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Changes in fibre type, number and diameter in developing and ageing skeletal muscle

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INTRODUCTION

The growth and ageing of mammalian skeletal muscle has been a topic of study for several decades, and many of the characteristics of these important processes are now reasonably well documented. The postmitotic nature of skeletal muscle tissue (Cowdry, 1952) means that its ability to effect repair or to regenerate is somewhat limited. In most muscles studied fibre number reaches a maximum value at, or shortly after, birth and remains unchanged throughout development (Goldspink, 1972). An increase in muscle size and weight is therefore restricted to changes in muscle fibre size as demonstrated in the mouse (Rowe & Goldspink, 1969) guinea-pig (Lieberman, Maxwell & Faulkner, 1972) and the rat (Chiakuluas & Pauly, 1965; Ontell & Dunn, 1978). Such increases are associated with an increase in myofibril number and size (Goldspink, 1970) thus increasing the contractile force of the tissue.

Studies as early as 1927 (Ruger & Stoessiger) showed that skeletal muscles deteriorate functionally in old age, a fact confirmed later by Critchley (1956), who associated it with a decrease in muscle mass. Wasting in muscles can be explained by either a decrease in fibre number or a decrease in fibre cross sectional area. Moore, Rebiez, Holden & Adams (1971) demonstrated a reduced mean fibre diameter in many muscles in human subjects older than 40 years. The exact relationship between fibre number and fibre diameter is not clear. Some muscles, in spite of losing fibres, tend to show an increased fibre diameter (Rowe & Goldspink, 1969). In some muscles there appears to be a selective loss of fibres of a given type. Tauchi, Yoshioka & Kobayashi (1971) observed reductions in number of 'red' fibres and in the volume of 'white' fibres in the tibialis anterior of the rat. Differential atrophy of fibre types has received more attention recently. Larsson, Sjodin & Karlsson (1978) found the human vastus lateralis to have a higher Type I to Type II fibre ratio in older subjects. However the ratio of Type IIA and IIB fibres remained unchanged.

Primary fetal muscle fibres are the first muscle fibres to appear. They are relatively large, have centrally located nuclei and are destined to become Type I or slow oxidative fibres (SO) in fully differentiated adult muscles. At a later stage a second period of primary myotubes results in a further population of fetal myofibres which are smaller and more numerous than the primary fibres. These become Type IIA or fast oxidative glycolytic fibres (FOG) and IIB or fast glycolytic (FG) fibres. The growth rate in the secondary population is such that they become significantly larger than the original primary fibres and this sometimes results in a bimodal distribution of fibres (Ashmore, Addis & Doerr, 1973; Ashmore, Robinson, Rattray & Doerr, 1974). Goldspink (1962)

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and Rowe & Goldspink (1969) studied the growth of normal mice at ages between 3 weeks and 24 weeks. They measured fibres from muscles with histochemically mixed populations such as biceps brachii, sternomastoid and tibialis anterior. Distribution analyses of these muscles for the youngest ages showed a normal distribution of fibre diameters. At later ages their histograms showed a distinct second peak which they suggested was a result of compensatory hypertrophy of some fibres brought about by the normal increase in body weight resulting in an increased load on the muscles. Other workers (Hegarty & Hooper, 1971; Hegarty & Naude, 1973) found similar results in mice using formalin-fixed sections. They concluded that the bimodal distribution of fibre size was an artifact resulting from a combination of slow penetration of fixatives and the onset of rigor mortis which allowed some fibres to become altered in size before the fixative had taken effect.

The present study describes age related changes in the three factors that determine muscle mass namely; fibre size, fibre number and fibre type.

MATERIALS AND METHODS

Male CFY Sprague–Dawley rats aged 21, 84, 185 (+/−3), 298, 508 and 732 (+/−16) days were purchased from the Wolfson Institute of Gerontology. The animals were raised and kept under controlled conditions from birth to death and are representative of a developing and ageing population (Alnaqeeb, Al Zaid & Goldspink, 1984).

Two hind limbs muscles were selected for study: the soleus muscle which is predominantly made up of slow motor units and the extensor digitorum longus muscle (EDL) which has a majority of fast contracting motor units. Both muscles have a simple fusiform structure and they are small enough to allow the total number of fibres to be determined from a single section taken from the belly of the muscle. Animals were killed with an overdose of pentobarbitone (Sagatal, May & Baker Ltd). The two muscles were removed rapidly, cleaned of superficial fat and connective tissue and weighed on a torsion balance to +/−0·5 mg.

As most fixatives result in shrinkage (Goldspink, 1961; Goldspink, Gelder, Clapison & Overfield, 1973; Eisenberg & Mobley, 1975) and reduce or inhibit enzyme activity, unfixed cryostat sections were used. This method has been shown to cause the least dimensional distortion (Goldspink *et al.* 1973; Hegarty & Naude, 1973). The dissected muscles were mounted on thin cork blocks in Tissue Tek II and then frozen in supercooled isopentane precooled by immersion in liquid nitrogen (−160 °C). Transverse sections 10 μm thick were cut in the belly region of each muscle on a cryostat (Bright Instrument Company) at −15 °C, air dried for 30–90 minutes at room temperature then stored until required at −20 °C.

Histochemical stains

Histochemical staining of myosin ATPase in these sections was based upon the method of Tunell & Hart (1977). This method was found to be more reproducible than the original method of Guth & Samaha (1969, 1970). It also produced good differentiation of the fast fibre types and required a shorter incubation period. Selected sections from muscles from different age groups were stained according to the acid and alkaline preincubation techniques of Guth & Samaha. Adjacent sections were also stained for succinic dehydrogenase (Nachlas *et al.* 1957). The myosin ATPase sections were cross referenced with succinic dehydrogenase to confirm the typing of the muscle fibres.

Muscle fibre diameters

The irregular fibre diameter was estimated as the mean of the orthogonal axes of each fibre (Song, Shimada & Anderson, 1963, modified by Schmitt, 1976). One hundred fibres of each type were sampled wherever possible. Measurements were made using a graduated eyepiece in a Leitz Ortholux microscope which was calibrated with a stage micrometer.

As both the EDL and the soleus muscles showed a mosaic distribution of the various fibre types, an organised scan technique of the whole cross section was used. This eliminated any area or subjective sampling errors that could arise because of the uneven distribution of size and fibre type (Pullen, 1977). The scan was carried out in such a way as to sample all the fibres along the deep to superficial axes of the muscle. The spacing between any two scans was wide enough to cover the whole muscle when 100 fibres of each type were measured.

Fibre diameters of the different fibre types were subjected to an analysis of variance followed by an *a priori* comparison. Frequency distributions of individual muscles and grouped muscles were accumulated and tested for significance.

Muscle fibre number

ATPase-stained sections were used to count the total number of each fibre type. Since sections were cut in the belly region of each muscle and because of the simple geometry of the muscles used, virtually all the fibres are known to be present in these sections (Rowe, 1967). In each section, every fibre was counted and allocated to one of the three fibre type categories; FOG, FG and SO. This reduced the risk of area sampling errors arising from the uneven distribution of the various fibre types (Pullen, 1977). To facilitate identification and counting of muscle fibre types, muscles were divided into small areas, each area was projected using a Leitz Microprojector. The final screen magnification was between 120 and 300 times.

Total fibre number within each muscle was obtained by adding together the individual fibre type numbers. The results of all counts were grouped according to fibre type and age group and subjected to an analysis of variance.

RESULTS

Animals older than 508 days did not show significant weight changes, although as expected younger animals showed significant rates of growth (Table 1). As far as the muscle weights were concerned the EDL increased rapidly in weight from weaning to 185 days and then remained stable. In senile animals (700 + days) however, significant weight losses in the EDL were detected (Table 1). The soleus presented similar results except that the weight gain in young animals continued up to 298 days followed, in senile animals, by a significance loss in weight (Table 1). It is interesting to note here that out of another five muscles tested for weight loss (Table 1) only two showed a significant age-related loss, namely the tibialis anterior and posterior muscles.

Using myosin ATPase staining it was possible to distinguish in the EDL all three major fibre types described by Peter *et al.* (1972). Under alkaline pre-incubation and pre-fixation conditions. FOG fibres showed the highest ATPase activity, SO fibres stained weakly for ATPase, whilst FG fibres stained at an intermediate intensity (Fig. 1*b*). Acid pre-incubation showed a complete reversal of alkaline pre-incubation effects in all fibre types in the adult animals. Acid pre-incubation in senile animals showed erratic and weak staining characteristics. ATPase fibres compared with succinic

Table 1. *Weights of selected muscles in the developing and ageing rat*

Age (days)	Body weight	EDL	Soleus	Muscle weight ±S.D. (mg) Biceps brachii	Palmaris longus	Tibialis posterior	Tibialis anterior	Extensor hallucis Proprius	Heart
21	55·9*± 2·5	20·5*± 3·5	20·8*± 1·8	—	—	—	—	—	230·8*±41·0
84	346·8*±19·3	165·8*± 8·0	172·0*±12·4	—	—	—	—	—	1050·0*±154·1
185	511·3*±64·0	246·1 ±37·2	236·2†±36·3	—	—	—	—	—	1396·0 ±143·5
298	—	237·4 ±24·2	263·0 ±16·9	—	—	—	—	—	1484·2 ±105·5
388	—	—	—	355·0±30·1	72·8±5·0	339·2±25·5	947·8± 52·6	28·6±1·9	—
508	585·4*±38·1	253·2†±14·1	264·0†±45·1	—	—	—	—	—	1376·0†±111·3
714+	606·6 ±77·0	228·6 ±34·8	232·3 ±31·5	321·4±25·2	71·6±1·8	227·2±46·1	718·0±205·8	32·0±5·3	1537·1 ±205·4

Probability levels * < 0·001, † < 0·05 compared to following age group.

Table 2. *Diameter and fibre number in the extensor digitorum longus of the developing and ageing rat*

Age (days)	No. of muscles	Diameter (μm)±S.D. FOG	FG	SO	Number±S.D. FOG	FG	SO	Total number	% Area FOG	FG	SO
21	4	19·0*±1·0	26·9*±1·3	20·7*±0·8	3057*±647	1905*±181	112±47	5074*±762	43·4	54·7	1·9
84	5	42·8†±0·9	60·1*±1·8	35·6†±1·3	2490†±200	1194*±115	125±62	3808*±313	50·4	47·9	1·7
188	5	50·6 ±1·7	69·9 ±3·1	38·3 ±1·7	2027 ±156	644 ±159	84±22	2775 ±126	60·7	37·9	1·4
299	5	48·1 ±4·8	71·4 ±3·1	38·2 ±2·1	1988 ±181	916 ±304	70±17	2974 ±232	49·0	49·8	1·1
508	5	51·7 ±8·7	73·7 ±8·5	40·3†±3·0	2096 ±279	687 ±221	81±24	2864 ±182	59·2	39·4	1·4
716+	8	48·3 ±5·2	75·9 ±7·2	43·0 ±2·3	2215 ±234	626 ±172	69±28	2990 ±333	58·0	40·5	1·4

Probability level * < 0·001, † < 0·05 compared with following age group.

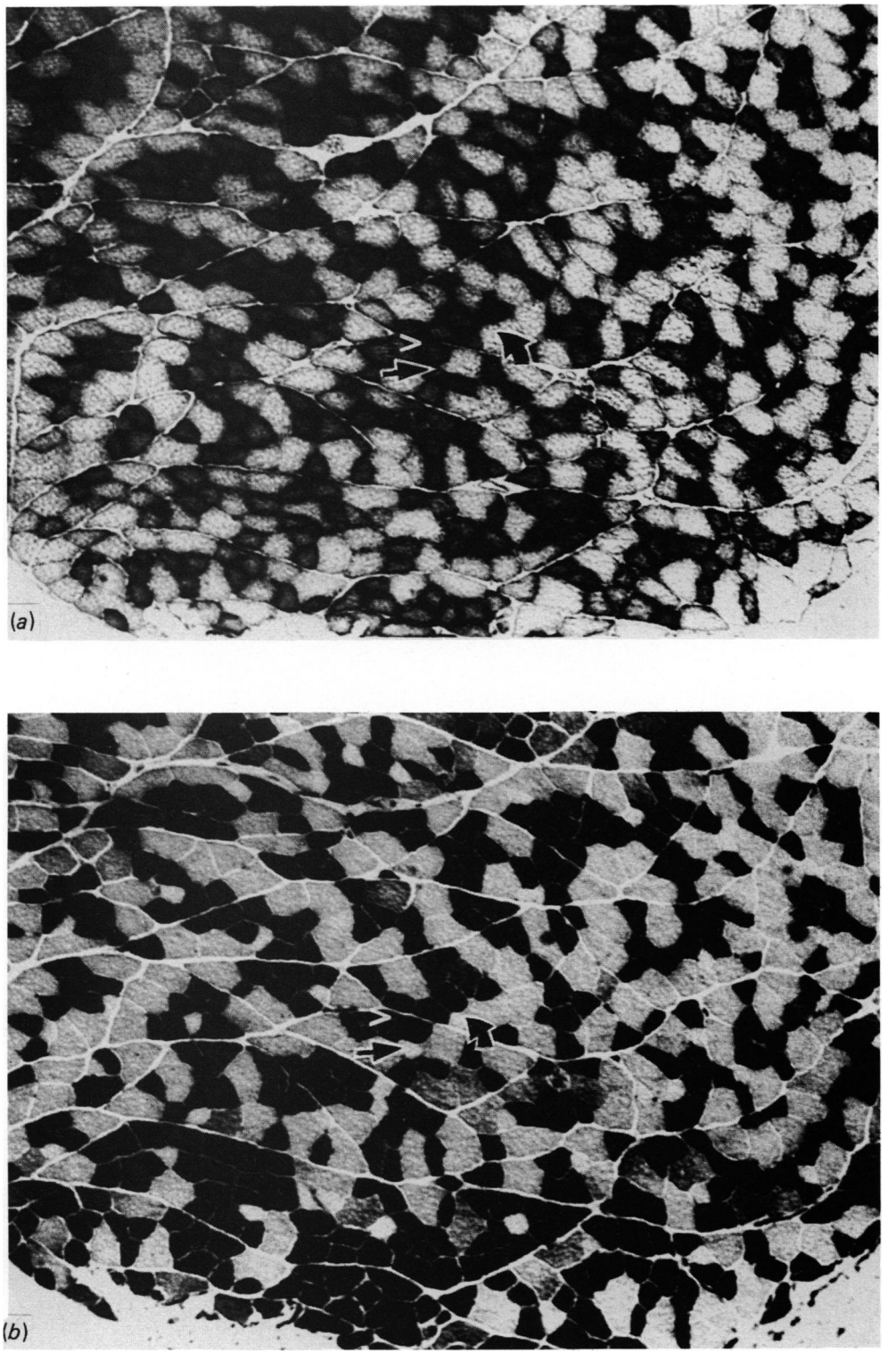

Fig. 1 (*a–b*). Staining characteristics of the extensor digitorum longus for succinic dehydrogenase (*a*) and alkaline ATPase (*b*). Three fibre types can be distinguished: slow oxidative (➙), fast glycolytic (➘) and fast oxidative glycolytic (➔). The two stains demonstrated a high correlation factor which allowed cross-referencing of fibre types. × 200.

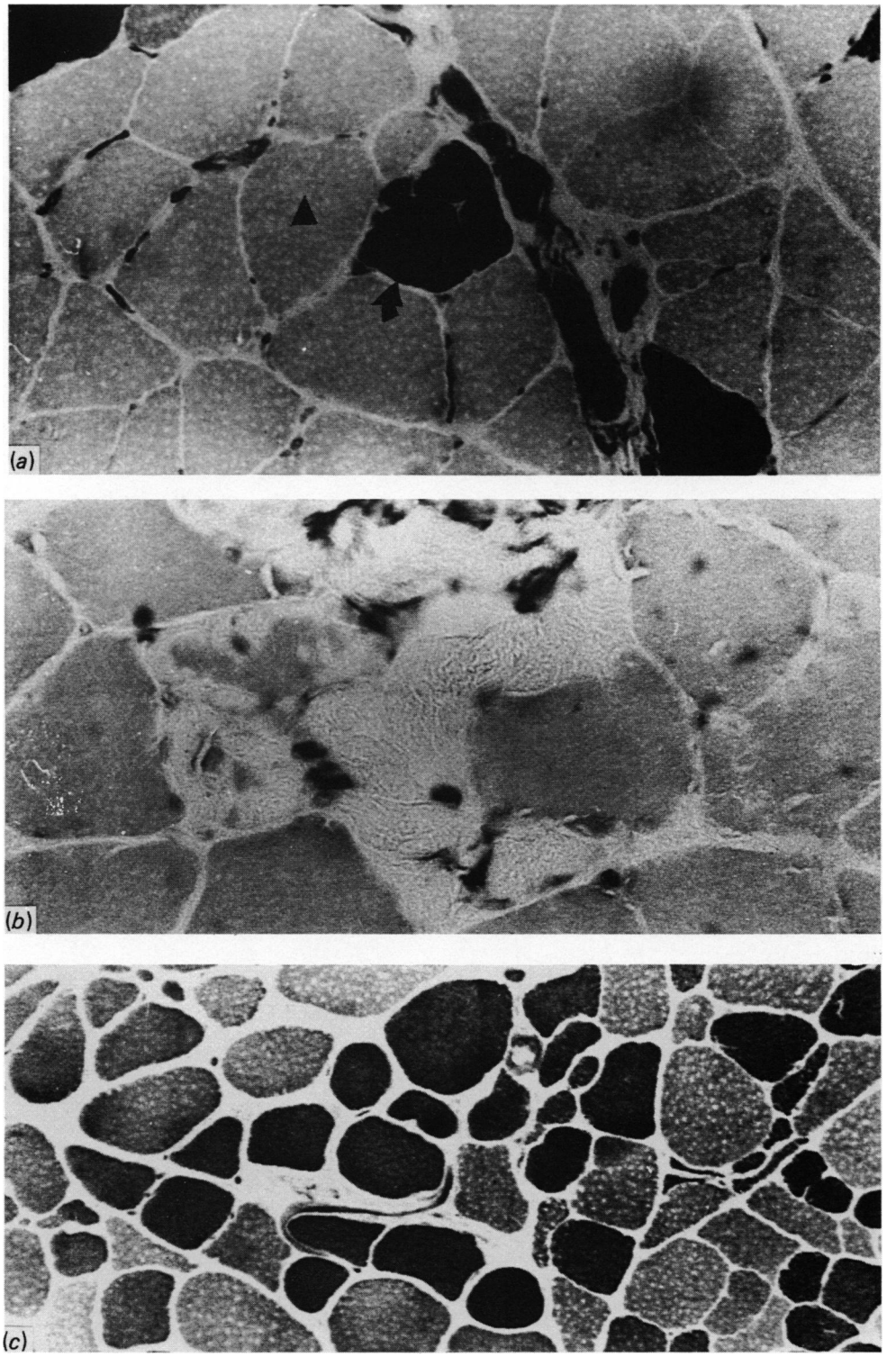

Fig. 2 (*a–c*). Senile muscles exhibit a variety of abnormal features. Longitudinal splitting occurred more frequently in senile muscles (*a*) as did fibre degeneration (*b*) and hypertrophy (*c*). The differential staining of the different fibre types continued into old age; the soleus showing both slow oxidative (▲) and fast oxidative glycolytic (⬆) fibres, and the extensor digitorum longus showing the two major fast types. (*a* and *b*) × 300; (*c*) × 135.

Table 3. *Diameter and fibre number in the soleus of the developing and ageing rat*

Age (days)	No. of muscles	Diameter (μm) ± S.D.		Number ± S.D.		Total fibre number
		FOG	SO	FOG	SO	
21	5	25·0* ± 2·3	32·2* ± 2·8	1345* ± 169	1395* ± 107	2740 ± 245
84	5	56·3* ± 2·8	65·6 ± 2·0	261 ± 67	2628 ± 97	2889 ± 97
188	5	65·4* ± 2·4	68·5* ± 3·1	166 ± 94	2578 ± 158	2753 ± 272
299	4	74·5 ± 3·9	79·8† ± 3·6	341 ± 150	2164 ± 419	2505 ± 276
508	4	74·1* ± 3·3	74·6 ± 2·5	323 ± 131	2237 ± 204	2561 ± 256
716+	5	47·6 ± 6·6	73·6 ± 4·4	299 ± 169	2226 ± 277	2525 ± 234

Probability level * < 0·001, † < 0·05 compared with following age group.

dehydrogenase-positive fibres demonstrated good correlation (Fig. 1*a*,*b*). Muscle fibres in the soleus were of two types only, SO and FOG (Fig. 2*a*). Their staining characteristics were similar to those of the EDL when stained for ATPase. SO fibres stained for succinic dehydrogenase showed a more diffuse stain than the SO fibres of the EDL. Again the reproducibility of staining of senile soleus muscles was poorer than those of younger animals.

All fibres in the EDL continued to increase in diameter from weaning up to 188 days (Table 2). FG fibres tended initially to grow faster than the two fibre types although the SO fibres in the EDL continued to grow even in senile animals. In contrast, in the soleus both fibre populations grew up to 299 days (Table 3); however after this age SO fibres remained unchanged but the FOG fibres continued to show a considerable reduction in diameter (35·8 %). It is important to note here that such large reductions in diameter may be due to fibre splitting (Fig. 2*a*). The percentage of split fibre is difficult to quantify, because this would have entailed the examination of many serial sections. Nevertheless the number of split fibres was observed to be higher in older muscles. Qualitatively, both muscles in the senile animal were alike in showing fairly extensive fibre degeneration and hypertrophy (Fig. 2*b*,*c*).

The size distribution of each individual fibre type approximated to a normal distribution. Although the FOG and SO fibres overlapped in their distribution within the soleus (Fig. 3) both had distinct modes with the SO fibre population initially possessing the highest modal diameter. However, because of the more rapid rate of growth of the FOG fibres the two peaks came to overlap in the mature animal (Fig. 3). The slow fibres were found to be less affected by the ageing process whereas the FOG population in senile animals showed a totally disrupted distribution with no really distinct single peak. The tails of both fast and slow populations in elderly and senile animals were exceedingly flat and covered a wide range of diameters (Fig. 3*f*). The tails correspond to hypertrophied fibres at one end and splitting fibres on the other end. This widening of the distribution of fibre sizes therefore reflects one of the detectable ageing changes in muscle.

In the EDL individual fibre types again showed a normal distribution during the first 188 days *post partum*. In the youngest muscles examined the three fibre types had mode diameter values in the order FOG, SO and FG, the latter being the largest (Fig. 4*a*). This changed to become SO, FOG and FG by 84 days (Fig. 4*b*,*c*) and remained so thereafter. As the animals aged the FG and particularly the FOG distributions deviated markedly from normal distributions giving rise to asymmetrical tails. The situation was most obvious in animals aged 508 and 716 days (Fig. 4*e*,*f*). Fibre diameter ranges in old and senile animals were extremely wide, this again reflecting the presence of hypertrophied and degenerating fibres.

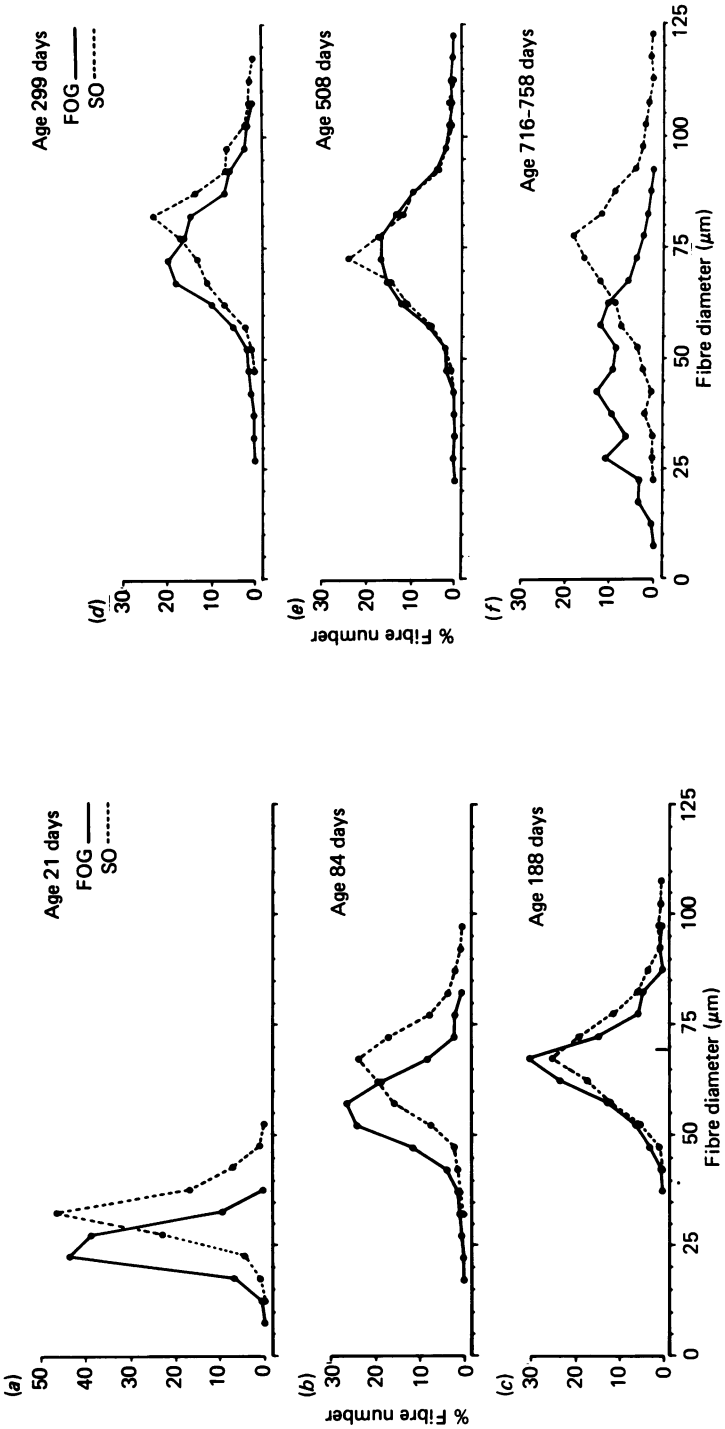

Fig. 3 (*a–f*). Frequency distribution of the fast oxidative glycolytic and slow oxidative fibre populations in the soleus of the developing and ageing rat. Frequencies are shown as a percentage of the specific fibre type.

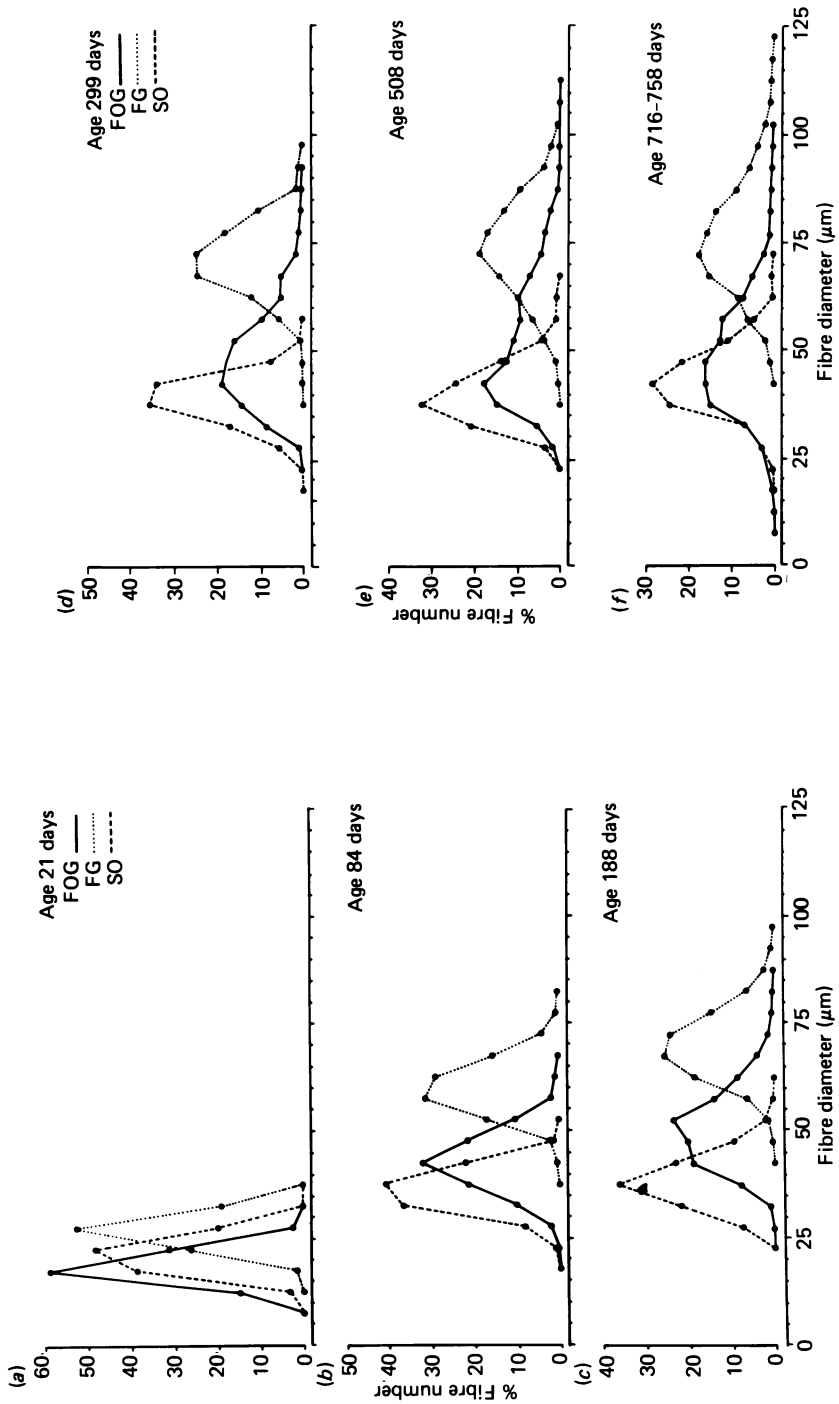

Fig. 4 (*a–f*). Frequency distribution of the fast oxidative glycolytic, fast glycolytic and slow oxidative fibre populations in the extensor digitorum longus of the developing and ageing rat. Frequencies are shown as a percentage of the specific fibre type.

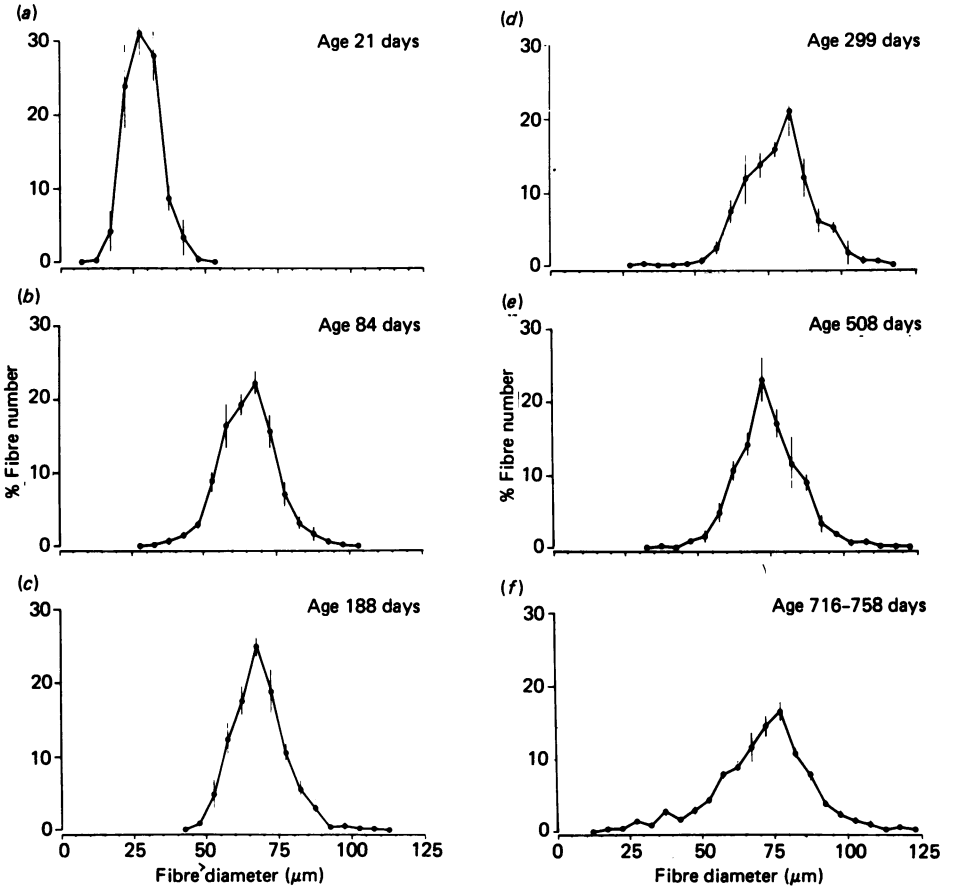

Fig. 5 (*a–f*). Composite frequency distribution of fibre diameters in the soleus of the developing and ageing rats. The distribution allows for the ratio of the two fibre populations occurring in the soleus.

Composite polygons were constructed from fibre type ratios and frequency distributions for the soleus and the EDL. These were constructed by estimating the contribution of each fibre type to the fibre diameter frequency distribution of the whole muscle; in other words, these polygons were based on the total number as well as the sizes of the different fibre types in the muscle at a particular age.

The soleus displayed the shift of peak frequency encountered earlier which was attributed mainly to the increase in the dominant SO fibres. At all ages the distribution was essentially a unimodal one (Fig. 5). The construction of the composite EDL polygons however showed that, as the differences between the mean fibre diameters increased, the normal distribution gradually transformed to a bimodal form (Fig. 6). Animals aged 299 days showed a well defined bimodal distribution with the two peaks corresponding to the modes of the FOG and FG fibre types. In old and senile animals the bimodality was progressively eroded until a skewed distribution with a peak and a plateau was left (Fig. 6). This is due mainly to the increased variability of FOG fibre diameters (Fig. 4*e*, *f*).

A transformation of fibre type was detected in the soleus muscle. The ratio of SO to FOG fibres showed an alteration from almost equal populations of both types (Table 3) to a muscle predominantly made up of SO fibres; the change was complete

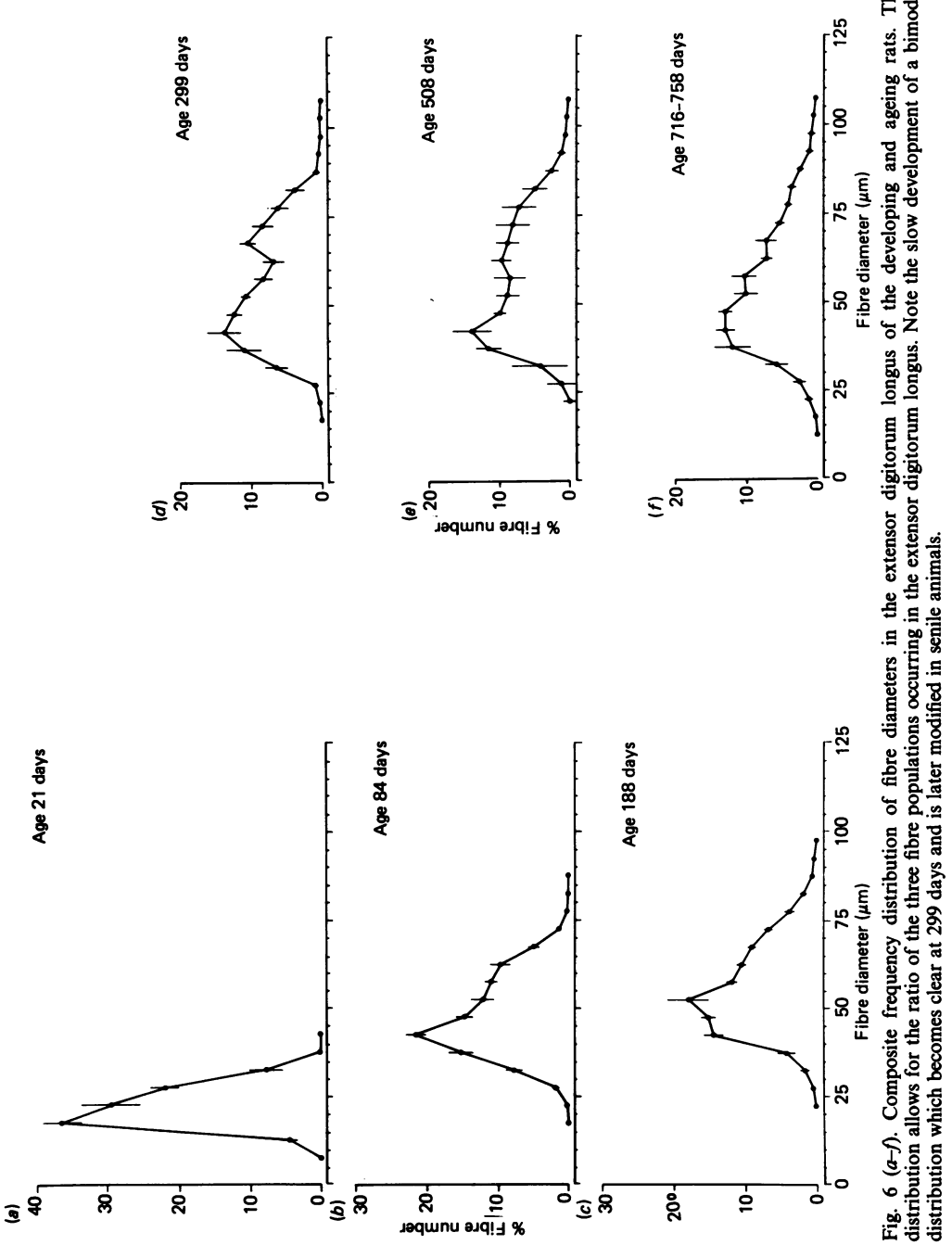

Fig. 6 (*a–f*). Composite frequency distribution of fibre diameters in the extensor digitorum longus of the developing and ageing rats. The distribution allows for the ratio of the three fibre populations occurring in the extensor digitorum longus. Note the slow development of a bimodal distribution which becomes clear at 299 days and is later modified in senile animals.

by 84 days *post partum*. Statistical analysis failed to detect any significant changes in the total fibre number in the soleus with increasing age (Table 3).

In contrast, the total fibre number in the EDL decreased dramatically between the ages of 21 and 188 days (Table 2). The decrease was due mainly to the loss of FG and FOG fibres which make up the largest percentage of all fibres, and to a lesser extent the loss of SO fibres.

DISCUSSION

CFY rats have been shown to increase in weight with age, reaching a maximum value at about 700 days after which they start wasting rapidly (Merry & Holehan, 1981). It has been shown here that this is associated with decreases in weight of some but not all muscles (Table 1). Skeletal muscles have been reported to waste at different rates during ageing (Rubinstein, 1960; Gutmann & Hanzlikova, 1976), the rate probably being modified by their activity (Muravov, 1969) which in older animals is rather limited. The bulkier muscles in this study wasted more markedly in extreme age, the tibialis anterior losing on average 24·2% of its normal adult weight.

A change in muscle weight and cross sectional area may be the result of a change in the number of muscle fibres within the muscle, a change in individual fibre diameter or a combination of both these factors. It was shown here that the increase in muscle cross sectional area during growth appears to result mainly from massive increases in the diameters of individual fibres. The soleus, with no change in fibre number, increased in weight and cross sectional area and the EDL, despite a significant loss of fibres, increased both in weight and cross sectional area. The loss of muscle fibres in early life has been reported by several workers (Ihemelandu, 1980; Layman, Hegarty & Swan, 1980). The reason for this loss is not known but it may be due to a continuation of a reduction in the number of neurons which is part of the development process.

The loss of muscle mass during ageing also seems mainly to be the result of a decrease in fibre number combined with a decrease in fibre size. In the soleus the mean fibre diameter of FOG fibres decreased by as much as 35·8% although some of this decrease appears to be due to splitting. SO fibres on the other hand decreased in size only marginally over the same period. More pronounced changes in SO mean fibre diameter were probably masked by the presence of some fibres which had undergone compensatory hypertrophy. The existence of such fibres could be seen in the frequency distribution of the SO population in senile animals compared with younger ones (Fig. 3). There, in a given population of fibres, the net effects of atrophy, splitting and hypertrophy determine the degree of loss of cross sectional area and the wide fibre size distribution which are characteristic of ageing.

Another aspect of ageing seems to be a shift towards more oxidative metabolism. In the EDL the most affected fibres were the FG fibres (Table 2) which lost as much as 22·7% of their cross sectional area between the adult and senile stages. FOG fibres increased in cross sectional area by 12·4% over the same period while SO fibres increased by 24·3%. However, the only real contribution was that of FOG fibres, since SO fibres occupied a very small percentage of the whole muscle.

The soleus, which acquired a high oxidative capacity by the time the animal was weaned, showed a continuous increase in SO fibres reaching a maximum of 95% of the total population well before adulthood. In view of the unchanged total fibre number with age, one is led to suspect a transformation process of FOG fibres into SO fibres. Caccia, Harris & Johnson (1979) reported the presence of transitional fibre types in the soleus; this fibre population is presumed to have characteristics intermediate between FOG and SO fibres. This observation is in line with the

demonstrated ability of muscles to adjust to the type of activity by selectively increasing one or more fibre types (Lieberman *et al.* 1972; Watt, Goldspink & Ward, 1984). Similar transformations were attainable in cross-innervated muscles (Dubowitz, 1967; Brooke, Williamson & Kaiser, 1971).

The construction of composite histograms for the EDL based on the three fibre populations revealed the existence of a bimodal distribution. The second peak which occurs during growth is generated by the very fast growing and large FG fibres. The occurrence of bimodality has been reported in several muscles (Rowe & Goldspink, 1969) most of which are mixed. This bimodality has been the subject of a controversy. Hegarty & Hooper (1971) claimed that this was a fixation and rigor mortis artifact arising from the rate at which a fixative penetrated the muscle and the postmortem state of that muscle. Goldspink *et al.* (1973) demonstrated that neither rigor mortis nor fixation produced differential size distributions. They showed that pre- and post-rigor mortis fibre diameters of fibres located near the outer surface and near the centre of the muscle showed similar percentage shrinkage, regardless of their position within the muscle. The present work has shown that bimodality is not an artifact and that it actually arises from the different growth rates of the fibre types and the differences in their diameters. The soleus which consists mainly of one fibre type did not exhibit any bimodality. If either rigor mortis or fixative penetration were responsible for bimodality, both the homogeneous soleus and the heterogeneous EDL would have shown similar bimodalities within their fibre distributions.

The reason for the preferential atrophy of the non-oxidative type muscle fibres during ageing is not understood. It is not known for instance whether this is due to decreased activity and hence fibre recruitment or an ageing change *per se*.

SUMMARY

Morphometrical and histochemical features of muscle undergo continuous changes with age. Two representative muscles were studied to determine the nature of these changes, the extensor digitorum longus and the soleus. Muscle fibre type ratios were found to change with age so that there were more oxidative types. In the extensor digitorum longus the cross sectional area occupied by fast oxidative glycolytic fibres increased, while in the soleus fast oxidative glycolytic fibres apparently underwent conversion into slow oxidative fibres. Muscle fibre diameter increased dramatically during early growth but later in senile animals there was evidence of both atrophy and splitting. In the extensor digitorum longus the uneven growth of the two dominant fibre types gave rise to a bimodal fibre diameter distribution. The soleus, which is composed of predominantly one fibre type, did not show bimodality. Senile muscles had a characteristic wide distribution of fibre diameters with ill defined peaks. Total fibre number in the extensor digitorum longus decreased in early life while total fibre number in the soleus remained unchanged.

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