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Varieties of fast and slow extrafusal muscle fibres in amphibian hind limb muscles

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INTRODUCTION

The skeletal muscle of frogs and toads is known to contain two distinctly different types of extrafusal muscle fibres: fast (or 'twitch') and slow fibres. Descriptions of the functional and structural properties of amphibian fast and slow muscle fibres abound (see reviews by Peachey, 1961 and Hess, 1970). Much of the early work on the function of the two types of muscle fibres indicated that each was organized into homogeneous motor units, fast motor units being innervated by large-diameter motor nerves and the slow motor units by small-diameter motor nerves (Tasaki & Mizutani, 1944; Kuffler & Vaughan Williams, 1953).

More recent evidence suggests that the simple dichotomy – fast fibres and slow fibres – may not be sufficient to enable one to describe the organization of skeletal muscle in the Anura. The population of fast muscle fibres is not composed of fibres with similar properties (Orkand, 1963; Nasledov, 1966; Lännergren & Smith, 1966; Engel & Irwin, 1967; Asmussen & Kiessling, 1970). However, the varieties of fast fibres are probably organized into motor units, each of which has a relatively homogeneous composition (Smith & Lännergren, 1968). Electrophysiological observations on single muscle fibres (Shamarina, 1962, 1963) can be interpreted as indicating that the population of slow fibres is not homogeneous. Moreover, the contractile properties of motor units show that two varieties of slow motor units exist (Smith & Lännergren, 1968).

Only one attempt (Barker, 1968) has been made to incorporate any of the recent findings into a scheme which describes the organization of skeletal muscle in the Amphibia. Clearly, much more information is needed on the types of muscle fibres present in amphibian muscle before any such scheme can be regarded as being valid. The present study presents a morphological base for the recognition of a number of varieties of fast and slow extrafusal muscle fibres in hind limb muscles of the Anura. This is part of a larger body of work whose object is to define the organization of skeletal muscle in frogs and toads.

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MATERIALS AND METHODS

Tissue preparation

Various hind limb muscles from the adult toad, *Xenopus laevis*, were used in this study. In addition, several hind limb muscles from a frog, *Rana pipiens*, and a toad, *Bufo woodhousei*, were examined for comparison. Hind limb muscles were dissected out intact in a chamber filled with oxygenated Ringer's solution. The Ringer's solution had the following millimolar composition: NaCl, 112; KCl, 2·5; $CaCl_2$, 2·0; $NaHCO_3$, 2·5.

Histochemical procedures

For histochemistry, several muscles of each type named in the Results were used. Male and female animals were examined in the months September to June. No difference between the sexes in the composition of any of the muscles was noted, consequently the sex of the animals is not given in the Results. The isolated whole muscles were removed from the Ringer's solution, dipped in talcum powder, and rapidly quenched in 2-methylbutane cooled to -70 °C with dry ice. Frozen sections, 8–10 μm thick, were cut in a cryostat at -20 °C. Myosin ATPase activity was demonstrated by the Guth & Samaha (1970) modification of the Padykula & Herman (1955) procedure. Best results were obtained by alkaline preincubation at pH 8·5 and incubation in the substrate at pH 9·4. Succinic dehydrogenase (SDHase) activity was localized by the nitro-blue tetrazolium method of Nachlas *et al.* (1957).

Light and electron microscopy

For electron microscopy, flexor tarsi and iliofibularis muscles from four male specimens of *Xenopus laevis* were pinned to cork boards under moderate stretch and fixed at room temperature for several hours in 5% glutaraldehyde in 0·1 M phosphate buffer, pH 7·3, with 0·2 M sucrose. Muscles were washed in phosphate-buffered sucrose, cut into small pieces, postfixed in 2% buffered OsO_4 for two hours, and dehydrated in a graded series of ethanols and propylene oxide. Specimens were embedded in Araldite 502 or in Epon 812. Transverse sections (1–2 μm thick) of each block were cut with glass knives, stained with a saturated solution of phenylenediamine in absolute methanol (Estable-Puig, Bauer & Blumberg, 1964), mounted under a coverslip, and examined by bright-field or phase-contrast microscopy. This method was used for initially distinguishing the extrafusal fibre types (Korneliussen, 1972). Identification of each fibre type was made on the basis of (1) mitochondrial distribution and staining intensity, (2) myofibrillar size and organization, and (3) relative fibre diameter. Thin sections of each block examined in this manner were cut with diamond knives, mounted on formvar-coated grids, and stained with uranyl acetate and lead citrate. Each block cut for cross-sections was then re-oriented appropriately for longitudinal sections and treated in a similar manner. Grids were examined in a Zeiss EM 9A or a Philips EM 300 electron microscope.

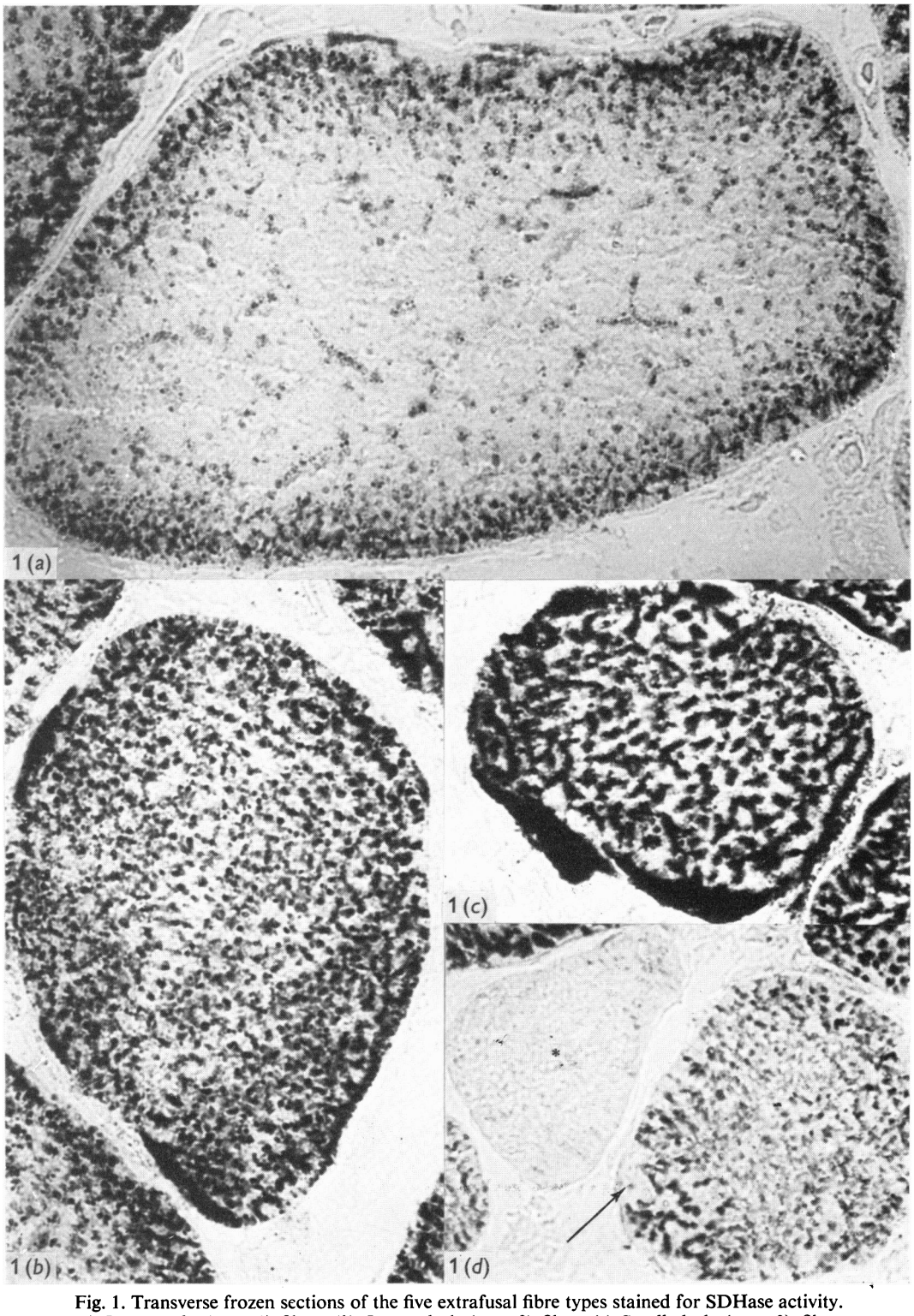
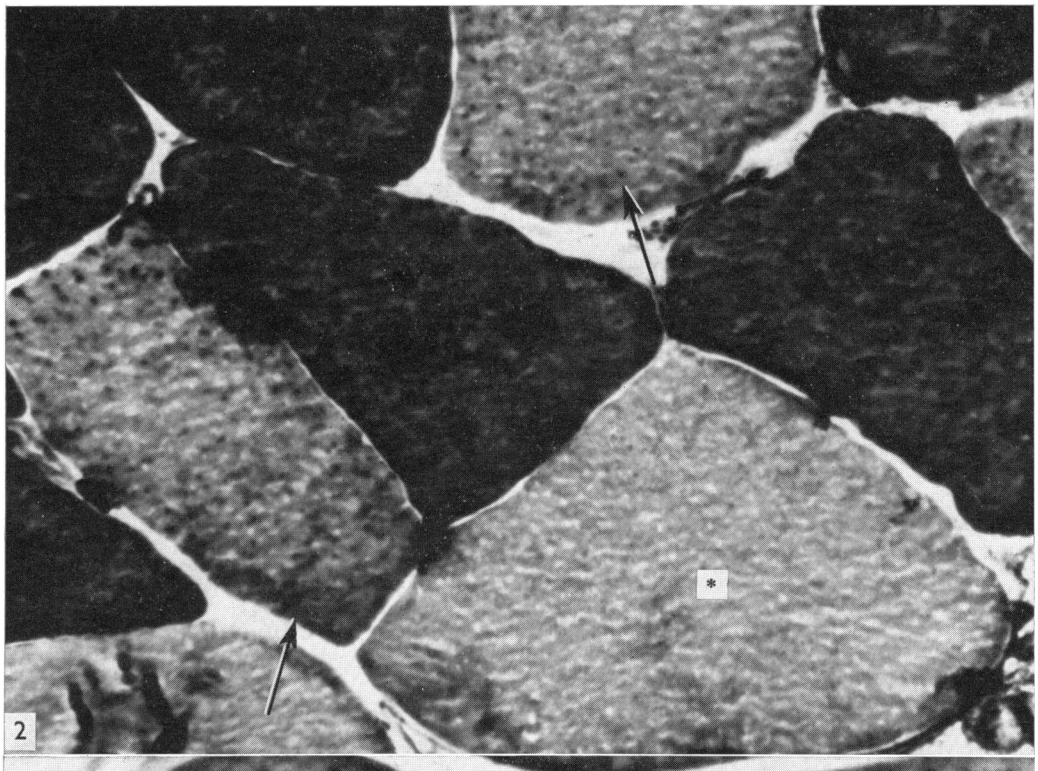


Fig. 1. Transverse frozen sections of the five extrafusal fibre types stained for SDHase activity. (*a*) Large pale (type 1) fibre. (*b*) Large dark (type 2) fibre. (*c*) Small dark (type 3) fibre. (*d*) Small pale (type 4) fibre (arrow), and small clear (type 5) fibre (asterisk). *Xenopus* flexor tarsi muscle. × 700.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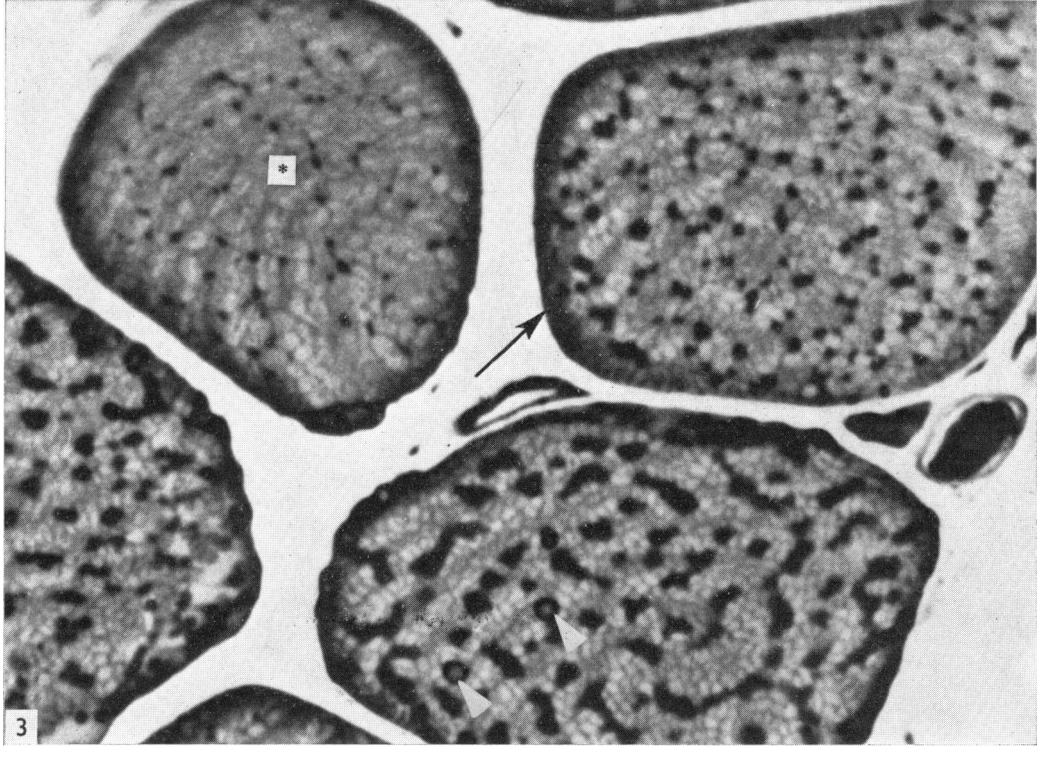
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Measurements

Frozen cross-sections of whole muscles stained for SDHase activity were examined, and the mean diameters (average of two diameters at right angles) of muscle fibres were measured using an ocular micrometer. The mitochondrial volume in fibres of each type in one muscle was estimated by point-counting stereological analysis of electron micrographs (Weibel, 1969). Cross-sections of each fibre type were examined, and only those micrographs containing all sarcomere levels were used. All micrographs examined had a final magnification of 26800. The mitochondrial volume was expressed as percentage of fibre volume: these percentage volumes represented average values for entire fibre cross-sections. No attempt was made to describe quantitatively the distribution of mitochondria within the muscle fibres.

Cross-sectional areas of myofibrils were obtained in the following manner. Cross-sections of each fibre type were photographed and printed on single-weight Kodabromide paper with a final magnification of 26800. A myofibril was defined as a group of myofilaments completely separated from its neighbours by sarcoplasm or sarcoplasmic reticulum. Cross-sectional profiles of a total of 50 myofibrils from each fibre type of the muscle flexor tarsi in one animal were traced, cut out, and weighed. Individual weights were converted to units of area by determining the weights of photographs of known areas; a carbon replica of a diffraction grating was used as the specimen in this case.

RESULTS

Histochemistry

Sections stained for myosin ATPase showed two clear categories of fibres: those which stained darkly and those which stained lightly. The latter group, however, could be subdivided, since a proportion of these fibres contained small isolated spots of reaction product (Fig. 2). Adjacent serial sections, when stained for SDHase, revealed that the group of fibres which stained darkly in the ATPase reaction could be further divided into three categories. The two varieties of ATPase-light fibres were also distinguishable in the SDHase preparations (Fig. 1). Thus the histochemistry allowed, in all, five varieties of extrafusal muscle fibres to be recognized. Each of these varieties was assigned a descriptive name based on the reaction for SDHase, a procedure which in part takes its precedent from previous work (Lännergren & Smith, 1966), and also, for the sake of brevity, a number (see Table 1). The numerical designation is used throughout this text. Not all the muscles studied contained all five varieties of fibres. The five types will first be

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Fig. 2. Transverse frozen section of *Xenopus* iliofibularis muscle stained for myosin ATPase. Fibre types 1, 2 (not shown), and 3 all stain darkly. Type 4 fibres (arrows) are lightly stained and appear pale. Type 5 fibres (asterisk) show almost no staining activity and appear clear. × 1500.

Fig. 3. Thick Epon transverse section of type 3 (below), type 4 (dark arrow), and type 5 (asterisk) fibres stained with phenylenediamine. Differences in myofibril size and in the distribution of mitochondria in each fibre type are evident. Lipid droplets (white arrowheads) are abundant in the type 3 fibre. *Xenopus* flexor tarsi muscle. × 2200.

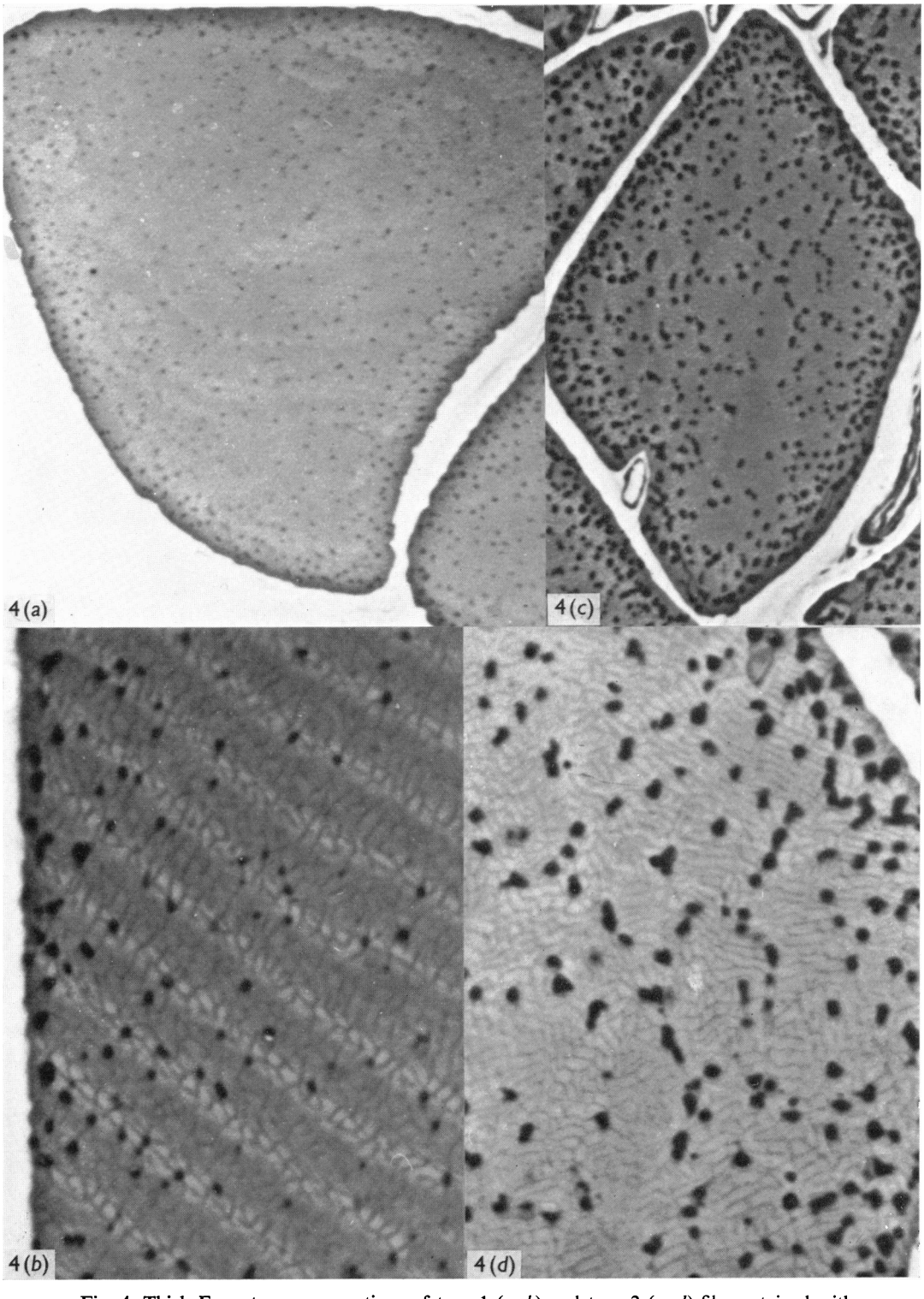


Fig. 4. Thick Epon transverse sections of type 1 (*a*, *b*) and type 2 (*c*, *d*) fibres stained with phenylenediamine. Compare with figure 3. *Xenopus* iliofibularis muscle. (*a*, *c*) × 900. (*b*, *d*) × 2200.

Table 1. *A summary of the characteristics of five types of muscle fibres in the hind limb muscles of* Xenopus laevis

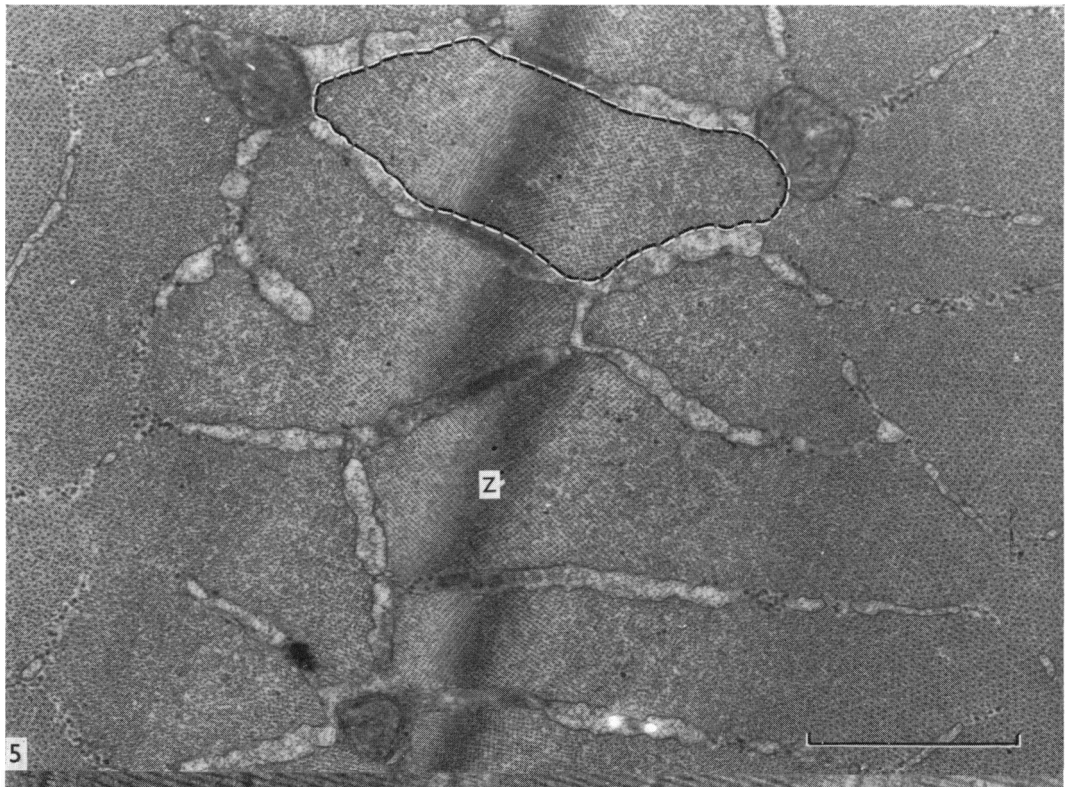
(The descriptive name for each type of muscle fibre (top row) is based on the reaction for SDHase. The numerical designation (second row) is used in the text and figure legends. Fibres of type 1 sampled from iliofibularis muscle. Fibres of types 2–5 sampled from the flexor tarsi. Where appropriate, values are given as mean ±s.d. Fibre diameters estimated from random selections of equal number, $n = 20$. All fibre diameter means are significantly different, $P < 0{\cdot}01$. For myofibril cross-sectional areas, $n = 50$ for each sample. Myofibril areas are statistically treated in Fig. 19.)

Muscle fibre types ...	Large pale	Large dark	Small dark	Small pale	Small clear
Numerical designation ...	1	2	3	4	5
Myosin ATPase staining	Dark	Dark	Dark	Pale	Clear
SDHase staining	Pale	Dark	Very dark	Pale	Clear
Muscle fibre diameter (μm)	169·0±17·8	145·0±17·6	76·7±12·1	60·7±5·4	44·0±7·7
Myofibril cross-section area (μm^2)	3·3±1·0	1·1±0·3	0·7±0·2	2·8±1·2	5·4±2·5
Mitochondrial volume (%)	2·5	11·1	17·4	4·1	1·3
Glycogen and lipid droplet content	Low	High	Very high	Low	Very low
Z band	Thin; square lattice; straight	Moderately thick; square lattice; straight	Moderately thick; square lattice; straight	Thick; square lattice; jagged	Thick; irregular lattice; jagged
M band	Straight	Straight	Straight	Irregular when present	Absent
Sarcotubular system	Well developed	Well developed	Moderately developed	Poorly developed	Poorly developed

described, and their distribution in various muscles of the hind limb will then be briefly outlined.

Type 1 fibres (large pale fibres) stained very slightly for the mitochondrial-bound enzyme SDHase. Most of the diformazan deposits in these fibres were located close to the sarcolemma, although sparsely scattered granules did occur throughout the cross-section of the fibre (Fig. 1*a*). Type 2 fibres (large dark fibres) were, in general, smaller in diameter, and contained many small diformazan deposits scattered throughout the sarcoplasm. While these fibres also stained more intensely just beneath the sarcolemma, they were readily distinguished from the type 1 fibre (Fig. 1*b*). Type 3 fibres (small dark fibres) were smaller than those of types 1 or 2 and exhibited intense staining. Dense plaque-like diformazan deposits were scattered throughout the sarcoplasm (Fig. 1*c*). Again, the largest of these deposits, and hence the greatest numbers of mitochondria, were always found immediately beneath the sarcolemma.

Fibres of types 4 and 5 were those which stained lightly for myosin ATPase. Type 4 fibres (small pale fibres) were small in diameter and contained small diffusely spread diformazan deposits (Fig. 1*d*). There was little or no tendency for the staining


Z
5


Z
t
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6

to be concentrated beneath the sarcolemma. Type 5 fibres (clear fibres) were also small in diameter, and contained few if any diformazan deposits (Fig. 1*d*).

The diameters of a random selection of each type of fibre are given in Table 1. Those of type 1 were selected from the iliofibularis muscle because of the low number of these fibres in the flexor tarsi. However, type 1 fibres in each muscle were of comparable diameter. In general it was not possible to relate precisely the diameters of the various fibre types in one muscle to those in another muscle. For example, in the flexor tarsi type 5 fibres were significantly smaller than type 4 fibres, but in the iliofibularis and anterior tibialis muscles the type 5 fibres were the larger of the two. Thus, while it was a useful adjunct to the recognition of the fibre types, the diameter of the fibres was not relied on heavily in this investigation.

The larger muscles of the hind limb could be divided into two categories on the basis of their fibre composition. The first category contained all five types of muscle fibres. Muscles of this type were: iliofibularis, semitendinosus, tibialis anterior, gastrocnemius, rectus internus, the adductors brevis, longus and magnus and the flexor tarsi. Without exception these three muscles showed a striking zoning pattern caused by a characteristic distribution and association of the various fibre types. Type 1 fibres were predominant in a peripheral zone furthest away from the side where the nerve entered the muscle. Deeper in the muscle, but still away from the site of nerve entry, the numbers of type 2 fibres increased. Close to the entry of the nerve trunk into the muscle, fibres of types 3, 4 and 5 were mixed together; generally, a zone of type 3 fibres surrounded fibres of types 4 and 5. Other workers have noted and shown photographs of the zoning of various amphibian muscles as demonstrated by various histochemical procedures (Lännergren & Smith, 1966; Engel & Irwin, 1967; Asmussen & Kiessling, 1970), and Asmussen & Kiessling (1970) have commented briefly on its significance. One of the zoned muscles, the flexor tarsi, requires additional description, since it was used extensively in this study. The flexor tarsi is a small (about 170 fibres) strap-like muscle which contained the five types of fibres in the following proportions: type 1, 6%; type 2, 38%; type 3, 32%; type 4, 11%; and type 5, 13%. The fibre types 3, 4 and 5 were located in a very superficial band across one side of the muscle, and consequently well-fixed specimens of these fibres could be obtained for electron microscopy.

The second category of muscles contained fibres of types 1 and 2 only. These muscles showed no zoning. The following muscles were of this type: sartorius, rectus externus, semimembranosus, triceps, rectus internus major and minor, rectus femoris anticus, extensor cruris brevis and peroneus. Some small hind limb muscles

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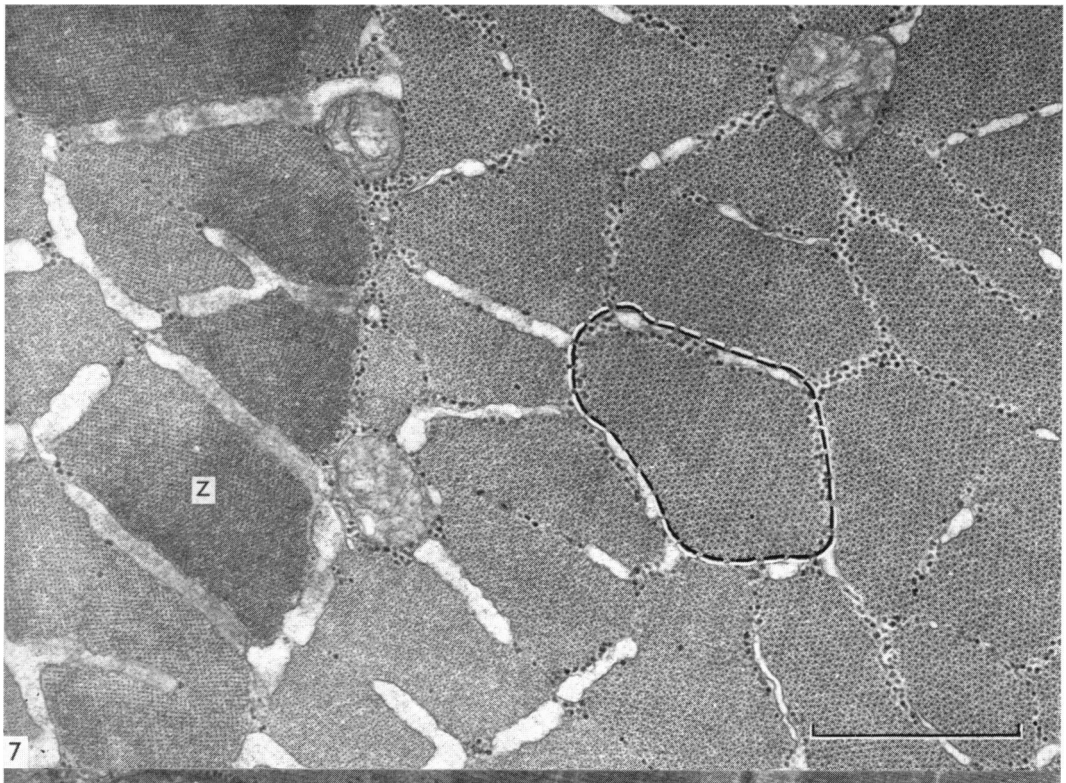
Fig. 5. Transverse section of a type 1 fibre. Myofibrils are large and well delineated by abundant elements of the sarcotubular system. The borders of a typical myofibril are indicated (broken line). Mitochondria, glycogen particles, and the interfibrillar sarcoplasm are sparse. Z band (Z). *Xenopus* iliofibularis muscle. Marker bar on this and subsequent figures indicates 1 μm. × 26800.

Fig. 6. Longitudinal section of a type 1 fibre. Well-defined M bands (M) are situated in the centre of each sarcomere. Z bands (Z) appear straight and narrow. Triads (t) occur regularly at each Z band level. Mitochondria (Mi) are small and rare. *Xenopus* iliofibularis muscle. × 26800.



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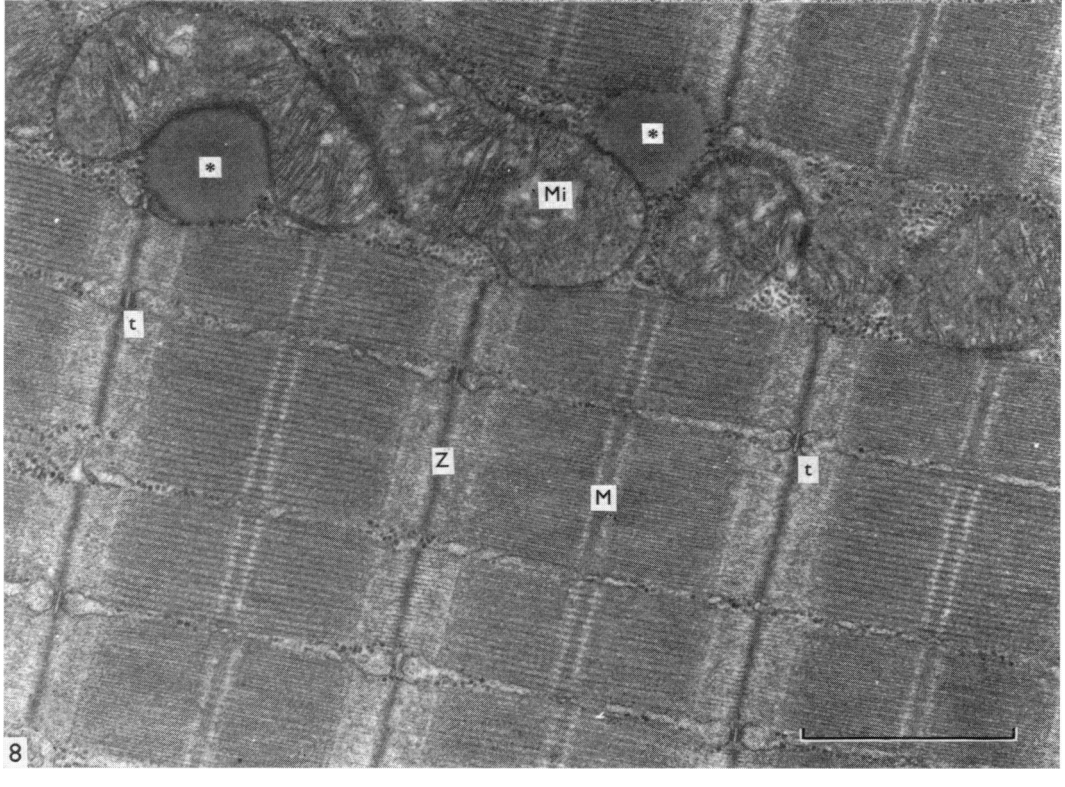

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contained fibres of types 2, 3, 4 and 5. Examples of this type of muscle are the extensor digiti IV longus and the pyriformis.

Observations similar to those described above were made on hind limb muscles from the frog *Rana pipiens* and the toad *Bufo woodhousei*. Thus, the results may be generally applicable to the tailless amphibia.

Ultrastructure of fibre types

Thick Epon sections stained with phenylenediamine were used to locate and identify the five fibre types which were subsequently sectioned for electron microscopy. In the thick sections the pattern of distribution and relative size of mitochondria in each fibre type were clearly evident (Figs. 3–4), and could be directly correlated with the results obtained with SDHase staining (compare with Fig. 1). In addition, distinct myofibrillar patterns could be seen (Figs. 3–4) and these features were also used for ultrastructural identification of each fibre type (Table 1).

Type 1 fibres

These characteristically had a well-developed sarcotubular system (i.e. sarcoplasmic reticulum, or SR, and T-system) which delineated the myofilaments into large, discrete myofibril units (Fig. 5). The mean cross-sectional area of the myofibrils was $3{\cdot}3 \pm 1{\cdot}0\ \mu m^2$ (see Table 1). Elements of the sarcotubular system were especially prominent at Z-band levels of each sarcomere where they formed an extensive network of interconnected tubules and cisternae (Fig. 5). Junctional couplings (triads) between SR cisternae and T-tubules were numerous, and occurred regularly at Z-band levels of each sarcomere (Fig. 6). The interfibrillar sarcoplasm was sparse, and contained few glycogen particles. Lipid droplets were rare. Mitochondria were rare, had poorly developed cristae, and occurred as isolated organelles oriented longitudinally. The mean mitochondrial volume was 2·5 %. Z bands were thin (about 37 nm) and straight in longitudinal section, and a well defined M band was present in the centre of each sarcomere (Fig. 6).

Type 2 fibres

These were similar to type 1 fibres in that they had a well-developed sarcotubular system and regularly occurring triads at each Z band level (Figs. 7–8). Myofibrils, although smaller than in the type 1 fibres, were well delineated at all sarcomere levels. The mean myofibril cross-sectional area was $1{\cdot}1 \pm 0{\cdot}3\ \mu m^2$. The interfibrillar sarcoplasm was more conspicuous, containing more abundant glycogen particles and lipid droplets. Mitochondria usually occurred in long interfibrillar rows and, in general, were larger, more numerous, and contained more abundant cristae

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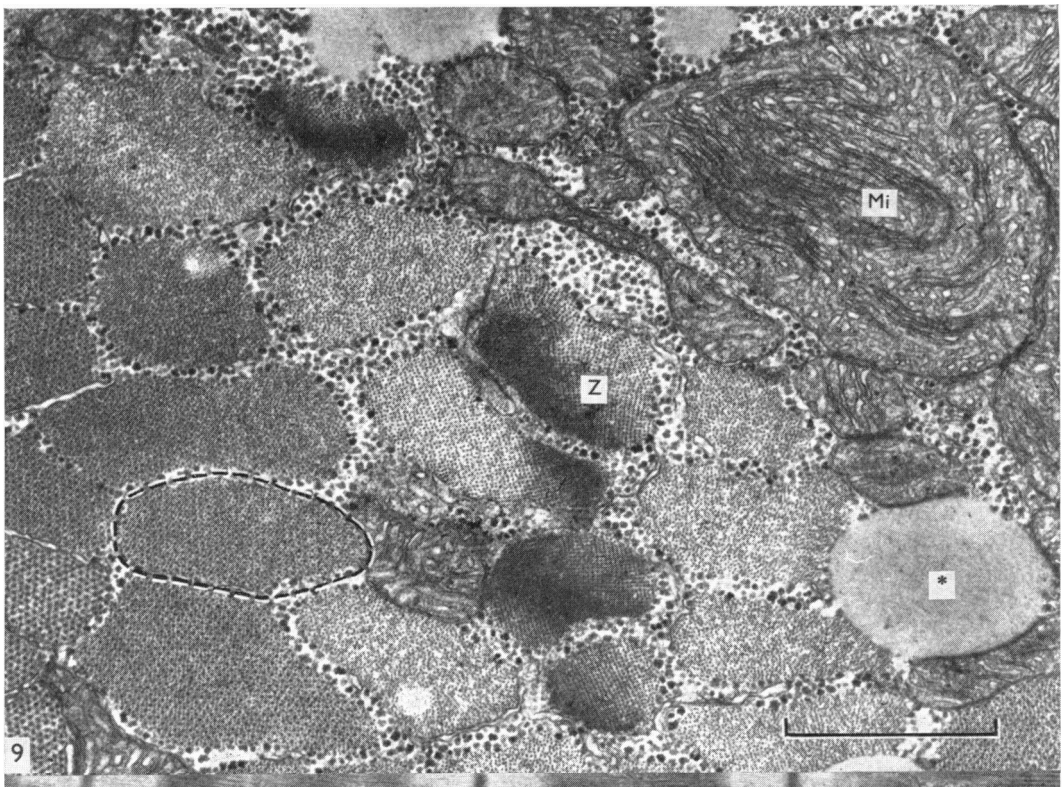
Fig. 7. Transverse section of a type 2 fibre. Myofibrils are smaller than those seen in the fibres of type 1, although they are similarly well delineated, especially at Z band (Z) levels. A typical myofibril is indicated (broken line). Mitochondria and glycogen particles are abundant. *Xenopus* iliofibularis muscle. × 26800.

Fig. 8. Longitudinal section of a type 2 fibre. Long interfibrillar rows of mitochondria (Mi), lipid droplets (asterisks), and glycogen particles are present. Z bands (Z) are straight, but appear wider than those seen in the type 1 fibre. M bands (M) and triads (t) are conspicuous. *Xenopus* iliofibularis muscle. × 21000.



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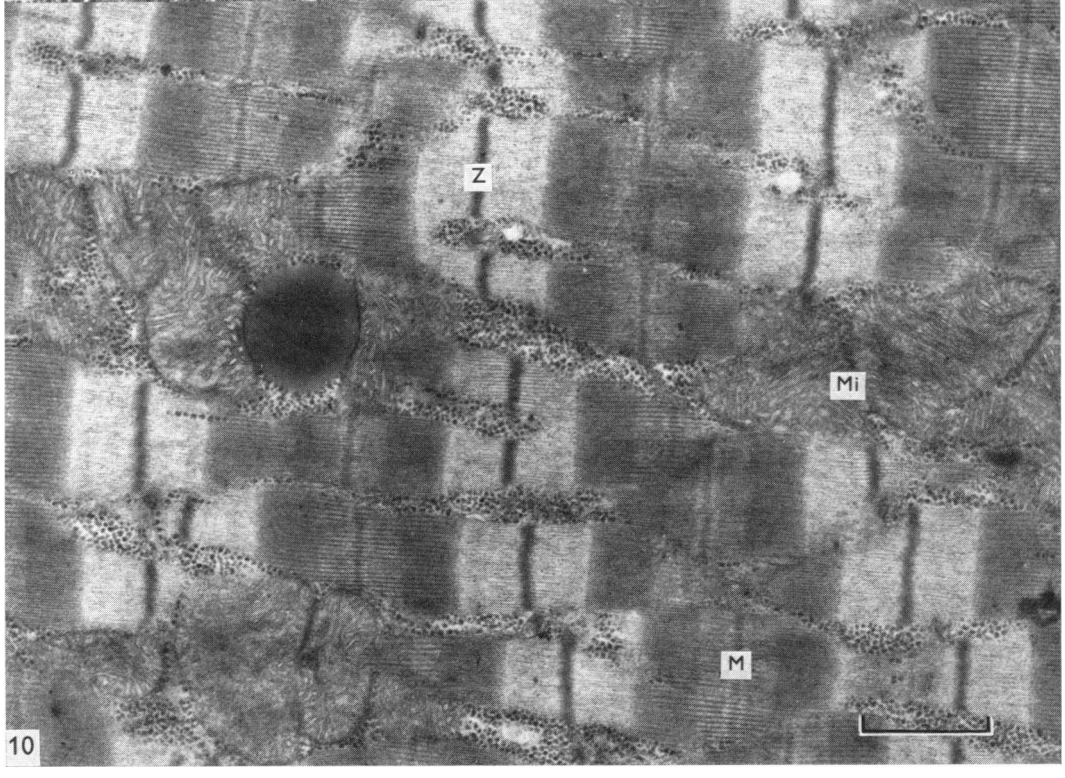

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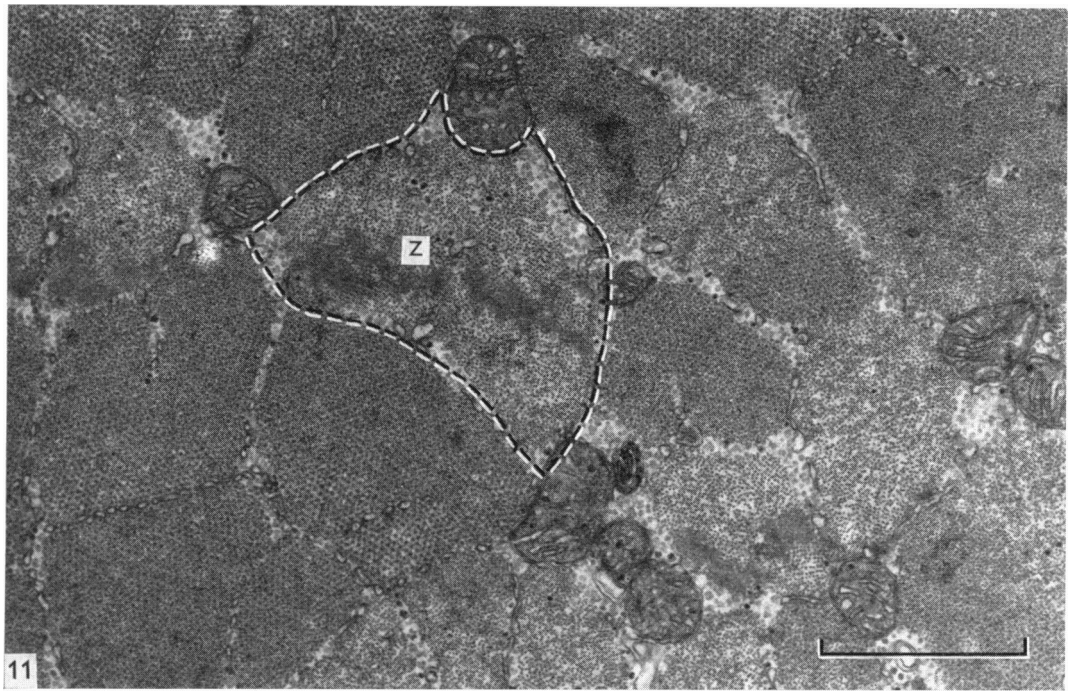


Fig. 11. Transverse section of a type 4 fibre. Myofibrils are large and poorly defined. The boundary of a typical myofibril is indicated (broken line). Mitochondria are small, and moderate numbers of glycogen particles occupy the interfibrillar spaces. Z band (Z). *Xenopus* flexor tarsi muscle. × 26800.

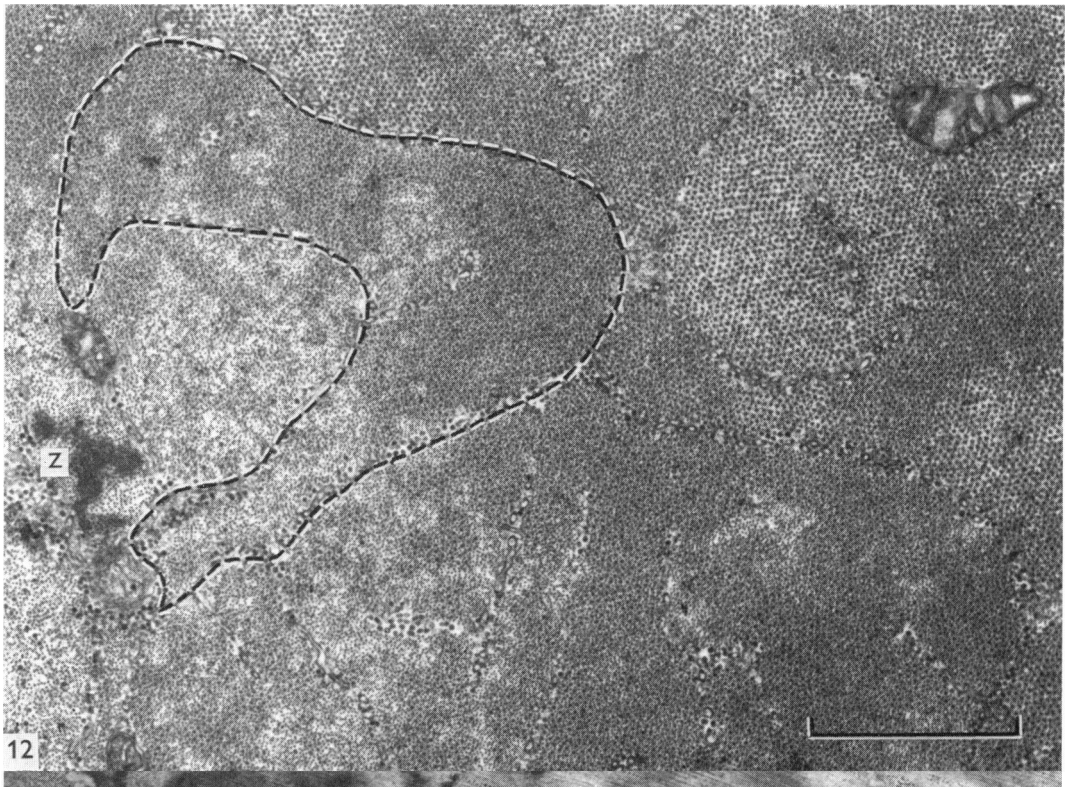
than those in type 1 fibres (compare Figs. 6 and 8). The mean mitochondrial volume was 11·1 %. A well defined M band was present in the centre of each sarcomere. The Z bands in the type 2 fibre, which also appeared straight in longitudinal section (Fig. 8), were generally wider (about 55 nm) than those in the type 1 fibre (Fig. 6).

Type 3 fibres

These fibres differed in several respects from the two preceding types. The sarcotubular system was less well developed, and triads were less frequently encountered (Figs. 9–10). Myofibrils were notably smaller and more circular; the mean cross-sectional area was $0{\cdot}7 \pm 0{\cdot}2\ \mu m^2$. The interfibrillar sarcoplasm was better developed and contained large amounts of glycogen and lipid droplets. Mitochondria were considerably larger and more numerous, and contained more

Fig. 9. Transverse section of a type 3 fibre. Myofibrils (one indicated by broken line) are much smaller than those seen in fibre types 1 and 2. Elements of the sarcotubular system are less abundant. Mitochondria (Mi), glycogen particles, and lipid droplets (asterisk) are especially prominent. In addition, mitochondria often appear very large, with extremely convoluted cristae. Z band (Z). *Xenopus* flexor tarsi muscle. × 26800.

Fig. 10. Longitudinal section of a type 3 fibre. Triads appear sporadically: Z bands (Z) and M bands (M) are straight and well defined. Mitochondria (Mi) and glycogen particles are aggregated into large interfibrillar clumps. *Xenopus* flexor tarsi muscle. × 16900.



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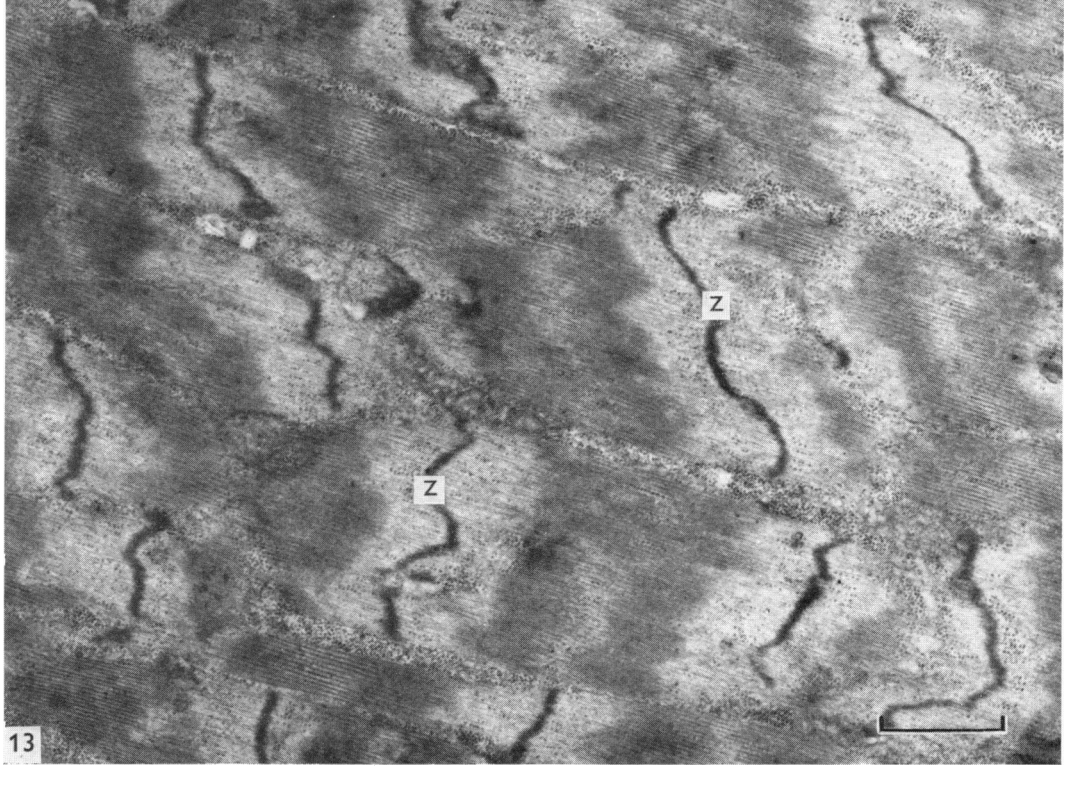

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abundant tightly packed cristae. They were often arranged into large interfibrillar or subsarcolemmal aggregates (Fig. 9). The mean mitochondrial volume was 17·4 %. The appearance of the Z band in the type 3 fibre was similar to that of the type 2 fibre, although its width was sometimes greater (about 78 nm). A well defined M band was also situated in the centre of each sarcomere (Fig. 10).

Type 4 fibres

These had myofibrils that at all sarcomere levels were large, tightly packed, and, because of a poorly developed sarcotubular system (Fig. 11), less clearly defined than in the fibres described above. The mean myofibril size was $2{\cdot}8 \pm 1{\cdot}2\ \mu m^2$. Mitochondria were small, sparse, and isolated. The mean mitochondrial volume was 4·1 %. The interfibrillar sarcoplasm was moderately developed, and lipid droplets were occasionally present (Fig. 14). A distinguishing feature of this fibre, not seen in fibres of types 1, 2 and 3, was the presence of an irregular banding pattern in each sarcomere. Z bands were jagged and thick (about 90 nm), and M bands, when present, were ill defined and irregular (Fig. 14).

Type 5 fibres

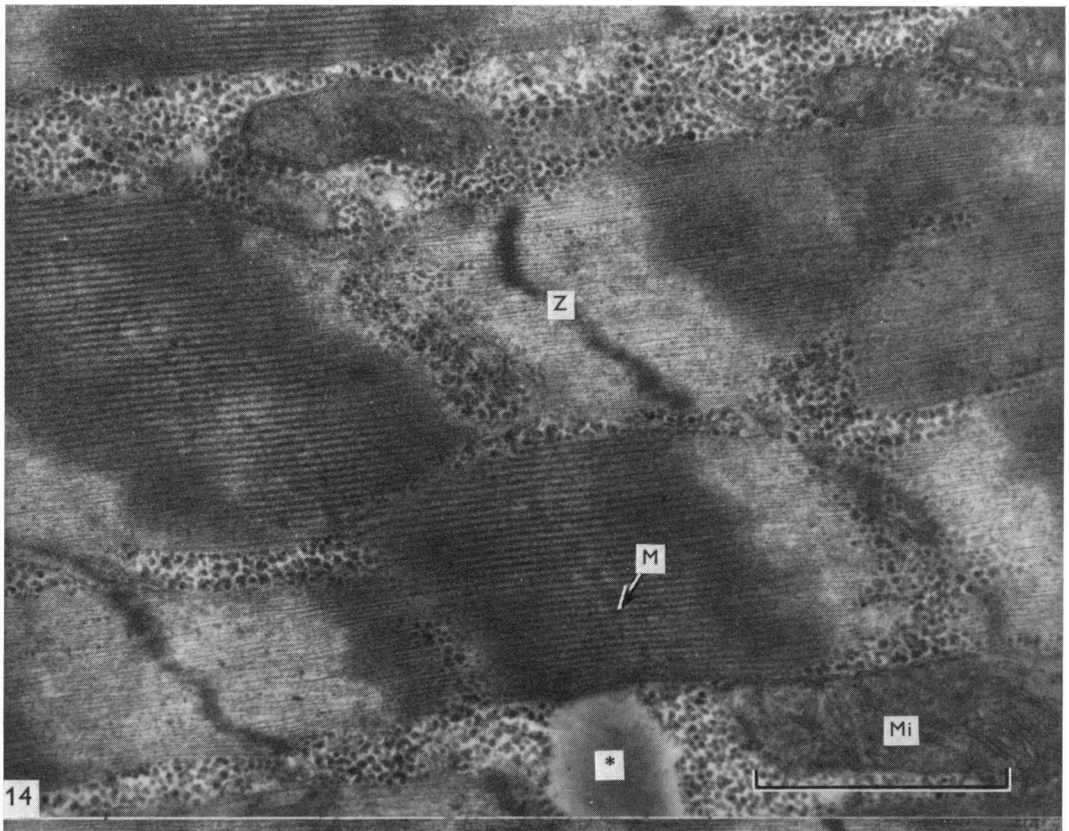
Fibres of this type were similar in many respects to type 4 fibres in that they had a poorly developed sarcotubular system and large, closely packed myofibrils (Figs. 12–13). The cross-sectional area of myofibrils in the type 5 fibre, however, was significantly larger ($5{\cdot}4 \pm 2{\cdot}5\ \mu m^2$), and myofibrils often appeared irregular or ribbon-shaped. Mitochondria were small and notably less abundant in this type of fibre. The mean mitochondrial volume was 1·3 %. The interfibrillar sarcoplasm was sparse, and in contrast to type 4 fibres, lipid droplets were lacking. In addition, glycogen particles in the type 5 fibre usually appeared as single (beta) units, instead of forming the multiple aggregates or 'rosettes' which were characteristic of the type 4 fibre (compare Figs. 14–15). Longitudinal sections of Z bands of the type 5 fibre were similar in appearance and width to those of the type 4 fibre, and were jagged (Figs. 13, 15). No M bands, however, were found in comparable longitudinal sections of the type 5 fibre (compare Figs. 14–15). Other differences between the fibre types are described below.

M bands and Z bands

The presence or absence of an M band in mid-H zone regions of the sarcomere is a criterion which has served to distinguish fast and slow extrafusal muscle fibres (Peachey & Huxley, 1962; Page, 1965; Hess, 1970). In the present study longitudinal sections of the five fibre types showed that well defined M bands were always present in sarcomeres of fibre types 1, 2 and 3 (Figs. 6, 8, 10), sometimes present

Fig. 12. Transverse section of a type 5 fibre. Myofibrils are ill defined, are larger than those seen in type 4 fibres, and may display irregular ribbon-shaped contours (broken lines). Mitochondria and glycogen particles are sparse. Compare with Fig. 11. Z band (Z). *Xenopus* flexor tarsi muscle. × 26800.

Fig. 13. Longitudinal section of a type 5 fibre. The striations are irregular and are not in register between myofibrils. Z bands (Z) are jagged, and the centres of each sarcomere are devoid of M bands. *Xenopus* flexor tarsi muscle. × 16900.



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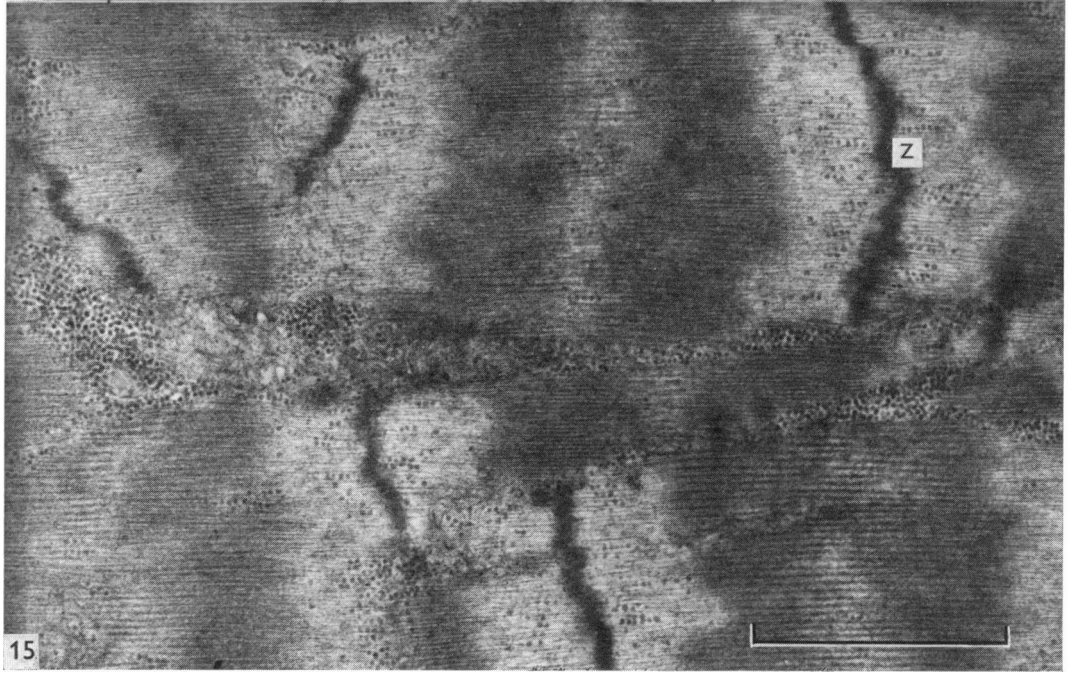

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in sarcomeres of fibre type 4 (Fig. 14), and absent in all sarcomeres of fibre type 5 (Fig. 15).

A comparison of Z bands in the five fibre types at high magnification revealed further differences in their structural organization (Figs. 16–18). Z bands in types 1, 2 and 3 appeared straight in longitudinal section (Figs. 6, 8, 10). The material in each Z band was organized into the typical square lattice pattern described by previous workers for fast muscle fibres (Knappeis & Carlsen, 1962; Page, 1965; Rowe, 1971; Kelly & Cahill, 1972). In addition, the I-band filaments in the region of each Z band were also organized in a regular array. Z bands of the type 4 fibre, although often appearing irregular or patchy at low magnifications (see Fig. 11), exhibited a square lattice pattern when viewed at higher magnification, the pattern being somewhat similar to that seen in fibre types 1, 2 and 3 (compare Figs. 16–17). Z bands of the type 5 fibre, as in the slow fibres described by Page (1965), were dense and patchy, and lacked any definite internal pattern. The I-filaments entered these Z-bands randomly (Fig. 18).

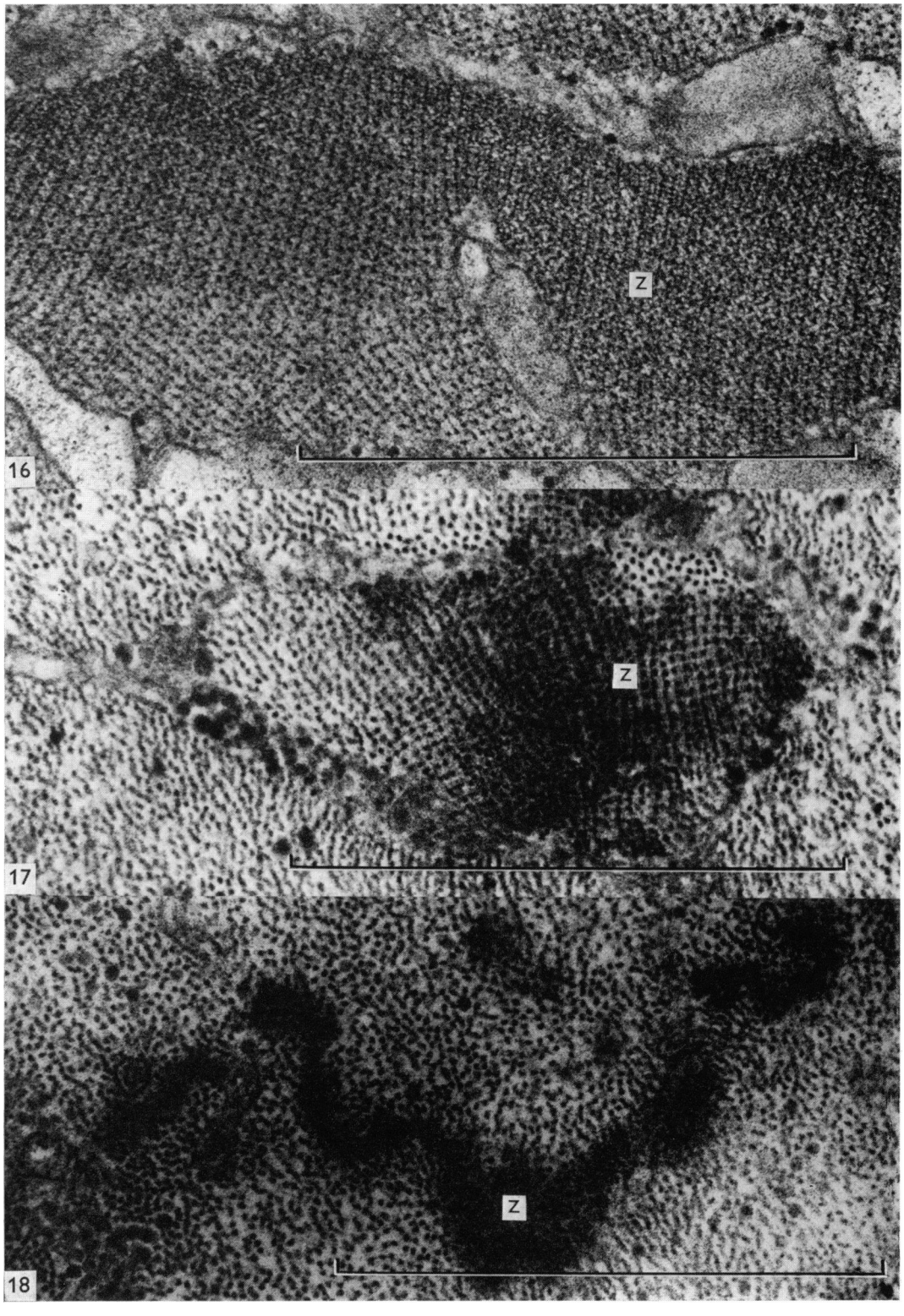
Myofibril sizes

Clear-cut qualitative differences between muscle fibres are obviously the most satisfactory way of categorizing fibre types. The size and arrangement of myofibrils has been treated as such a qualitative difference between fast and slow fibres (Krüger, 1952; Peachey, 1961; Hess, 1970). However, Page (1965) has suggested that myofibril topography may not always be a reliable indicator of whether a frog muscle fibre is of the fast or slow variety. The measurements presented in the present study (Table 1) suggest that the question of myofibril size should be examined more closely. In order to determine whether the mean cross-sectional areas of myofibrils in the five types of muscle fibre were different, confidence intervals for the mean of each set of 50 measurements were calculated. Figure 19 shows each of the five mean values plotted with the 98 % confidence interval for the mean. Examination of such a diagram allows one to appreciate immediately that the mean cross-sectional areas of myofibrils in fibres of types 2, 3, 4 and 5 were different. The cross-sectional areas of myofibrils in fibres of types 1 and 4 were not different on this basis. However, these fibres showed other structural differences which have been described above.

DISCUSSION

That all skeletal muscle fibres in a given animal, or even in a given muscle, are not exactly similar in structure or function is a fact no one disputes. However, this fact may be viewed from a number of hypothetical positions, each

Figs. 14 and 15. Higher magnification micrographs of a type 4 fibre (Fig. 14) and a type 5 fibre (Fig. 15) showing some of their structural differences. Sarcomeres of the type 4 fibre occasionally exhibit irregular ill-defined M bands (M), while they are never seen in comparable sections of sarcomeres of the type 5 fibre. Glycogen particles, mitochondria (Mi), and lipid droplets (asterisk) are more common in the type 4 fibre. Both fibres contain jagged Z bands (Z) and a poorly developed sarcotubular system. *Xenopus* flexor tarsi muscle. × 32000.


Z
16
Z
17
Z
18

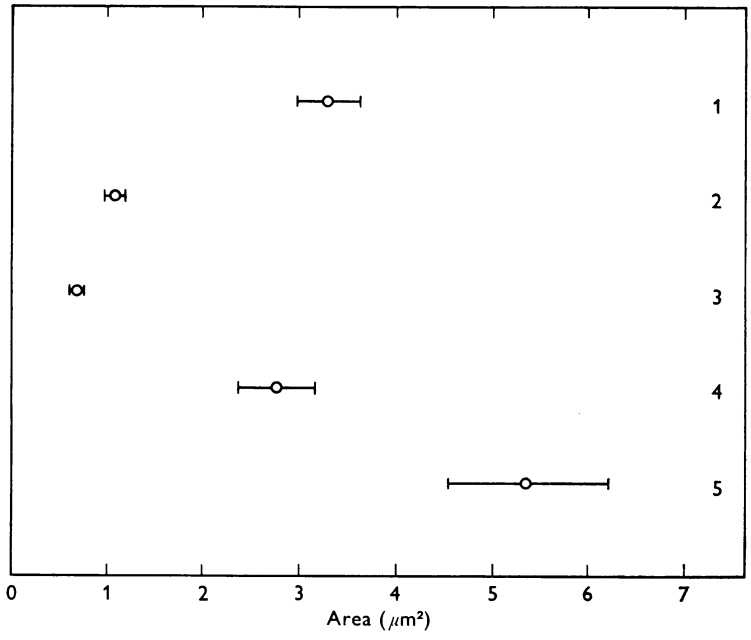


Fig. 19. The mean of 50 determinations of the cross-sectional areas of myofibrils in each of the fibre types 1–5 is plotted as a circle. Surrounding each mean value is the 98% confidence interval for the mean. The vertical separation of the points allows each value to be assigned to one of the five fibre types. The numerical designation of fibre type on the right hand of the diagram is the same as that given in Table 1.

of which attributes a different significance to the observed variations. The most distinctly different kinds of hypothesis that might be applicable are: (1) that all the properties of muscle fibres may be described in terms of a continuum, and (2) that a finite number of different fibre types exists. The second hypothesis has been given a number of specific forms, two of which will be considered here: (2*a*) that two types of muscle fibre exist, and (2*b*) that there is an, as yet, undetermined number of distinctly different types of muscle fibres.

The first hypothesis supposes that the full spectrum of muscle fibres is composed of cells whose properties grade smoothly from one extreme of the range to the other. The idea in its pure form is not one which is popular, although it has been considered in reviews of the literature (Huxley, 1964; Peachey, 1968). This possibility has been noted with respect to the contractile properties of motor units in *Xenopus laevis* (Smith & Lännergren, 1968) and some work on frog muscle could be interpreted in these terms (Shamarina, 1963; Nasledov, 1966; Zhukov & Leushina, cited

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Figs. 16–18. Transverse sections through Z band regions of type 1 (Fig. 16), type 4 (Fig. 17), and type 5 (Fig. 18) fibres. Comparable regions through fibre types 2 and 3 are not included here since they conform to that seen in Fig. 16. Note the regular square lattice pattern present in Figs. 16 and 17. In Fig. 18, the filaments at the Z band are randomly arranged. *Xenopus* flexor tarsi muscle. × 85500.

by Nasledov, 1966). We consider that this is not a particularly useful idea to apply to the problem of the organization of skeletal muscle; it generates predictions which are both conceptually and experimentally cumbersome. The 'continuum' hypothesis, however, is worth stating explicitly since it may describe the variation of some (but probably not all) properties of muscle fibres. In addition, it is often included as an implicit element within hypotheses which suppose that there is a finite number of different types of muscle fibres.

In a recent review, Hess (1970) advocated hypothesis 2*a* above. He contended that vertebrate muscle fibres can be divided into two kinds: slow and fast. He reviewed the evidence that the slow muscle fibre does not, under normal circumstances, propagate an action potential, exhibits slow graded contractions, and has a distinctive ultrastructure, the type example of which is seen in the frog (Page, 1965). The fast muscle fibre, on the other hand, does propagate an action potential, and usually contracts in a fast all-or-nothing fashion (a 'twitch'). Again, the ultrastructural features of fast muscle fibres are characteristic and similar in a wide range of vertebrates. Hess (1970) claimed that any fibres which do not exactly fit the currently accepted descriptions merely show 'variation from the typical' (cf. hypothesis 1). He went on to expand this opinion into an investigative philosophy with the statement 'A description of the way in which certain muscle fibers may vary in their morphology from the general categories of twitch and slow fibers, rather than a delineation of more fiber types (here Hess referred to hypothesis 2*b*), should prove most advantageous in promoting the knowledge of the structure of muscle fibers.' While this point of view presents some difficulties, such as just how one defines one's datum (i.e. the 'typical' muscle fibre of each type) it does, nevertheless, represent a commonly accepted view of non-mammalian skeletal muscle.

Hypothesis 2*b* – that there is a finite number, greater than two, of types of muscle fibres – is a view which is commonly held for mammalian muscle. For instance, the consensus of histochemical, ultrastructural and functional evidence points to the existence of three kinds of fast (or twitch) muscle fibres in mammalian limb muscles (Henneman & Olson, 1965; Padykula & Gauthier, 1967; Kugelberg & Edström, 1968; Edström & Kugelberg, 1968; Gauthier, 1969; Burke *et al.* 1971). However, over-emphasis of the significance of a single property or a limited group of properties has led to the description of a large number of fibre types; Padykula & Gauthier (1967) have commented on the resulting confusion. Thus this point of view, by association with the confused state of the literature, is in danger of lacking credibility, and the idea has been rejected in recent reviews (Sandow, 1970; Hess, 1970). We argue to the contrary that, providing the different types of muscle fibres are members of discrete functional units, this last point of view offers the most useful approach to the understanding of the organization of skeletal muscle. The hypothesis that there is a finite number of types of muscle fibres which differ from each other qualitatively or in some stepwise quantitative manner, and which are organized into homogeneous motor units, is a view which is susceptible to rigorous definition and experimental testing. This approach is useful in that it leads not only to a precise formulation of the organization of skeletal muscle but also to testable propositions regarding the organization of the spinal cord and perhaps higher nervous centres. In fact, this is an approach commonly adopted in the

investigation of mammalian neuromuscular organization (Henneman & Olson, 1965; Burke *et al.* 1971). We, therefore, interpret our results along these lines: the categories of amphibian fast and slow muscle fibres represent a useful division of muscle fibre types, but each of these categories may be subdivided into a number of fibre types which are likely to be functionally discrete.

Using histochemical and ultrastructural criteria we have described five different kinds of muscle fibres in hind limb skeletal muscles of the Anura. Types 1, 2 and 3 (Table 1) may be regarded as belonging to the general category of fast muscle fibres. Their ultrastructural features are characteristic of fast fibres and they show histochemical similarity in their reaction for myosin ATPase. A division of the fast group of fibres into three types has been anticipated by other recent work. Fibre types 1, 2 and 3 were clearly recognized in histochemical investigations (Lännergren & Smith, 1966; Engel & Irwin, 1967; Asmussen & Kiessling, 1970). These workers all took the final point of view that the population of fast muscle fibres could be divided into two broad categories: large fibres with few mitochondria and small fibres with many mitochondria. However, Table 1 in each of the papers (Lännergren & Smith, 1966; Engel & Irwin, 1967; Asmussen & Kiessling, 1970) shows that in fact each group of investigators independently recognized the three types of fibres. Evidence also exists that the type 1 and 2 muscle fibres are functionally different variants of the fast fibre (Lännergren & Smith, 1966) and that each type may be arranged into motor units which are homogeneous (Smith & Lännergren, 1968). In addition, each type of motor unit may be innervated by motor axons with a characteristic range of diameters (Smith & Lännergren, 1968). The distribution of the muscle fibres among different hind limb muscles provides evidence that the three types of fast fibres may be functionally independent. Some muscles, such as the sartorius, contain fibres of types 1 and 2 but none of type 3 (see also Lännergren & Smith, 1966; Engel & Irwin, 1967; Asmussen & Kiessling, 1970). Large muscles, such as the iliofibularis and gastrocnemius, contain types 1, 2 and 3, while small muscles, such as the extensor digiti IV, contain types 2 and 3 only. The results of Nasledov (1966) are pertinent. Using electrophysiological criteria, he classified all the fibres of the sartorius muscle as type 'a', and the fast fibres of the tonus bundle of iliofibularis as a mixture of types 'a' and 'b'. We may suppose then that Nasledov's 'b' fibres correspond to our type 3 fibres, and hence his work supplied evidence that these differ functionally from either type 1 or type 2. From data on the size of motor units and their susceptibility to fatigue (Smith & Lännergren, 1968) one may surmise that fibres of type 1 are organized into large motor units serving motions that are fast and powerful but not long lasting, hence their presence in the major muscle masses of the hind limb. Fibres of type 2 differ in that they are probably organized into motor units of smaller size which do not fatigue very rapidly (Smith & Lännergren, 1968). The functional organization of fibres of type 3 is completely unknown, but it is possible that their function may relate to small movements or maintained postural effort since they occur only in muscles which also contain slow muscle fibres.

We regard types 4 and 5 as being varieties of slow muscle fibres. Both have the very irregular pattern of striation characteristic of amphibian slow fibres (Page, 1965). In addition, their myofibrils are large and closely packed, their mitochondria

are small and sparse, and they both show very little staining in the histochemical reaction for myosin ATPase. These fibre types, however, have clear qualitative structural differences in the organization of the Z band and in the presence or absence of a recognizable M band. In *Xenopus laevis* two types of slow motor units have been described which differed markedly in their speeds of contraction (Smith & Lännergren, 1968). One, which was supplied by motor axons in the range 4–5 μm, gave very slow contractions typical of those described by earlier workers (Tasaki & Mizutani, 1944; Kuffler & Vaughan Williams, 1953). Since direct identification of the fibre type responsible for slow non-propagated contractions has been achieved (Peachey & Huxley, 1962), one may with fair confidence identify fibres of type 5 with the slower of the two slow motor units. The other slow motor unit described by Smith & Lännergren (1968) contracted quite rapidly but still in a graded fashion. These were supplied by motor axons in the range 6–10 μm. We feel it is probable that these motor units were composed of fibres of type 4.

In summary, the work described in this paper, taken together with that of others, supplies information sufficient to justify the formulation of the following hypothesis: skeletal muscle fibres in the Anura may be separated into five functionally and structurally distinct classes consisting of three types of fast muscle fibres and two types of slow muscle fibres.

SUMMARY

Histochemical and electron microscopic studies of hind limb skeletal muscles in toads and frogs were undertaken with the intention of describing the varieties of muscle fibres present. Frozen sections stained to demonstrate myosin ATPase revealed two categories of fibres: darkly staining and lightly staining. The lightly staining group could be further divided into two categories. Sections serial to these, when stained for succinic dehydrogenase, showed that those fibres which stained darkly for myosin ATPase could be separated into three groups. The two varieties of ATPase-light fibres could also be distinguished in the sections stained for SDHase. Correlated observations using thick Epon sections stained with phenylenediamine, and of thin sections of the same material viewed in the electron microscope, similarly revealed five varieties of fibres. The fibres differed in myofibrillar size and organization, the appearance of the M and Z bands, glycogen and mitochondrial content, and the organization of the sarcotubular system. The results are discussed in the light of the current understanding of the organization of amphibian skeletal muscle. The hypothesis is presented that the skeletal muscle of frogs and toads is composed of five types of muscle fibres, three of which are varieties of fast muscle fibres and two are varieties of slow fibres.

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