammalian nuclear-chain fibres (Cooper & Daniel, 1956; Jones, 1966a).

The arrangement of the myofibrils in the intrafusal muscle fibre has been carefully examined because Boyd (1962) has made differences in myofibrillar density a criterion for distinguishing nuclear-bag from nuclear-chain fibres, stating that in transverse sections, the myofibrils are tightly packed in the former, which are poorer in sarcoplasm, than in the latter. Therefore the nuclear-bag fibres show the 'Fibrillenstruktur' while nuclear-chain fibres show the 'Felderstruktur' of Krüger (1952). However, Barker (1962) questioned the validity of myofibrillar density as a distinguishing feature and has reported that the myofibrillar pattern changes along the individual intrafusal muscle fibres so that both patterns occur in the two types of fibres. In the present study all the intrafusal muscle fibres examined with the LM appeared to show 'Fibrillenstruktur', but closer examination with the EM showed that both 'Fibrillenstruktur' and 'Felderstruktur' occur in the same muscle fibre and their presence depends on the precise level of sectioning. This is in agreement with the recently reported study of the fine structure of the intrafusal muscle fibres of the snake (Fukami & Hunt, 1970).

In lacking nuclear-bags, the tortoise intrafusal muscle fibres resemble those of the frog, the long thin capsulated fibres of lizard and snake spindles and the nuclear-chain fibres of the mammalian spindle. It might be expected that the intrafusal muscle fibres of the tortoise would most resemble those of the lower vertebrates, but this does not seem to be the case. The study of the fine structure shows that although there is a resemblance between the large twitch intrafusal muscle fibres of the frog and those of the tortoise, the latter lacks the specialized reticular zone seen in the sensory region of the frog intrafusal fibres (Katz, 1961; Karlsson, Andersson-Cedergren & Ottoson, 1965). Although the tortoise intrafusal fibre is similar in gross structure to those with long thin capsules in lizards and snakes, they differ in their fine structure; snake intrafusal muscle fibres lack M lines, have rounded centrally placed nuclei, and triads are rare or absent.

A close similarity in almost all features to the tortoise intrafusal muscle fibres is shown by the mammalian nuclear-chain fibres. The only difference is that the large vesicular equatorial nuclei of the nuclear chain fibres are lacking in intrafusal muscle fibres of the tortoises. But Cooper & Daniel (1949) in their study of the human extraocular muscle spindles were unable to distinguish two types of intrafusal muscle fibres on the basis of nuclear distribution

and that 'The striking group of rather vesicular nuclei, which appear almost to replace the intrafusal muscle fibre in the equatorial region in other somatic muscle spindles, are not so obvious in the intrafusal fibres of human eye muscle spindles.' (page 7). Thus these fibres bear a closer relationship to the intrafusal muscle fibres of the tortoise than do the nuclear-chain fibres of the other mammalian somatic muscle spindles. However a study of their fine structure is necessary before it can be said that the similarity is complete.

Comparisons of the intrafusal muscle fibre and capsular structure of muscle spindles of human extraocular muscles and tortoise limb muscles show that these structures are similar in detail in two phylogenetically very widely separated species. Some light may be thrown on this, at first sight surprising, resemblance by Barker's (1968) observation that the mammalian extraocular muscles are phylogenetically very ancient.

Sensory Innervation

Work on mammalian muscle spindles (e.g. Barker, 1948 - rabbit; Boyd, 1962 - cat; Cooper and Daniel, 1963 - man; Jones, 1966b - opossum) has shown conclusively that there are two types of sensory nerve fibres as identified by diameter, and the shape and location of the endings on the intrafusal muscle fibres. The other salient features of these authors' work may be summarized as follows:

Large Group Ia and medium sized Group II nerve fibres supply the primary and secondary sensory nerve endings respectively. The terms 'primary' and 'secondary' were first used by Ruffini (1898). Each spindle is supplied by only one Group Ia afferent nerve fibre which never supplies more than one spindle and ends on the mid-equatorial region of both nuclear-chain and nuclear-bag fibres in regular spirals or rings. On the other hand, one or more Group II afferent nerve fibres supply the spindle, and in contrast to Group Ia fibres, may supply endings on two separate spindles. Unlike the branches of Group Ia fibres, the branches of the Group II fibres travel for a considerable distance between the capsular layers before terminating in irregular spirals, or occasionally in sprays. The secondary endings occupy the juxtaequatorial regions of the intrafusal muscle fibres on one or both sides of the primary ending and occur predominantly on nuclear-chain fibres. Primary and secondary endings are similar in their fine structure, Adal (1969).

Chicken muscle spindles are supplied by both large and small sensory nerve fibres (de Anda & Rebollo, 1967), giving both primary and secondary endings (Barker, 1968).

In the snakes and lizards there are two types of nerve fibres, distinguished by their diameters and mode of termination, but only one of which is found in each monofibrillar

spindle (Regaud & Favre, 1904; Barker, 1968; Proske, 1969). The thinner nerve fibres, terminate in short simple endings immediately after entering the short thick capsulated spindles, while the thicker nerve fibres, branch extensively and run for some distance parallel to the intrafusal muscle fibres of the thin long-capsulated spindles before terminating in an extensive ending. Both types of ending have the same fine structure (Fukami & Hunt, 1970).

According to Hines,(1930), there is only one type of sensory innervation of the spindles of the American alligator, and each spindle receives only one sensory nerve fibre.

In the frog spindle, each capsular region is supplied by only one sensory nerve fibre which supplies only one type of sensory ending. These are fine, straight, varicose threads running parallel to the intrafusal muscle fibres (Cajal, 1888; Dogiel, 1890; Gray, 1957).

The present investigation clearly shows that the tortoise muscle spindles, like those of the frog and alligator are supplied by only one type of sensory innervation as judged by the unimodal distribution of the nerve fibre diameter and shape of sensory endings, and are therefore simpler than the mammalian, avian and snake and lizard spindles. The form of the sensory ending in the tortoise spindle most resembles the mammalian secondary endings in that they do not form regular spirals or rings, and in that the parent nerve fibre may supply

more than one spindle. They also resemble the sensory endings of the thin long capsulated spindle of the snakes and lizards in that they branch extensively. The varicose endings of the tortoise spindle differ from those of the frog in that they meander over the intrafusal muscle fibres.

According to Huber and de Witt (1897) the muscle spindles of the tortoise E. meleagris and C. pecta, are innervated by 1-3 sensory nerve fibres, whereas the present study clearly shows that in T. graeca the spindle is supplied by only one sensory nerve fibre which divides into 2-3 main branches at some distance from the spindle. It seems probable that Huber and de Witt did not trace the nerve fibres far enough back to see the point of branching of the parent nerve fibre, and assumed that each branch is a separate nerve: this is supported by the fact that the maximum number of nerves (3) that they reported as entering the spindle corresponds to the maximum number of branches of the parent nerve fibres seen in the present study. Further support for the existence of a single type of sensory ending is adduced from the findings of Huber & de Witt (1897) and Giacomini (1898) that there is only one type of sensory ending which Giacomini described as flower sprays and is apparently the same as that seen in the present study.

It was not possible to confirm with the EM that the tortoise muscle spindle has only one type of sensory ending, although some endings have mitochondria and some do not, and

some lie in shallow troughs with a lip made by satellite cells, while others lie in deep troughs with sarcolemmal lips. However it is not suggested that these differences are of sufficient magnitude to separate two types of endings since such variations could have resulted from different planes of cutting or differences in the position at which the sensory terminal is sited on the intrafusal muscle fibres. In any case, in the cat spindle where two types of sensory endings have been definitely established, no ultrastructural differences have been found between primary and secondary nerve endings on either nuclear-chain or nuclear-bag fibres (Adal, 1969; Corvaja et al., 1969). It seems probable that distinction between different types of sensory endings can be made only on the basis of low power examination to determine their configuration and position upon the intrafusal muscle fibres.

The fine structure of tortoise sensory endings and those of other vertebrates so far studied (Katz, 1961 - frog; Landon, 1966 - rat; Adal, 1969 - cat; Fukami & Hunt, 1970 - snake) are similar in many respects. viz. aggregations of mitochondria, two types of vesicles, the fluffy cytoplasm and degree of separation of the membranes of the sensory terminal and the muscle fibre.

The sarcolemma and the plasma membrane of the sensory endings show periodic patches of increased density with associated accumulations of dark filamentous material crossing the gap between the membranes, similar to those described in the frog spindle (Katz, 1961) but absent from the spindles of the rat (Landon, 1966) and cat (Corvaja et al, 1969). Similar patches of dark filamentous material have been seen in relation to the sensory endings of the snake spindles (Fukami & Hunt, 1970) but they are only present in the subsarcolemnal cytoplasm, and not in the sensory ending.

The cytoplasmic continuity between the intrafusal muscle fibres and the sensory endings which was occasionally observed in the present study has not been described in any other species, although Katz (1961) has described 'local adhesions' between these membranes. The possibility that this cytoplasmic continuity may be an artifact must be borne in mind pending further ultrastructural examination.

Motor Innervation

Barker (1968) has amply reviewed the motor innervation of vertebrate skeletal muscle with particular emphasis on the innervation of muscle spindles. The following account is largely based on that review and consequently few references to the original papers will be given.

With few exceptions, mammalian skeletal muscle fibres are focally innervated by plate endings. The muscle spindles derive their motor innervation from two sources; firstly the β skeletomotor fibre gives collaterals supplying plate endings (p1); and secondly there is the purely fusimotor γ supply. The latter gives two types of endings, plates (p2), and diffuse multiterminal trail endings. These three types of ending are not segregated in their distribution to the different types of intrafusal muscle fibres, i.e. nuclear-bag and nuclear-chain fibres can receive the three types of endings, (see also Barker, Stacey & Adal, 1970).

Of the mammalian spindles so far studied, only those of the extraocular muscles have no γ supply and, like those of other somatic muscles of the lower vertebrates, are supplied by diffuse grape endings as well as focal plate endings. These endings are derived from collaterals of both types of skeletomotor fibres, and in the sheep, at least, are not segregated in their distribution to the intrafusal muscle fibres.

In the birds, so far studied, the fusimotor plate and grape endings are supplied by collaterals from skeletomotor nerve fibres, except in the case of chicken posterior latissimus dorsi muscle in which the skeletomotor nerve fibres supply plate endings only. In this particular case, the intrafusal grape endings are supplied by special fusimotor nerve fibres comparable to the mammalian γ system.

In reptiles, the snake and lizard spindles are always supplied by collateral motor nerve fibres. In the lizard the short thick capsulated spindles are always supplied by grape endings, while the motor innervation of the long thin capsulated spindles depends on the pattern of innervation of the muscle in which they are located. So that in purely plate innervated muscle, the spindle receives plate endings only, and in muscles supplied by both grape and plate endings the spindles receive either plate or grape endings (Proske, 1969).

Szepsenwol (1960) described typical and atypical plate endings in the lizard Anolis cristatellus. The typical plate endings have a Doyere's eminence. In the atypical plate endings, the terminal axon is applied to the surface of the muscle fibre and runs in linear fashion parallel to its long axis and in some cases the terminal axon gives 'two branches which run in opposite directions to the surface of the intrafusal muscle fibre.' (Szepsenwol, loc.cit. page 28). This author stated that such atypical plate endings can be differentiated from grape endings by their 'linear' arrangement.

In the spindle of the frog, the large and small intrafusal muscle fibres are supplied by collateral plate and grape endings respectively.

According to the present study the tortoise spindles do not have an independent γ innervation but are supplied by collateral fusimotor nerve fibres terminating in plate and grape endings. This confirms the observation of Giacomini (1898)

who reported both grape and plate endings in the muscle spindles of the tortoise Testudo graeca although he did not say whether they were collaterals. The simple plate endings seen in the present study, are very similar to the atypical plate endings reported in the lizard A. cristatellus (Szepsenwol, 1960), and can be differentiated from grape endings by their linear arrangement parallel to the long axis of the intrafusal muscle fibre.

In this study it has not been possible due to technical difficulties to say whether both endings occur on the same intrafusal muscle fibres, as is the case in the mammalian muscle spindle, or whether they are segregated as in the lower vertebrates. However, it was possible to demonstrate that the tortoise intrafusal muscle fibres have a multiple plate innervation like those of other species (e.g. Gray, 1957 - frog; Szepsenwol, 1960 - lizard; Barker et al., 1970 - cat).

Physiological considerations

The structure of the capsule is interesting because of the absence of the fluid-filled space which occupies the greater part of the volume of the spindle capsule of other vertebrates. The absence of this fluid-filled space in the

tortoise spindle raises the question of its function in the spindles of other species, and three functions have been proposed.

Firstly, Merrillees (1960) suggests that the capsular cells partake in active transport of ions in both directions in order to maintain a constant environment around the sensory nerve endings and intrafusal muscle fibres. He also suggests that the flocculent precipitate seen in the electron micrographs of the fluid-filled space of spindle capsule represents 'a protein or polysaccharide complex which could have ion-exchange or other binding properties,' which would aid the buffering properties of the intracapsular fluid (Merrillees, loc.cit., p.735). The capsular cells of the tortoise contain the caveolae and pinocytotic vesicles supposed by Bennett (1956) to be indicative of active transport, but the lack of intracapsular fluid suggests that either the tortoise capsule is less efficient in regulating the environment around the sensory endings and intrafusal muscle fibres, or it has some other mechanism. It is however, possible that the tortoise spindle is capable of functioning over a wider range of environmental conditions. Indeed, preliminary experiments with an isolated muscle-nerve preparation have shown that it is extremely resistant to great fluctuations in temperature and the spindles continue to respond to stretch for as long as 3 days after isolation. This is perhaps not surprising in view of the fact

that the tortoise is a poikilothermic and phylogenetically ancient vertebrate.

Secondly, Bridgman & Eldred (1964) make the suggestion, based on their own experimental observations on cat spindles, that the fluid-filled capsule may be part of a pressure-sensing device possessed by the spindles. Clearly, a similar mechanism cannot exist in the tortoise spindles, although the possibility that they are pressure-sensitive by a different mechanism is not ruled out.

Thirdly, Houk, Cornew & Stark (1966) suggest that the purpose of the fluid-filled capsule is to isolate the sensory nerve endings from mechanical disturbances caused by the surrounding muscle fibres. Since the tortoise fusimotor nerve fibres are collaterals of the skeletomotor nerves, contraction of the intrafusal muscle fibres would probably coincide with contraction of the extrafusal muscle fibres. Thus, in those species with fusimotor supply independent of the skeletomotor supply there would be a need for a much more efficient isolating mechanism, because movement of the extrafusal muscle fibres relative to the intrafusal muscle fibres could occur. However this raises the problem of the significance of the fluid-filled capsule in the other lower vertebrates which, like the tortoise, have an exclusively collateral fusimotor innervation.

If these ideas about the function of the capsule were essentially correct, it therefore appears that the tortoise is less well equipped in all these respects.

In all species so far studied it has been possible to record from spindles a frequency discharge related to the rate of stretch (dynamic response) and a frequency discharge related to the magnitude of the stretch (static response) (e.g. Matthews, 1931; Katz, 1950; Cooper, 1961; Fukami, 1970).

It has been suggested that the dynamic response arises in sensory endings which lie on regions of the intrafusal muscle fibres which are more or less devoid of myofibrils (e.g. the amphibian reticular zone and the mammalian nuclear bag), because at these regions the intrafusal fibre is thought to be predominantly elastic as compared to the viscoelastic polar regions (Matthews, 1931; Katz, 1961; Matthews, 1964). This idea is supported by mathematical models (Houk et al., 1966; Crowe, 1968).

If these hypotheses about the origin of the dynamic response are true, the muscle spindles of the tortoise are unlikely to be dynamically sensitive, because no such structural variations are seen at the sensory region. However, the presence of two structurally different fusimotor endings and the close approximation of the capsule to the intrafusal muscle fibres, together with the variation in the diameter of the intrafusal fibres at the capsular region may provide a system capable of providing a dynamic response.

In any case, Ottoson & Shepherd (1970) have shown that there is no difference in the stretch properties of the

reticular and polar zones of the frog spindle. Therefore the question of whether the tortoise spindle is dynamically sensitive must await the outcome of neurophysiological experiments. Such experiments will be of great interest because, if the tortoise muscle spindle is without a dynamic response, then it will raise interesting questions about the evolution of the muscle spindle and the role of the dynamic response in the control of muscle movements. If, on the other hand, the tortoise spindle does respond to the dynamic phase of stretch, great doubt will be thrown on current theories of the physiology of the muscle spindle.

V SUMMARY

1. Over 150 spindles in the extensor digitorum brevis I muscle of the tortoise Testudo graeca have been studied with the light and electron microscopes.

2. The spindles are evenly distributed in the muscle and are of almost the same length as the extrafusal muscle fibres.

3. The spindles are multifibrillar with an approximately central capsule. Only 3 'double tandem' spindles were found.

4. The capsule, composed of a single cell type, does not enclose a 'fluid-filled' space, but binds the whole spindle together and provides each intrafusal muscle fibre with an individual sheath.

5. The tortoise spindle lacks the elastic fibres present in mammalian and amphibian spindle.

6. The intrafusal muscle fibres are differentiated from the extrafusal muscle fibres only by their smaller diameter and the capsule, and are uniform in structure throughout their length apart from characteristic variations in diameter and histochemical activity at the capsular region. These variations are associated with the presence of the sensory endings and a slight increase in nuclear density.

7. The intrafusal muscle fibres could not be categorized into more than one type according to length, diameter,

nuclear arrangement, histochemical activity or fine structure.

8. The nuclei are flattened, peripherally placed and do not form equatorial aggregations.

9. Each spindle is supplied by only one sensory nerve fibre. All the sensory nerve fibres are of the same type and give only one type of ending which is intracapsular, irregular and varicose.

10. Occasional apparent cytoplasmic continuities between the sensory nerve endings and the muscle fibres are present.

11. The fusimotor plate and grape endings are derived from collateral branches of thick and thin skeletomotor nerves respectively, and are confined to the spindle poles.

12. The intrafusal muscle fibres have multiple plate innervation.

13. A less detailed study of other tortoise muscles confirmed these results.

14. The tortoise spindle is compared with those of other species and the physiological implications of its relatively simple morphology have been discussed.

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