THE UNIVERSITY OF HULL

AGE RELATED

CHANGES IN SKELETAL MUSCLE

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Majed A. Alnaqeeb, B.Sc. (Kuwait)

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CHAPTER I

GENERAL INTRODUCTION

Theories of Ageing

Ageing is a continuous process. Once initiated it will continue at different rates amongst various living structures. It is, in fact, an inherent characteristic of life, just as is death.

'If we were to view "natural death" as the termination of existence due to the uniform senescent atrophy of the organism, leading to a uniform depression and ultimately simultaneous extinction of all it's functions, then "natural death" in all probability never occurs', Robertson (1923).

Theories on ageing have been put forward since the mid-nineteenth century. A good number of these theories were based on nothing more than general observations, with little or no experimentation.

Comfort (1979) divided the earlier theories of ageing into fundamentalist theories, and epiphenomenalist theories. The first group treated senescence as an inherent property of living matter, while the later theories related it to a particular physiological system or condition. To the fundamentalists belongs the theories of "wear and tear". Weisman (1882), Magnus-Levy (1907), Pearl (1928) and Warthin (1929), suggested that senescence is the manifestation of general wear and tear of the body. Mechanochemical deterioration of cell colloids was held responsible for

ageing changes by Bergauer (1924), Lepeschkin (1931), Szabo (1931 a,b) and Dhar (1932).

Pathological and histochemical changes involving changes in a multitude of systems and tissues were considered; Demange (1886) and Savill (1896) sited the vascular system as the major factor, while the nervous tissue was suggested as responsible for senescence by several workers, (Muhlmann 1900, 1927, Vogt and Vogt 1946, Bab 1948) as was the endocrine system (Lorand 1904, Findley 1949, Steinach 1920, Vornoff 1920, 1929).

Epiphenomenalist theories exhibit a wide variety of interesting causes behind ageing; intestinal flora and slow poisoning by bacteria toxins (Metchnikoff 1904, 1907, Lorand 1929, Metalnikov 1937). Accumulation of metabolites due to incomplete metabolism and the progressive increase of inactive breakdown products (Eisig 1887, Molisch 1938 and Lansing 1942), and the accumulation of toxic waste (Montgomery 1906), heavy water (Hakh and Westling 1934, Griffiths 1973) and even cosmic rays were also theorized as senescence inducing factors.

Calorimetric experiments by Rubner (1909) gave to a concept relating the rate of living to the length of life. Loeb (1908), Pearl (1928) and Robertson (1923), held similar views. Hansemann (1893) and Orton (1928), identified reproduction as the major contributor to senescence and death in what is known as the depletive theory.

Theories deriving from the continuous morphogenesis and cytomorphosis as bases of senescence were proposed by several investigators (Roux 1881 , Delage 1903, Warthin 1929 and Jennings 1913). Minot (1908) adopted a most unusual concept of ageing 'In young cells the amount of cytoplasm in relation to the amount of nuclear substance is least, but during development it increases and undergoes differentiation, thus senescence'. This holds true if ageing is considered in the light of a continuous increase of the organisms susceptibility to change and its inability to renew,

increase in number or volume, with age. Needham (1949), assigned to multicellularity accompanying differentiation the cause of ageing changes. Roux (1881) and Lewes (1887) believed that competition amongst the multicellular organisms was the pitfall. Muhlmann (1900) advanced the relationship between volume and surface area, and the possible starvation of cells farthest away from the surface as a possible factor.

Weismann (1882, 1884, 1892) cited natural selection as the limiting factor in multicellular organisms. Bernstein (1898) indicated the probable existence of internal conditions that limit growth increasingly and progressively until death. Conditions such as the gradual change of organic molecules that will consequently affect the metabolic activity (Buhler 1904).

Autocatalysis with accumulation of end products that reduce the velocity of growth and enhance senescence with time was suggested by Loeb (1908) and supported by Robertson (1908a, 1913, Blackman 1909). Child (1911, 1914) showed that senescence is due to a decrease in metabolic rate and substrate metabolism relationship, a view shared with Conklin (1912). McCay (1952) showed a definite positive relation between diet restriction and longevity.

Cessation of somatic growth was suggested by various theories as the cause of senescence (Minot 1908, Carrel and Embeling 1921, Brody 1924 and Lansing 1948, 1951). 'Senescence was considered a correlate to the evolution of determinate growth and a final absolute size', Bidder (1925).

Newer theories of ageing based on more reliable data were by now being put forward. Enzyme performance and deterioration was suggested by Meyer et al (1940), Kirk (1959), Mandel (1961), Bertolini (1962) and Ghirighelli and Gerzeli (1963) as being a possible factor. Protein coagulation (Bancroft et al 1934a, b, 1935) and thermal denaturation by a sudden upsurge of localized heat (Sinex 1957, 1960) of large molecules was proposed as a factor in the ageing process. Selye (1959, 1962) indicated

that calcium deposition is a probable cause.

Medvedev (1961) supported later by Orgel (1963) suggested a theory based on the accumulation of erroneous nucleic acids, the transcription and translation of which results in erroneous proteins that serve no value. They may even contribute to the manufacture of catastrophic enzymes that can destroy the organism.

A mutation theory of ageing was put forward by Danielli (1956) and Failla (1957, 1958). It was suggested that mutation in somatic cells occur with time and these affect the overall condition of the organism. Mutations that hit DNA strands will presumably induce ageing, mainly because of the low repairability, particularly if the site of the mutation is in a control mechanism (Sinex 1974). Lamb and Maynard-Smith (1964) and later Harris (1971) demonstrated that mutations can be induced by ionizing radiation which will further mimic ageing. Genetic material can be altered by way of crosslinks between the two strands of the DNA helix (von Hahn and Verzar 1963). The inability of the strands to split causes the loss of functional genetic material. Partial repair of such damage is possible only if one strand is affected (Howard-Flanders and Boyce 1966). Cross linkage of non-genetic material produces large insoluble molecules which continue to increase with age, (Strehler et al 1959 , Bjorkerud 1964), Bjorksten et al 1962). Cross linking, may therefore, contribute towards ageing by hindering the normal processes and increasing useless proteins. The production of large mutant, "alien" molecules can trigger autoimmunity, a theory suggested by Walford (1962, 1969). Autoimmunity increases with age (Hallgren et al 1973) which might suggest a relationship between self-destruction and the manifestation of ageing in the form of damaged tissue. The decrease in antibody activity and immune function (Makinodan and Peterson, 1964) may explain the increase in related diseases and viral infections. Continuous exposure of the immune system to viral infections and the production of antiviral antibodies may pave the way to autoimmunity

(Alder 1974). Autoimmunity combined with the viral infection and the general decline of other related responses contribute towards the ageing process.

Direct (Lack 1965) and indirect (Williams 1957) selection pressures on a species act to reduce life span and induce senescence in favour of a more beneficial condition. This theory of programmed senescence was an attempt to establish why organisms age (Wilson 1974). This was first suggested by Weismann (1891) however, it has received support from Medawar (1957), Williams (1957), Emerson (1960), Smith (1962), Wynne-Edwards (1962) and Guthrie (1969). Even if a single gene evolved to control senescence in some species, it is likely that the other, indirect selective pressure will have led to other deletrious gene effects being present at the time of programmed senescence (Wilson 1974). This fact allows more than one system to influence age changes. Free radicals are such a factor. Free radicals are highly reactive groups, with a high oxidative ability (Pryer 1973), and they occur spontaneously (Commoner et al 1957, Harman and Piette 1966, Ingram 1958). Their high reactivity induce randomness and malfunction that accumulate throughout life in both nuclear (Brooks et al 1973, Medvedev 1964) and cytoplasmic (Packer et al 1967) structures. Hayflick (1974) and Sinex (1974) suggest that cytoplasmic changes are the major sites affecting ageing.

Gelfant and Smith (1972) introduced a model based on the existence of cells in one of two states; either cycling, i.e. dividing, or blocked state, i.e. non-dividing. Either state is triggered by conditions within the organism. Ageing in such a model is represented by the progressive shift towards the blocked state. A fact supported by the inability of ageing non-cycling cells to proliferate and become cycling again (Gelfant and Grove 1974, Aldelman et al 1972). This ties in nicely with the already demonstrated limited life span of dividing cells (Heilbrunn 1943, Hayflick 1965, Hayflick and Moorhead 1961).

In general Shock (1974) sees ageing as the sum effect of gradual deterioration of physiologic control mechanisms and increased disharmony of the various systems. The more complex a control system the higher the deterioration rate with age (Birren 1965, Welford 1965). The inability of the elderly to adjust to changes in the environment further supports the concept of disharmony (Krag and Kountz 1950, 1952, Rothbaum et al 1974).

It is evident that no single theory as yet can explain all facets of the ageing process. The structural manifestations of ageing are probably the final changes in a long sequence of molecular events. These events nevertheless are likely to be similar in the different organised structures of higher mammals. Data obtained for muscle tissue is therefore, hopefully, representative of the body as a whole.

Ageing in Muscle

Studies as early as 1927 (Ruger and Steossiger) showed that skeletal muscles deteriorate functionally with old age. A fact confirmed later by several workers (Ufland 1933, Norris et al 1953, Cathcart et al 1935, Burke et al 1953, Fisher and Birren 1947). Reduction in muscular strength in elderly persons was further confirmed by Critchley (1956), who associated it with muscular wasting. By comparing aged muscles with young muscles it is apparent that they may waste by as much as 400% (Korenchievsky 1961, Yiengst et al 1959, Andrew et al 1959). Furthermore, muscles being postmitotic tissue cannot divide or regenerate to any appreciable extent (Cowdry 1952).

To understand better the changes that accompany ageing in muscle and how they lead to reduced muscular vigor we have to study the basic structure and biochemistry of ageing muscle.

Neuromuscular changes associated with ageing

Senescence is "a process of unfavourable progressive change" (Lansing 1951). The rate at which age changes progress within the body or even

within one system is highly variable. The neuromuscular system is no exception to this.

Muscles with different functions are known to age at different rates (Rubinstein 1960, Gutmann and Hanzlikova 1976). This is probably a reflection of their activity (Muravov 1969) and intrinsic characteristics, (Bass et al 1975, Fujisawa 1975) for example, whether they are fast contracting or slow contracting. Physiologically skeletal muscles appear to have a mixture of slow and fast motor units (Close 1967). The ratio of the two types of units determine whether the whole muscle is slow or fact contracting.

In an attempt to understand the sum effect of deteriorative changes that lead to senile atrophy, a closer examination of the underlying trends is necessary.

i. Physiological changes

It has long been established that muscles in senile animals are less able to develop force as compared to muscles in younger animals (Quetelet 1836, Ufland 1933).

Investigations into the decline of muscle strength and coordination have revealed the involvement of the nervous and vascular systems. Oxygen diffusion through the plasma was reported to decline with age due to the increase in protein and cholesterol concentration (Chisholm et al 1971). This could induce hypoxia and be partly responsible for a change in the enzyme profile of some muscles with age.

The increase in oxygen uptake with age was demonstrated in resting and active thoracic flight muscle of some insects (Tribe 1966, 1967) this could be evidence for the uncoupling of oxidation phosphorylation with age (Tribe 1967). This also applies to human beings as Norris et al (1957) reported a reduced efficiency with which the elderly people "burn" oxygen at low work loads. This to some extent conflicts with the findings that elderly people have reasonably good powers of endurance providing the

exercise is of low intensity. When subjected to 40% maximum isometric tension Larsson and Karlsson (1978) found that endurance was actually higher in older people when expressed on a relative basis. However, it must be borne in mind that the maximum isometric tension was much reduced in the older subjects (Larsson and Karlsson 1978) and, therefore, in absolute terms the younger subjects would probably fatigue less rapidly if exercised at similar levels. It has been noted that the successful competitors in very long distance races (50 to 100 miles) are often older men, aged 40 to 60 years.

The decline of muscular strength in both fast and slow muscles have been equally well documented (Gutmann and Hanzlikova 1976). Slow and fast muscles are uniformly slow at birth (Banu 1922, Denny-Brown 1929 , Buller et al 1960a and 1960b, Close 1964, Buller and lewis 1965, Mann and Salafsky 1970, Kelly and Rubenstein 1980). The later development of the muscles has been shown to be influenced by neural activity (Buller et al 1960a,b, Gutmann and Syrovy 1967) of the motorneurons and possibly by a trophic influence (Barnard et al 1970).

As far as the contractile properties are concerned, the general response of the senile muscle is that of prolonged contraction and prolonged relaxation. The extensor digitorum longus, composed chiefly of fast motor units, shows an increased contraction time, relaxation time and latent period. The levator ani and the diaphragm, both very specialised muscles show similar trends; although the latter muscle is continuously used throughout the animal's lifetime (Gutmann and Melichna 1972). The change in the contractile properties of the slow soleus muscle appear to be controversial, Syrovy and Gutmann (1970) reported an unchanged contraction time of old muscles when compared with young ones. This fact was further confirmed in a later report (Vyskocil and Gutmann 1972). In a later study Gutmann and Syrovy (1974) showed a decrease of contraction time for the aged soleus muscle. However, the results for the latent

Period and relaxation times were consistant in that they showed a definite prolongation with age (Gutmann and Syrovy 1974).

Studies on motor units within the soleus indicate a progressive shift towards slow motor units with age (Kugelberg 1976), which raises the question of the involvement of more than one factor in determining the overall performance of the muscle.

The number of motor units in a senile muscle is reputed to fall with age (Campbell et al 1973). This means that the surviving units will need to bear the extra work which leads to further complications such as hypertrophy of the remaining units. If the loss of the motor units is not compensated by hypertrophy of the remaining units, then this will result in a reduced force output.

Nerve conduction velocities are also apparently impaired in the elderly (Campbell et al 1973). This is possibly due to the loss or diminution of motor neurons with low threshold and rapid conduction (Peterson and Kugelberg 1949). Minature end-plate potentials (m.e.p.p's) have also been shown to change with age. They increase in early life (Kelly 1978) and then decrease to a comparatively low level with extreme age (Kelly 1978, Vyskocil and Gutmann 1972, Gutmann et al 1971). This is an indication of the reduced resting activity of the muscle end-plate, which may be a reflection of either a decrease in the number of transmitter release sites in the nerve terminal membrane or change in the interaction of acetylcholine with the receptors. Unlike Kelly (1978), Schwarz et al (1966) reported an increase in muscle membrane potential at mid age and a decrease in later life in albino rats.

Deteriorative changes are not restricted to skeletal muscles. Cardiac muscles show a progressive decrease in functional activity with age (Bradfonbrener et al 1955, Harris 1975). Heart rate tends to increase with age (Goldberg 1978). Spontaneous heart rates as measured in vitro showed a more significant decrease than in vivo rate (Goldberg 1978),

hence the myogenic contractility seems to be more affected than the pacemaker and the adrenergic control of the heart.

ii Histochemical changes

The heterogeneity of skeletal muscles is well documented. Since the early stages of this study it was clear that no one single criteria was sufficient to allow accurate identification of the various fibre types. Several classifications of muscle fibre types are in current use. Two of the more popular are those of Brooke and Kaiser (1974), and Peter et al (1972). Basically, both classifications distinguish between the slow and fast contracting fibres; in addition to that, they divide fast contracting fibres into two main categories based on their oxidative capacity. Brooke's fibre type division are; type I, type IIB and type IIA. In the same order, and according to the second classification, they would be: slow twitch oxidative, fast twitch glycolytic and fast twitch oxidative glycolytic; some people also distinguish type IIC fibre, but this is believed to be an embryonic fibre type. Cluster analysis of muscle fibre types and different enzyme staining reactions show a high degree of correlation (Spurway 1978, 1978). By using microanalytical techniques on single muscle fibres, Lowry et al (1978) also concluded that each fibre type had a characteristic range of enzyme activity.

There is good evidence that these fibre types have different physiological functions. Motor units are made up of one type of fibre only (Kugelberg and Edstrom 1968). However, more than one type of motor unit may be found in the same muscle (Close 1967). Kugelberg (1976) correlated the rate of contraction of a motor unit to the type of fibres it contains as demonstrated histochemically. Type II fibres in the soleus were shown to have a contraction time (twitch time to peak) of 15-26 ms, whilst type I fibres were shown to be slow with a contraction time of 27-40 ms . Burke et al (1973) demonstrated the relation between fibre types and their fatigue resistance; fast contracting, fatigue sensitive fibres

were found to possess low succinic dehydrogenase whilst fatigue resisting fibres were of a high succinic dehydrogenase activity.

The same authors (1973) showed that the fibres of a particular motor unit are usually interspersed with the fibres of other motor units, and they are not necessarily concentrated in the same region of the muscle.

The relation between histochemical fibre types and contraction times of muscles was further confirmed with cross-innervation studies. Muscles with slow contracting rates were shown to alter both their histochemical profiles and contraction rates when innervated with a nerve originating from a fast muscle and vice versa (Dubowitz 1967).

Now that histochemical fibre typing has been placed on firmer grounds, it is possible to look at histochemical changes in ageing muscle and relate them to physiological changes. It is now appreciated that histochemical profiles of muscles continue to change with age and that the general activity of the muscle alters the rate at which changes occur. In some muscles the alteration in the fibre type profile starts a few weeks after birth (Maxwell et al 1973, Alnaqeeb and Goldspink 1980), although in most muscles studied the rate of change is slow.

The diaphragm in the guinea pig exhibits a decrease in red fibre number with age (Lieberman et al 1972). A similar shift is evident in the ratio of slow oxidative fibres to fast oxidative in the soleus; the slow type showing a continuous increase (Maxwell et al 1973). The rat soleus behaves not unlike that of the guinea pig in that there is a continuous transition between type II and type I fibres. This change is consistant with the increased contraction time of the ageing muscle (Kugelberg 1976). Caccia et al (1979) reported an intermediate fibre type with a lower ATPase activity. This they claim represented fibres which were undergoing transition from type II to type I.

The extensor digitorum longus, although showing a reduced ATPase activity per fibre, does not show a marked shift in fibre type as is the

case in the soleus (Bass et al 1975).

The limited work on human subjects shows similar trends. Type II fibres in the vastus lateralis were found to decrease, although the ratio of sub-types of type II fibres remained unchanged (Larsson et al 1978). The quadriceps exhibited similar changes (Larsson and Karlsson 1978).

The general impression one gets when studying the histochemistry of ageing muscle, is that of a decreased number of fast type fibres and an increase in the number of fibres with oxidative capacity.

iii. Morphological changes

(a) Morphometric changes: Morphometric changes within the muscle correspond well with the reduction in weight. A reduction in weight can be explained by either a decrease in fibre number or a decrease in fibre cross-sectional area.

Moore et al (1971) using several human muscles, demonstrated a reduction in fibre diameter in most muscles in subjects older than 40 years. The superior rectus however appears to be resistant to fibre diameter changes. The dog pectineus loses fibres with age (Ihemelandu 1980). The exact relation between fibre number and fibre diameter is not clear. Some muscles in spite of losing fibres, tend to have an increased fibre diameter. There are several examples of these sorts of muscle in which fibre loss is accompanied by compensatory hypertrophy, e.g. soleus, extensor digitorum longus and biceps brachii (Rowe 1969).

In some muscles there appears to be a selective loss of specific fibre types with age (Tauchi et al 1971, Larsson et al 1978). The decrease in cross sectional area is of a similar nature (Larsson and Karlsson 1978) type II fibres being the most susceptable to the ageing process.

Satellite cells which are thought to play a role in muscular hypertrophy (Schiaffino et al 1976) show a significant decrease in number from a healthy 4.6% in young animals to 2.4% in senile ones (Snow 1979,

Allbrook et al 1971).

(b) Ultrastructural changes: At the muscle fibre level considerable disorganisation is evident. The regular cross striations of the healthy muscle are sometimes replaced with longitudinal ones at irregular intervals. This reflects the disarray of the sacromeres. The myofibrils tend to exhibit a patchy appearance and irregularities. Endomysial fibrosis manifests itself in senile muscle, usually to a moderate extent (Fujisawa 1974). Although a qualitative study has yet to be carried out the thickness of degenerating fibres shows inconsistancy along their longitudinal axis and this is presumably a consequence of the factors mentioned above.

Ultrastructurally, myofibrils start changing well before the distruptions are observable at light microscope level (Fujisawa 1975). Gutmann et al (1971) reported a reduction in myofibril size with age.

Changes in the membrane systems have also been described including a thickening of the sarcolemma which appears to be associated with an increase in collagenous material (Gutmann et al 1971). Gutmann et al also reported general disorganisation and disintegration of the myofilaments accompanied by a proliferation of the sarcoplasmic reticulum. The myofibrillar degeneration appears to be limited to fibres rich in subsarcolemmal mitochondria. Fibres with no subsarcolemmal mitochondria exhibited streaming of Z-bands (Fujisawa 1975). Interestingly the T-system in these fibres showed increased proliferation with age (Gutmann et al 1971) and perhaps this is a compensatory mechanism to ensure the spread of the excitation potential.

In addition to the general disintegration reported earlier at the myofilament level, Steenis and Kroes (1971) reported aggregation of muscle nuclei associated with a swelling of the cytoplasm which was more granular and more hyaline in nature. Lipofuchsin "the age pigment" showed an increase with age, and was exclusively located in the subsarcolemmal region (Orlander et al 1978). Orlander reported a reduced mitochondrial fraction

with age.

The diminution in the number of large nerve fibres and shift towards smaller diameter axons was demonstrated as early as 1940 (Cottrel, Semenowa-Tjan-Schanskaja 1941). Also the axons of ageing nerves are more prone to demylenation and vacuolation. This is probably proceeded by swelling and dilation of myelin sheaths and the invasion of macrophages (Steenis and Kroes 1971).

The increase in the rate at which lesions of the nervous system occur is directly related to age (Berg et al 1962). The dedifferentiation of end-plates of both fast and slow muscle appears to be consistent with old age, such dedifferentiation is accompanied with erratic cholinesterase activity and some actual degeneration of end plates (Gutmann and Hanzlikova 1965).

Ultrastructurally, Fujisawa (1976) observed an increased number of vesicles that have a different identity to the synaptic vesicles. Neurofilaments and mesaxons with degenerative changes and swollen terminal axons were found to have lost their rounded shape.

Gutmann et al (1971) reported an increase in synaptic vesicles with an increased number of junctional folds. Synaptic clefts were larger than those in young animals but the basement membrane was thicker. Neurotubules and neurofilaments were found to occur in peripheral axons. These have not been observed in younger animals. Collagen fibrils were more abundant in older animals.

Structural changes in both the muscle fibre and the neuromuscular junction correspond well with the reduced function of the locomotion system. Some of these changes give the impression that repair processes are taking place; however, it is apparent that these processes are not completely successful.

iv. Collagen in muscle

The replacement of deteriorating ageing muscle tissue with connective

tissue was reported by Bick (1961). Several muscle diseases in which wasting occur are known to undergo similar changes (Lowry et al 1942 Dreyfus et al 1954, Weinstock et al 1958, Dam et al 1952). Various muscles apparently accumulate collagen at different rates throughout life. Schaub (1963) reported an increase of collagen in the hind leg and the abdominal musculature with age. Cardiac muscle show a similar trend (Schaub 1964b, Knorring 1970). Indeed, according to Mohan and Radha (1980) cardiac muscle shows the greatest increase of collagen with age, followed by the fast extensor digitorum longus and the slow soleus the least. Whether this has functional implications is hard to know, especially when bearing in mind the results published by Saski et al (1976) who reported no change in total collagen, but a shift towards an increased residual and TCA soluble fractions.

Cross-linking of ageing collagen molecules (Verzar 1956) is thought to reduce its breakdown rate, thus leading to the building up of residual collagen. "Old" collagen has been proved to be more resistant to degrading enzymes (Mohan and Radha 1980). In addition the activity of such enzymes is reduced with age in all types of muscles. The hydrolysis time of collagen in vitro increases with age of the molecule (Harrison and Archer 1978).

The amount and nature of collagenous material in skeletal muscle is important as it is probably the main factor in determining the stiffness or passive tension of the muscle.

v. Biochemical changes

The inability of the senile muscle to keep pace with the demands that it is subjected to, appear to result from reduced enzyme activity. RNA concentrations show a rapid decrease during the first few weeks after birth in the mouse and thereafter continue to decrease but at a slower rate. This is reflected on the microsomal activity (Srivastava 1969) and the reduction in amino acid incorporation with age (Narayanan and Eapen 1975).

It is, therefore, not surprising that enzyme levels decrease as these proteins are turned over rapidly.

The interprtation of biochemical changes particularly regarding muscle enzyme levels is often rather difficult. Muscle mitochondria of old animals are known to retain their activity, and to continue producing adenosine triphosphates at high rates if measured under optimum conditions (Gold et al 1966 , Kment et al 1966, Weinbach 1960). Froklis et al (1968) and Forklis (1969) noted that heart muscle tends to shift to a more anaerobic metabolism. Bass et al (1975) reported a decrease in the glycolytic activity of the fast extensor digitorum longus, in the form of reduced triosephosphate dehydrogenase (TPDH), lactase dehydrogenase (LDH) and C-glycerol-3-phosphate dehydrogenase (GPDH) activity with age. The slow soleus showed a decrease in TPDH and a decrease in the aerobic enzymes; malate dehydrogenase (MDH) and citrate synthase (CS). On the other hand Ermini (1976) reported a reduced oxygen consumption associated with a reduced aerobic activity. The reduced aerobic activity reduces adenosine triphosphate production by 25-35% (Ermini 1976). Both white and red muscles show a decreased creatine phosphate production and an unchanged creatine phosphokinase activity (Ermini 1976).

Hence it is apparent that enzyme activity changes with age, this appears to be a selective process as not all systems are affected to the same extent. The enzyme which is central to the force production of muscle is myosin ATPase. It has been shown that there is a good correlation between the specific activity of this enzyme and the speed of contraction (Barany 1967). During ageing it would be expected that parallel changes in these two parameters would occur. This seems to be borne out by the work of Syrovy and Gutmann (1970). However, Ermini (1976) reported that neither Ca^{++} nor Mg^{++} activated myofibrillar ATPase was affected by age. This is in contrast with the work of Rockstein and Brandt (1962) and later Gutmann et al (1974) who reported various changes. Gutmann

and Syrovy (1974) reported an increase in Ca^{++} activated myosin ATPase in the soleus with age, although in the extensor digitorum longus the same enzyme decreased in activity over the same period. The situation is therefore rather confusing and the results seem to depend on the particular laboratory in which the work was carried out, the technique and the particular muscle used.

Acetyl choline esterase activity was found to decrease in the older animals when compared to the young ones (Gutmann et al 1968). This decrease was usually associated with the appearance of lesions.

The conflicting evidence concerning some enzymes activities is probably due to the complex makeup of mixed muscles and dependance of the enzyme activities on the activity of the animal. This indicates that ageing in muscle is the sum effect of many extrinsic and intrinsic influences.

In the present study an attempt is made to correlate and clarify histochemical, physiological and physical changes associated with the developping and ageing skeletal muscle with specific reference to the extensor digitorum longus as a representative fast muscle and the soleus as a slow muscle in the rat.

CHAPTER II

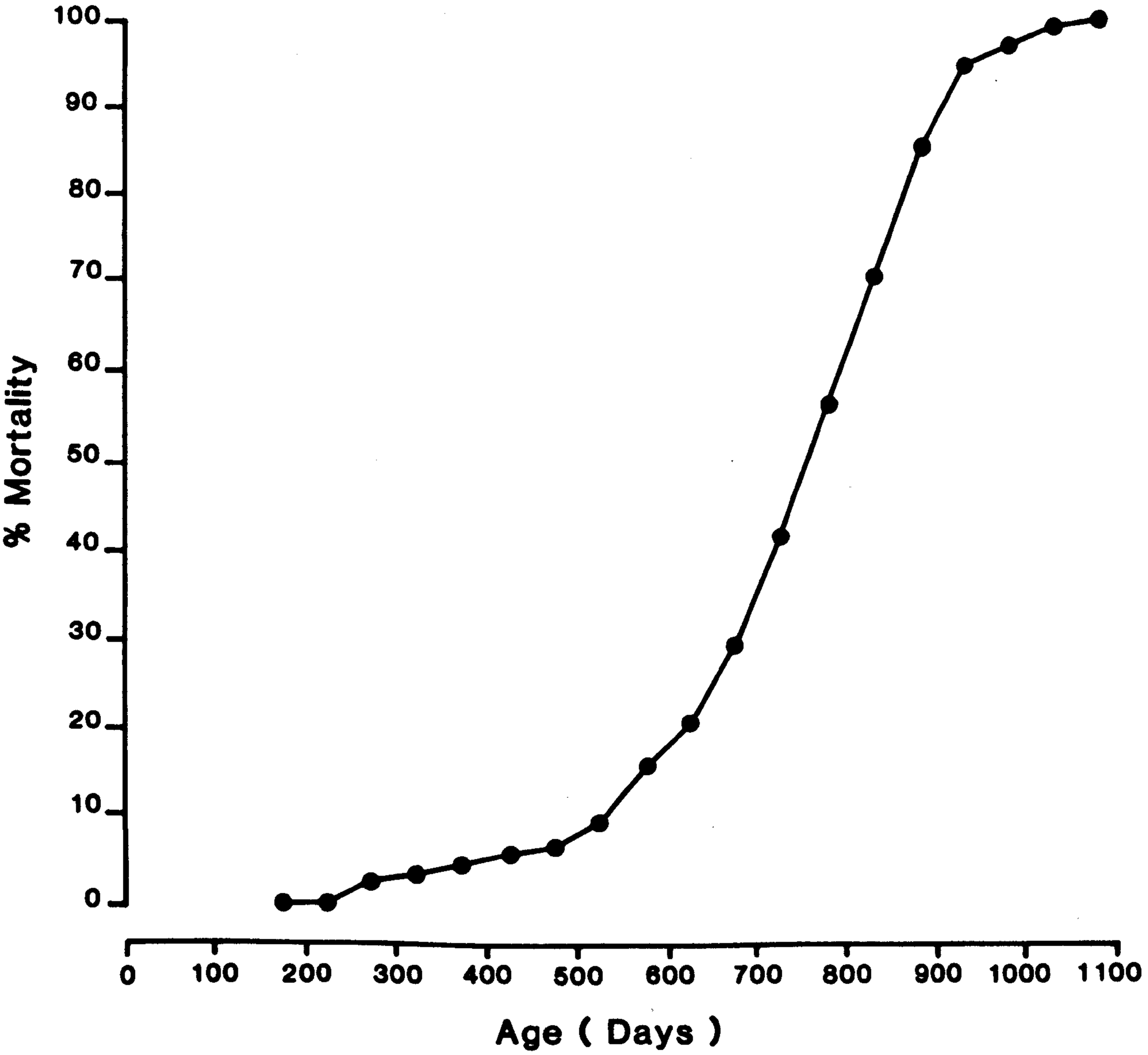
GENERAL MATERIALS AND METHODS

Animals

Male CFY Sprague-Dawley rats were chosen for this study. This strain loses 50% of the initial population by 694 days and has a maximum life span of 1056 days. The mortality rate beyond 700 days continues at a high rate, and less than 10% of the original population survives past 900 days (Merry and Holehan 1981). The mortality curve of this strain is typical of a population exhibiting senescence (fig. 2.1) and compares well with mortality curves for man (Comfort 1979).

The ages investigated were between 21 and 760 days, the exact grouping being different in each experiment. The animals were reared in the Wolfson Institute from a stock originally obtained from Caworth Europe, Huntingdon - now known as Haking and Churchill, Huntingdon. Conditions under which animals were kept were monitored closely. The temperature was 21 $\pm$ 1^{o}C while the humidity was between 35 and 45%. The photoperiod was 12 hours light from 8.00 to 20.00 hours. Animals were raised in litters of 8 to increase reproducibility and reduce competition, and were weaned 21 days post partum. All animals were kept in RC1 type cages supplied by North Kent Plastic Cages Ltd., Home Gardens, Dartford. Animals weighing 150g or less were kept at a density of 8 animals per cage

Fig. 2.1 Mortality curve of CFY Sprague-Dawley rats (Merry and Holehan 1981). The curve is typical of a population exhibiting senescence.


% Mortality
100
90
80
70
60
50
40
30
20
10
0
0
100
200
300
400
500
600
700
800
900
1000
1100
Age (Days)

whereafter the number was reduced to 4 animals per cage. Older animals were housed in cages with an extended lid to allow freer animal movement. Food and water was available ad libitum. The diet was based on forumla 41B (Appendix 1) supplied by Bradshaw, Driffield.

Choice of muscles

Muscles selected had to satisfy the following requirements:

(a) A simple fusiform design with fibres running longitudinally from one tendon to the other, thus allowing the assessment of the number of fibres in a cross section and accurate fibre diameter measurements. Furthermore, the simple alignment of fibres with well developed tendons increases the accuracy of tension measurements.

(b) A reasonable muscle size, so that rapid freezing of the whole muscle is possible.

(c) An accessible position for surgical exposure of the muscle in order to facilitate the in vivo stimulation and recording of isometric muscle contraction.

(d) A well-defined function and speed of contraction.

On these bases two hind limb muscles were selected; the m. soleus and the m. extensor digitorum longus. The soleus is a posture controlling muscle made up mainly of slow contracting motor units (Close 1967). The EDL on the other hand is a fast muscle with a majority of fast contracting motor units and a small number of slow contracting ones (Close 1967).

Statistical analysis

The diversity of experimental techniques in this study required the use of more than one statistical approach. The use of Hull University ICL 1904 S -George 3 computer system allowed accurate and rapid analysis of data using tailor made Fortran computer programmes designed by Dr. P. Zelnik and myself. (Appendix 3).

(a) General statistics

In cases where the arithmetic means and standard errors of the mean

were required, they were calculated in the usual manner (Sokal and Rohlf, Box 4.2 and 7.1,1969). Standard deviations were obtained using Sokal and Rohlf machine calculation formulae, Box 4.2 (1969). The frequency of occurrence was tested to construct distribution tables for fibre diameters. The test was built around a Fortran NAGF library programme, routine GØ1AEF (Mark 5, 1974). All programmes were of a single precision design.

(b) Analysis of variance

This analysis attempts to establish whether the variation in the means of the samples under investigation is due to the presence of an added component, i.e. the treatment. If such a variation is present the null hypothesis is rejected, and the various groups are assumed to come from different populations. The significance of the variation is qualified using an F-Test. The advantage of this test over other techniques is that it allows the analysis of more than one factor in each experiment simultaneously.

Model I Anova

This model of the analysis of variance assumes that the variation between the samples to be tested is due chiefly to the treatment with no allowance to any other influencing factors or any significant random variation within the tested samples. The detection therefore of any variation within the tested groups can only arise from the fact that the samples tested do not belong to the same population. This type of analysis was used in testing the influence of various age groups on the experimental factors, e.g. age versus fibre number or age versus muscle weight. The formulae for this analysis is based on Sokal and Rohlf, Box 9.1 (1969).

Model II Anova

Model II anova estimates a random component that is different in each group tested and establishes its contribution to the variation of the means. If the random component's contribution is higher than that

of the treatment then the means do not reflect reliable differences between the various populations tested. The analysis used was of the Two-level Nested Anova with Unequal Samples (Box 10.4, Rohlf and Sokal 1969).

Both Anova models were followed by a priori comparisons. This test was carried out regardless of the preliminary Anova, for although an Anova will indicate a possible variance difference, the difference cannot be attributed to any one treatment. The a priori test was a preset comparison between preselected pairs of groups carried out regardless of their apparent significance. The significance of the comparison was established using an F-test, which is in this case mathematically equivalent to a T-test. The a priori comparison formulae used in our computer programmes were those of Rohlf and Sokal, Box 9.8 (1969).

(c) Linear regression analysis

Using the NAGF library routine GØ2CAF (Mark 5, 1974) data showing linear relationship between the independent variable and the dependent variable were fitted to a straight line of the form: $y = a + bx$ or $\log y = a + bx$. Whenever the calculated correlation coefficent was of a value greater than 0.9, the regression was considered to be reliable and was used to compare groups of fitted regression lines. The steepness of the fitted line was used as an indicator of the rate at which the dependent variable progressed.

CHAPTER III

MORPHOMETRIC AND HISTOCHEMICAL CHANGES IN DEVELOPING AND SENILE MUSCLE

The postmitotic nature of skeletal muscle tissue makes it's ability to grow by division or even regenerate, limited (Cowdry 1952). At birth or shortly after birth muscle fibre number reaches it's maximum value and remains unchanged during development (Goldspink 1972). Increases in muscle weight and size is therefore restricted to changes in muscle fibre diameter, as demonstrated in the mouse (Rowe and Goldspink 1969) guinea pig (Lieberman et al 1972) and the rat (Chiakulas and Pauly 1965, Ontell and Dunn 1978). Such increases are associated with an increase in myofibril number and size (Goldspink 1970) thus increasing the contractile force of the tissue.

In general, muscles waste with age. The decrease in muscle weight has been related to reductions in fibre number (Rowe 1969). Gutmann et al (1968, 1971) cited reductions in fibre number as the main cause of impaired contraction power. Selective degeneration of muscle fibres classified broadly as "Red" and "White" have been reported: Tauchi et al (1971) observed reductions in numbers of "Red" fibres and in the volume of "White" fibres in the anterior tibialis of the rat. Leiberman et al (1972) recorded a similar decrease in the "Red" fibre population in the diaphragm of the guinea pig

In the above mentioned studies muscles were regarded as having a homogenous population of fibres, or were at best divided into only "white" or "red" muscle fibres; a classification which fails to recognise intermediate fibres. However, the differential behaviour of the different muscle fibre types during development and ageing has received some attention during more recent years. The human vastus lateralis was found by Larsson et al (1978) to have a higher type I to type II fibre ratio in older subjects, however, the ratio of type IIA to IIB fibres remained unchanged. In the latter study small biopsy samples were obtained from which fibre ratios were calculated, hence total fibre numbers could not be accurately estimated nor could any allowance be made for area sampling errors that may arise due to uneven occurrence of ageing changes or fibre type distribution.

The developing and ageing muscle has been shown to change with exercise. Forced activity alters the oxidative enzyme content of young muscles (Howells and Goldspink 1974) and different muscle fibre types respond differently to exercise (Goldspink and Ward 1979). Ageing muscles react to exercise up to a threshold age showing positive increases in weight (McCafferty and Edington 1974). The diaphragm; a continuously active muscle appears to be able to maintain the mosaic appearance of ATPase stained sections in old age (Bass et al 1975). The soleus on the other hand is a postural muscle with moderate sustained activity, it apparently suffers a reduction in the number of type II fibres with age, but the main population of fibres, that is to say the slow oxidative ones hardly change in size (Bass et al 1975). Unfortunately, the contribution of each fibre type to the total cross-sectional area of the muscles was not quantified. It appears that the function and activity of the muscle plays an important role in determining the degree of muscle atrophy. The exact relation between muscle function and selective fibre atrophy in the form of fibre number and/or fibre diameter reductions is not entirely clear.

Fragmented evidence indicates a selective process of atrophy affecting different fibre types at different rates. An attempt has been made in this section to understand and correlate the changes in the number, size and appearance of the different fibre types with development and ageing.

Many of the features displayed in the senile muscle correlated well with denervation atrophy. As mentioned in the introduction disorders in nervous supply and changes in nerve morphology increase with age (Berg et al 1962, Caccia et al 1979). Gutmann et al (1968) suggested that neural disorders and end-plate disruptions are responsible for muscle atrophy. This view was supported by Steenis and Kroes (1971) and further substantiated by ultrastructural studies on terminal axons of rat skeletal muscles (Fujisawa 1975, 1976). Whether such changes are peculiar to specific fibre types or whether they are of a more generalised nature has not been investigated. An examination of nerve fibre number within nerves supplying the muscles under study was carried out to establish the nature of changes associated with denervation atrophy, if any such changes actually occur.

It is by now apparent that any attempt to investigate developmental changes and senile atrophy should take into account the plasticity of muscle, for although the end result of two functionally different muscles can appear to be similar; for example, loss of contractile strength in senile animals, the underlying mechanisms can follow separate trends.

The use of histochemical techniques to determine the metabolic characteristics of individual fibres have been subjected to extensive study. By using enzymes that are representative of different metabolic pathways it is possible to distinguish different types of muscle fibres. Studies suggested the existence of three or four major fibre types (Spurway 1978, Nemeth et al 1979). Types additional to these may arise from time to time because of the dynamic nature of muscle and the fact

that enzyme activity is readily changed (Guth and Yellin 1971). Exercise or increased work load has been shown to alter the relative abundance or activity of enzyme systems causing the muscle fibre to display uncharacteristic histochemical staining (Sjøgaard et al 1978).

In some histochemical stains procedural variations have been shown to affect adversely the stain quality and reliability. Myosin ATPase was at one stage subjected to close scrutiny to establish its specifity (Guth 1973, Schiaffino and Bromioli 1973, Brooke and Kaiser 1974). The use of cross-referencing and well-controlled staining conditions and buffers has led to considerable reduction in variability and improved reproducibility. The very dependence of this stain on the relative denaturation of different myosin ATPase "types" under controlled conditions makes it susceptible to errors arising from poor control procedures.

Histochemical procedures nevertheless offer the only convenient way of distinguishing muscle fibre types on a reasonably large scale so that statistically valid measurements can be made and be applied to muscle studies with a reasonable degree of confidence although caution must still be displayed when interpreting the results.

DNA content estimates were also conducted in this study in an attempt to establish the nature of fibre loss in young extensor digitorum longus muscles. DNA content reflects both the activity and abundance of nuclei in an organ. When related to protein content, it is possible to verify whether fibre loss is due to degeneration of individual fibres or whether the loss is due to the fusion of adjacent fibres which gives rise to an apparent reduction in total fibre number.

MATERIALS AND METHODS

1. Body weights

Animals sacrificed for the various experiments were weighed to $\pm$ 0.1 grams. The weights were used to construct a growth rate curve. Ages examined were 21, 84, 183, 188, 508 and 758 days. The sample size for

each age was 6 animals. The results were subjected to a Model I analysis of variance which was followed by group comparisons.

2. Muscle weights

In addition to the soleus and the extensor digitorum longus, 6 other muscles were investigated to establish the general trend. Two muscles were from the fore-limb; the palmaris longus and the biceps brachii, and three from the lower hind limb; the anterior and posterior tibialis and the extensor hallucis proprius. In addition cardiacmuscle was measured. The soleus, extensor digitorum longus and the heart were weighed in animals aged 21, 84, 183, 188, 298, 508, 716 and 758 days. The remaining 5muscle groups were only weighed at 388 $\pm$ 2 and 714 $\pm$ 6 days.

In all cases muscles were dissected out, with both tendons intact and were cleaned free from fat and connective tissue before being weighed on a torsion balance to $\pm$ 0.5mg. The heart was cut open and was rinsed in saline to remove the blood before being weighed.

All treatments were tested statistically with either a T-test or a Model I analysis of variance, the latter being used when more than one group of animals was involved.

3. Histochemical stains

The main histochemical technique used in this study was myosin ATPase. This technique utilizes the senstivity of the myosin molecule to both pH changes and formaldhyde. The resulting denaturation permits the identification of the three major fibre types, namely, slow oxidative, fast oxidative glycolytic and fast glycolytic. Although the exact locality of the stain reaction and deposit sites have been the subject of debate (Guth 1973), Schiaffino and Bormioli (1973) showed using selective extraction procedures, that Ca^{++}activated ATPase reaction products are due primarily to the direct action of myosin ATPase.

The original Padykula and Herman (1955) method has been modified and improved several times with new techniques being introduced (Hayashi and

Frieman 1966, Guth and Samaha 1969, Meijer 1970, Khan et al 1972b, Tunell and Hart 1977). The modified techniques increased the reliability and reproducibility of the stain and the identification of a larger number of fibre types.

In this investigation the basic technique of Guth and Samaha (1970) and Tunell and Hart (1977) was used with some small modifications. The latter method was preferred because of the increased differentiation between the two fast fibre types and the reduced incubation times. Both stains were cross-related using serial sections, and were found to be totally compatible.

I Preparation of muscles

As most fixatives cause muscle shrinkage (Goldspink 1961, Goldspink et al 1973, Eisenberg and Mobley 1975) and reduced or inhibited enzyme activity, freeze-fixation was adopted. Freezing causes the least dimensional distortion (Goldspink et al 1973, Hegarty and Naude 1973) and retains the enzyme activities of the muscle fibres.

Muscles were dissected out, cleaned of excess fat and connective tissue then cut in half in the belly region. Both halves were mounted on a thin cork in such a way that two sections of the belly region were obtained. The preparation was surrounded with Tissue Tek II (Raymond A. LAMB, London) and then quenched in supercooled isopentane. Isopentane has a very low boiling point and is superior to liquid nitrogen as a freezing agent because the latter forms an insulating layer of gas bubbles around the tissue; under these conditions the formation of damaging ice crystals in the muscle due to slow cooling is reduced, thus avoiding the risk of artefact formation. Small muscles especially those requiring extra support were sandwiched between cool liver strips before being frozen.

Sections were cut at a thickness of 10 μm in a Bright Cryostat (Bright Instrument Co., Huntingdon) at -15° to -25°C. Mounted sections

were allowed to dry at room temperature for 30-90 min before being stored until required in a deep freeze. At such a low temperature enzyme acitivities are known to decrease at a very slow rate (Dubowitz and Brooke 1973).

II Myosin ATPase (Based on Guth and Samaha 1969, 1970)

(a) Alkali preincubation

Sections were fixed at 3°C for up to 5 minutes in buffered formaldhyde made up from 5% fresh paraformaldhyde solution, 0.9M $CaCl_2$, 0.25M Sucrose and 0.2M sodium cacodylate adjusted to pH7.2. The fixative was rinsed 2 x 1 min in 0.1M Tris-HCl containing 18mM $CaCl_2$ (pH7.8). Excess solution was blotted and the slides placed in the alkaline preincubation medium for 15 - 20 min . The alkaline preincubation solution contained 0.1M 2-amino-2-methyl-1-propanol and 18mM $CaCl_2$ adjusted to pH10.4. Sections were rinsed 2 x 1 min in Tris-$CaCl_2$ and then incubated for ATPase.

(b) Acid preincubation

Acid preincubated sections were either fixed as decribed above or processed with no fixation through the following stages (unfixed sections do not show differentiation into the two fast types). Acid preincubation was carried out in 0.2M barbiturate-acetate buffer for 15 - 20 min . The exact pH was manipulated between 4.3 - 4.6 to obtain the best differentiation (Brooke and Kaiser 1970). Sections were rinsed in 0.1M Tris-$CaCl_2$, blotted dry then stained for ATPase.

(c) ATPase staining

Sections were incubated at 37 $\pm$ 0.5°C for 30 - 40 min in a freshly prepared medium of 18mM $CaCl_2$, 0.15M 2-amino-2-methyl-1-propanol, 5mM KCl and 1.52mg/ml Adenosine Triphosphate disodium salt. The solution was adjusted to pH9.4 at the exact incubation temperature. Sections were rinsed 3 x 30 seconds in a 1% $CaCl_2$ solution then placed for 3 min in a freshly prepared 2% cobalt chloride solution. After a quick rinse of

4 x 30 seconds in 0.1M 2-amino-2-methyl-1-propanol the sections were placed in 1% yellow ammonium sulphide for 3 minutes, then washed under running water for 5 minutes, dehydrated in graded alcohols, cleared and mounted in De Pe X.

III Mysoin'ATPase (Based on the method of Tunell and Hart 1977)

Sections were fixed for 5 - 10 min in a preincubation medium consisting of 0.1M glycine, 2% $CaCl_2$ and 10% freshly prepared formaldehyde (40% stock). The pH was adjusted to 7.35. Sections were washed 4 x 30 seconds in saline then incubated at 37°C $\pm$ 0.5 in a medium containing 0.1M glycine, 2% $CaCl_2$ and 1.52mg/ml Adenosine Triphosphate (disodium salt). The pH of the incubation solution was 9.35 at 37°C and incubation times were between 30 and 45 minutes. Sections were washed in 1% $CaCl_2$ for 4 x 30 seconds and placed in 2% fresh cobalt chloride for 3 minutes. After a wash in saline for 4 x 30 seconds, section were placed in 2% ammonium sulphide for 1 - 2 min . Sections were then placed under running water for 10 minutes before being dehydrated in graded alcohols, cleared in xylene and mounted in De Pe X.

IV Succinic dehydrogenase

Succinic dehydrogenase was employed as a confirmatory cross-referencing enzyme. Serial sections were stained for both succinic dehydrogenase and ATPase. The correlation between the two stains was used as an indicator of the reliability of the ATPase stain.

Succinic dehydrogenase is a substrate-specific oxidative enzyme which is part of the Kreb's cycle. A colourless tetrazolium salt which acts as an electron acceptor is used. On reduction the salt is converted into a formazan which is insoluble and coloured. The intensity of the newly formed formazan indicates the oxidative activity of the muscle fibre.

The particular method used in this study is based on that of Nachlas et al (1957): Unfixed frozen sections were incubated at 37°C $\pm$ 0.5 in a medium containing 0.05M phosphate buffer, 0.05M sodium succinate

and 0.5mg/ml Nitro Blue Tetrazolium. The pH was adjusted to 7.6. Incubation times were variable between 5 - 30 min . The tetrazolium salt concentration was increased in some cases to 1mg/ml to increase the deposition intensity without increasing incubation times. Sections were then washed in 0.9% saline for 2 x 1 min and post-fixed in formol saline (10% formalin in 0.9% saline) for 10 minutes. Sections were rinsed in 15% alcohol for 5 minutes and then mounted in a water miscible media like glycerine jelly.

Appropriate controls were used in all staining techniques to verify the specifity of the stain. A further safeguard was the staining of all sections in the same batch thus reducing variability between the various age groups. This forced incubation times that were a compromise between those best for the youngest age group with high enzyme reactivity and those best for the oldest age group with low enzyme reactivity.

4. Muscle fibre diameters

The method used to measure fibre diameters in this study was that proposed by Song et al (1963) and modified by Schmitt (1976). To estimate the diameter of an irregular muscle fibre, the mean of the orthogonal axes was obtained, i.e. the maximum and minimum diameters were measured at right angles. This method appears to produce more consistant results that approximate to the true cross-sectional area without sacrificing speed.

The number of fibres sampled was 100 of each fibre type, whenever possible. This number gives a good statistical sample size, and was used by other workers (Goldspink 1962, Faulkner et al 1971). The total sample in each muscle exceeded 100 because of the number of fibre types within each muscle.

Measurements were obtained through a graduated eye piece on a Leitz Ortholux microscope. The cross-hair eye piece was calibrated using a one millimeter stage micrometer (Graticules Ltd., Tonbridge, Kent). The magnification at which the readings were obtained varied with the age

group investigated. In general the magnification was high enough to almost fill the field of vision with a single fibre. The resolution of the cross-hair was better than 0.5 μm.

Although both the extensor digitorum longus and the soleus showed a mosaic distribution of the various fibre types, an organised scan technique of the whole cross-section was used to select the fibres for measurement. This eliminated any area sampling errors that could arise because of possible size and fibre type concentrations (Pullen 1977). The scan was carried out in such a way as to sample fibres along the deep to superficial axes of the muscle. The spacing between any two adjacent scans was wide enough to cover the whole muscle by the time 100 fibres were measured.

Ages investigated in this experiment were 21, 84, 188, 299 $\pm$ 1, 508, 716 and 745 days. Results were analysed using a nested analysis of variance followed by an a priori comparison. Frequency distributions of individual muscles and grouped muscles were accumulated and tested for significance.

5. Muscle fibre number

ATPase stained sections were used to count the number of each fibre type. Since sections were cut in the belly region of the muscle and because of the simple geometry of the muscle used, all fibres could be assumed to pass through the sections examined. Every fibre was counted and allocated to one of the three fibre type categories, this reduced the risk of area sampling errors arising from uneven distribution of the various fibre types (Pullen 1977). To facilitate the identification and counting of muscle fibre types, muscles were divided into smaller areas, each area was projected using a Leitz Microprojector with objective lens of x4, 6.3 and 10 magnifications and a x4 eye-piece. The final screen magnification was between 120 and 300 times.

Total fibre number within each muscle was obtained by adding up the individual fibre type number. The results of all counts were grouped

according to fibre type and age group and were subjected to a Model I analysis of variance.

6. Nerve fibre number

Male animals aged 21, 387 ± 2 and 714 ± 6 days were investigated. Nerves feeding the left extensor digitorum longus and soleus were examined. Because of the fragility and the small size of the nerves, resin embedding was employed in preference to paraplast. A soft resin was used (Luft 1961).

Solutions and embedding medium

(a) Gluteraldhyde fixative

0.2M phosphate buffer	45ml
5.4% glucose	10ml
25% gluteraldhyde	35ml

(b) Osmium tetraoxide fixative

0.2M phosphate buffer	4.5ml
5.4% glucose	1.0ml
2.0% osmium tetraoxide	4.5ml

(c) Phosphate buffer wash

0.2M phosphate buffer	225ml
H_2O	225ml
5.4% glucose	50ml

(d) Embedding medium (Luft 1961)

EPON 812	44ml
Dodecenylsuccinic Anhydride (DDSA)	30ml
Methyl Nadic Anhydride (MNA)	23ml
Benzyldimethylamine (BDMA)	1.5ml

Procedure

Nerves were carefully dissected and tied at both ends with fine threads under a dissection microscope. The nerves were then stretched to the approximate original length on thin cork and were placed in gluteraldhyde fixative for 16 hours at $4^{o}C$. They were then washed in

phosphate buffer 3 x 20 min . Sometimes samples were stored at this stage for up to 4 days. This allowed the accumulation of a suitable sample size for further processing. Nerves were then fixed in osmium fixative for one hour then washed in water 3 x 1 min. Dehydration was carried out immersing the tissue for 15 min in each of a series of 70, 90, 95 and 2 x 100% alcohols. Nerves were placed in propylene oxide for 2 x 15 min and then progressed through a mixture of 50% propylene oxide: 50% resin and 25% propylene oxide: 75% resin for one hour each. Fresh resin was used for the final stages and nerves were carried through 3 x 1 hour changes before orientation in flat silicone rubber moulds. Embedded nerves were cured for 72 hours at 60°C.

Blocks were cut on a Reichert Om U2 Ultramicrotome at a thickness of 0.5 to 1 µm. Sections were placed on glass microscope slides and stained with 1% Toluidine blue in 1% Borax on a hot plate for variable times in the order of seconds. A series of sections over a few millimeters of nerve were collected to reduce sampling errors.

Sections were examined under a Leitz Ortholux microscope. Micrographs were obtained on Pan X film at a negative magnification of x100. The number of nerve fibres was counted from enlarged micrographs. Nerve types were not distinguishable, thus only total axon counts were obtained. Data were analysed using a Model I analysis of variance and an a priori comparison.

7. Nuclear DNA content

Muscles were assayed for DNA using the method described by Munro (1969). Ages investigated were 21, 90 and 244 days. Because of the small muscle sizes several muscles were pooled together to increase the sample size.

(a) Extraction of DNA

Muscles were homogenised in 0.2M perchloric acid at 0°C for 30 seconds in a Polytron homogeniser (Kinematica GMBH, LUZERN-SCHWEIZ) at speed six. The homogenate was allowed to stand for 10 minutes at 0°C,

then was centrifuged at 1000g and 0°C for 15 min. The supernatant was discarded and the whole sequence was repeated two more times. The precipitate was suspended in an equal volume of 0.6N perchloric acid and heated to 90°C for 20 min. The mixture was centrifuged at 1000g at room temperature and the supernatant retained. The precipitate was washed twice in 0.6N perchloric acid and the accumulated supernatant was used to estimate DNA. The precipitate was used to estimate the protein content using the Biuret method.

(b) DNA assay

Equal volumes of 0.4% indole and concentrated HCl were mixed. The mixture was added to an equal volume of DNA sample. The mixture was heated for 10 min at 100°C and then allowed to cool to room temperature. Four volumes of chloroform were added to one volume of the previous mixture and then shaken vigorously. The layered chloroform was pipetted out and the procedure repeated twice with the final pipetting replaced with a centrifugation at 1000g for 10 min. The supernatant was read at 490nm against a control. Controls contained 0.6N perchloric acid and were treated in exactly the same way as samples were treated. DNA content was estimated using precalibrated standardization curves of DNA sodium salt (type III, from Salmon).

(c) Protein hydrolysis

Protein precipitate retained from the DNA extraction was washed in methanol for 10 min and the supernatant discarded. The precipitate was dried until moist in a water bath. Hydrolysis of the protein was achieved by using 1N NaOH and heating the mixture at 100°C for 10 min. When cool the solution was assayed for protein content using the Biuret test.

(d) Biuret test

The Biuret reagent was prepared by dissolving 1.5g $CuSO_4$ $5H_2O$, 6.0g NaK tartrate, 1g KI and 30g NaOH in 750 ml H_2O. The volume was

brought up to 1000ml. Four millilitres Biuret solution was added to 1ml protein solution, the mixture was allowed to stand for 15 min and was then read at 550nm against a control blank of 4ml Biuret solution and 1ml 1N NaOH. The readings were calibrated against an assay curve of Bovine Serum Albumin (Fraction V, 96 - 99% Albumin, Sigma, U.S.A.).

RESULTS

1. Body weights

All animal ages showed a weight increase with age (Table 3.1). The analysis of variance showed that during the earlier ages the rate of weight increase was highly significant averaging 4.6g day^{-1}. However, between the ages of 508 and 716 days, the rate of increase declined reaching a low non-significant increase of 0.08g day^{-1}.

2. Muscle weights

Results in Table 3.2 show that the extensor digitorum longus increased rapidly in weight from weaning to an age of 185 days ($P < 0.001$). Changes thereafter were not significant up to 508 days. However, in senile animals there was a significant decline ($P < 0.05$) as compared with the extensor digitorum longus of animals aged 508 days.

The soleus increased in weight (Table 3.2) at a rapid rate between 21 and 185 days ($P < 0.001$). The rate became slower but was still significant at the 5% level in animals aged 298 days. As was the case with the extensor digitorum longus, the mean weight of the soleus in 716 days old animals was lower than for the preceding 508 days group ($P < 0.05$).

The heart on the other hand continued to increase in weight throughout the ages examined (Table 3.2). The weight increase was significant up to 185 days ($P < 0.001$). The unexpected decrease in the heart's weight in animals aged 508 days is not significant when related to the preceding age group, namely 298 days, but the increase which followed between the ages of 508 and 716 days was significant ($P < 0.05$).

Table 3.1 Body weights of developing and ageing male CFY rats

Age (Days)	Number of Animals	Mean Body Weight (g) ± S.D.	Probability <
21	10	55.9 ± 2.5	0.001
84	5	346.8 ± 19.3	0.001
185 ± 3	10	511.3 ± 64.0	0.001
508	5	585.4 ± 38.1	n.s.
758	6	605.6 ± 77.0	

Table 3.2 Weights of the developing and ageing soleus, extensor digitorum longus and heart

Age (days)	Extensor Digitorum Longus (mg)				Soleus (mg)				Heart (mg)			
	No.	Weight	± S.D.	Pr. <	No.	Weight	± S.D.	Pr. <	No.	Weight	± S.D.	Pr. <
21	9	20.5	3.5	0.001	9	20.8	1.8	0.001	9	230.8	41.0	0.001
84	5	165.8	8.0	0.001	5	172.0	12.4	0.001	5	1050.0	154.1	0.001
185 ± 3	10	246.1	37.2	n.s.	10	236.2	36.3	0.05	10	1396.0	143.5	n.s.
298	5	237.4	24.2	n.s.	5	263.0	16.9	n.s.	5	1484.2	105.5	n.s.
508	5	253.2	14.1	0.05	5	264.0	45.1	0.05	5	1376.0	111.3	0.05
716 – 758	9	228.6	34.8		9	232.3	31.5		9	1537.1	205.4	

Out of the five muscles examined for age-related weight changes at the selected ages of 388 and 714 days (Table 3.3) only two showed a significant decrease in senile animals at the 5% level, namely the anterior tibials and the posterior tibialis. Although the biceps brachii and the palmaris longus decreased in a similar fashion, the decrease was not statistically significant. The extensor hallucis proprius showed an insignificant increase in weight in senile animals when compared with those of the younger groups.

3. Muscle fibre types

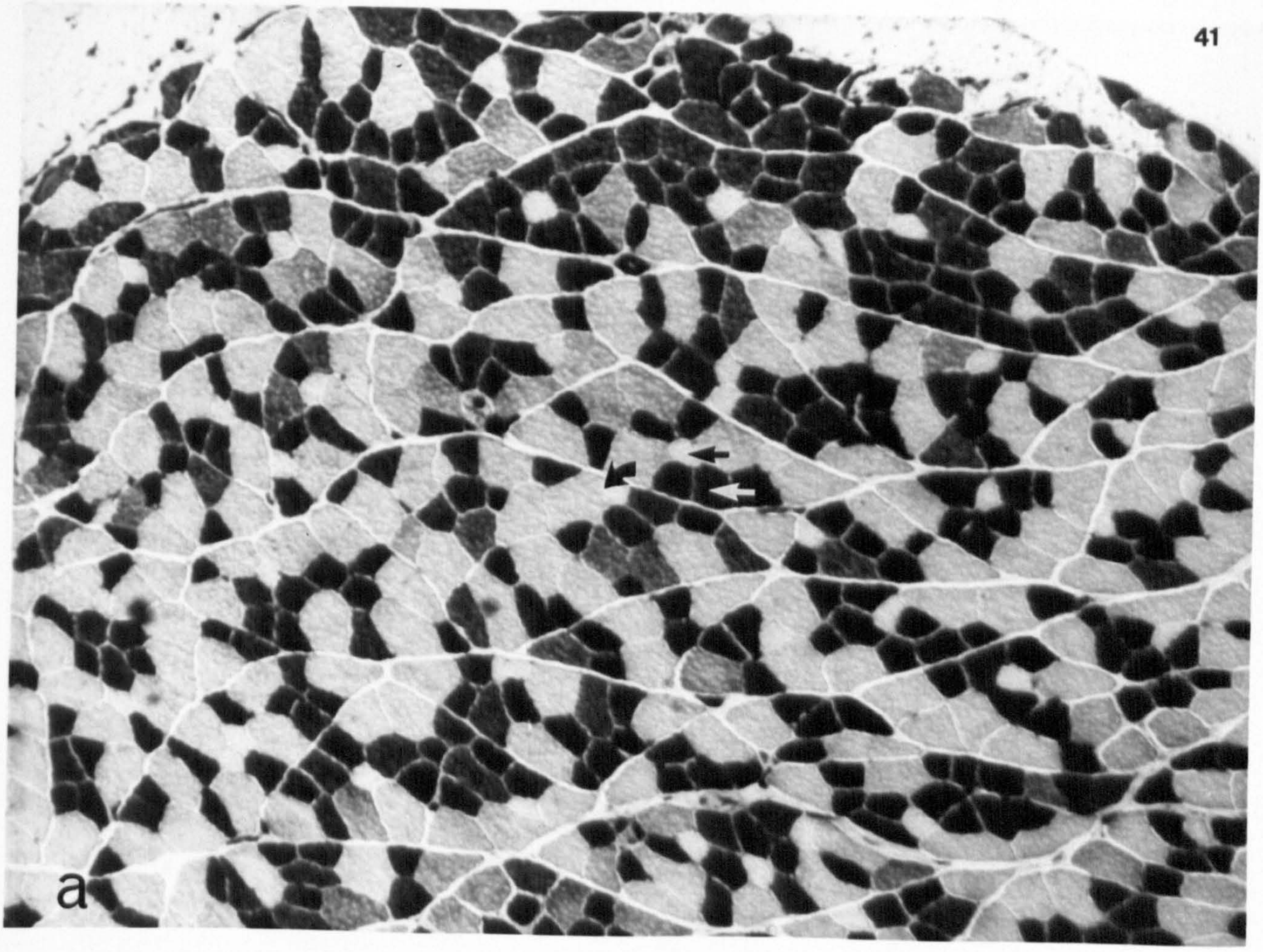
Using myosin ATPase staining it was possible to distinguish in the extensor digitorum longus all three major fibre types described by Peter et al (1972). Under alkaline preincubation and prefixation conditions, fast oxidative glycolytic fibres (FOG) were of the highest ATPase activity. Slow oxidative (SO) fibres stained weakly for ATPase, whilst fast glycolytic fibres (FG) stained at an intermediate intensity with slight or no stain gradation (Plate 3.1a). Acid preincubation produced an exact reversal of ATPase staining intensities in all fibre types in the adult animals. Acid preincubated senile muscles showed extremely poor staining characteristics with a high degree of irreproducility. Fibres stained for ATPase and cross-references using succinic dehydrogenase demonstrated a high correlation factor (Plate 3.1a,b). The characteristics of the different mammalian muscle fibre types are summerised in Appendix 2.

Muscle fibres in the soleus were of two types only; SO and FOG (Plate 3.2a). Their staining characteristics were similar to those of the extensor digitorum longus when stained for myosin ATPase. SO fibres stained for succinic dehydrogenase showed a more diffuse stain when compared with the SO fibres of the extensor digitorum longus. Nevertheless both stains were highly reproducible

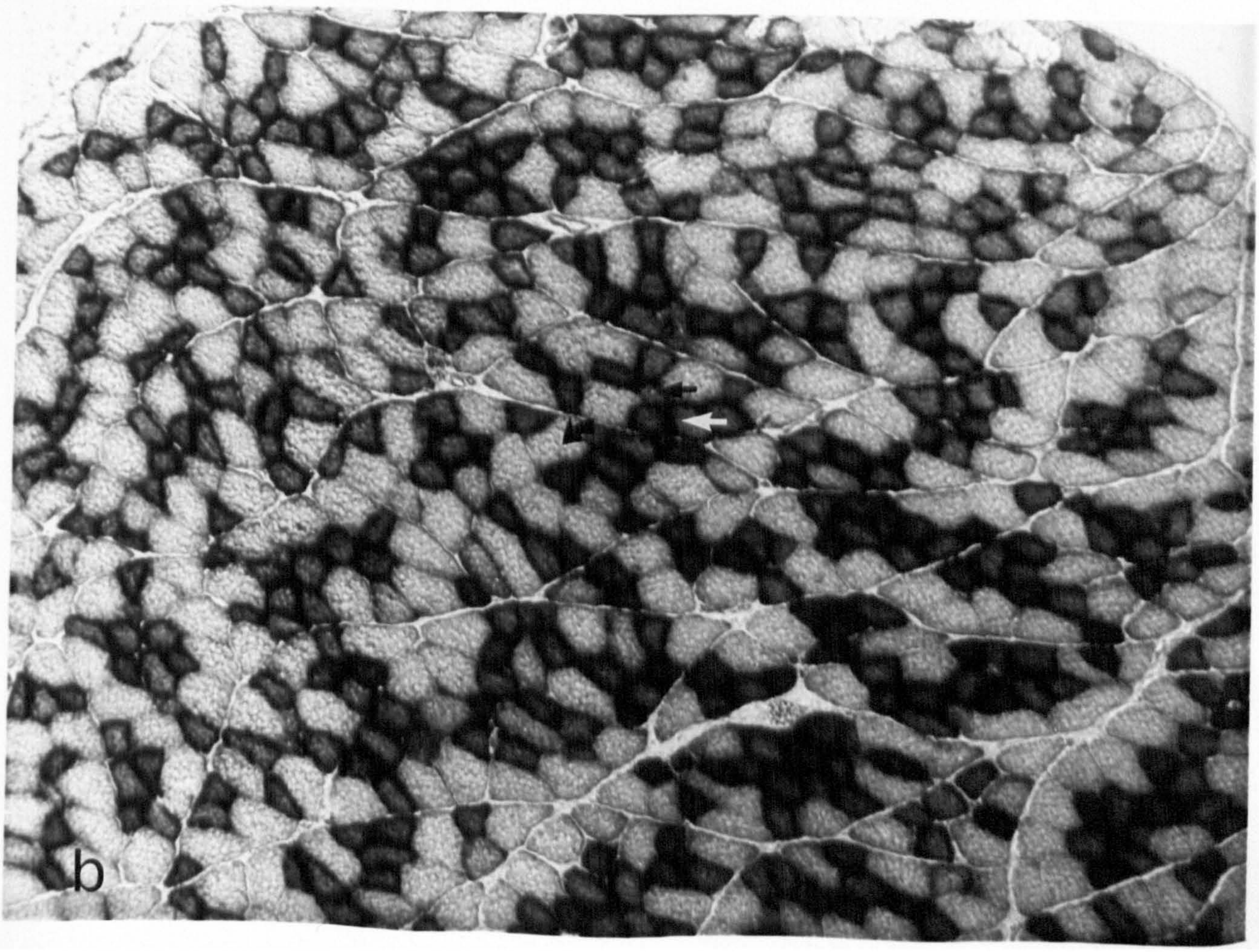
Table 3.3 Weights of five skeletal muscles in the ageing rat

Age	388 ± 2 days			714 ± 6 days			
Muscle	No.	Mean Wt. (mg)	± S.D.	No.	Mean Wt. (mg)	± S.D.	Pr. <
Biceps Brachii	5	355.0	30.1	5	321.4	25.2	n.s.
Palmaris Longus	5	72.8	5.0	5	71.6	1.8	n.s.
Posterior Tibialis	5	339.2	26.5	5	277.2	46.1	0.05
Anterior Tibialis	5	947.8	52.6	5	718.0	205.8	0.05
Extensor Hallucis Proprius	5	28.6	1.9	5	32.0	5.3	n.s.

Plate 3.1 Staining characteristics of the extensor digitorum longus for alkaline ATPase (a) and succinic dehydrogenase (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 (magnification x200).

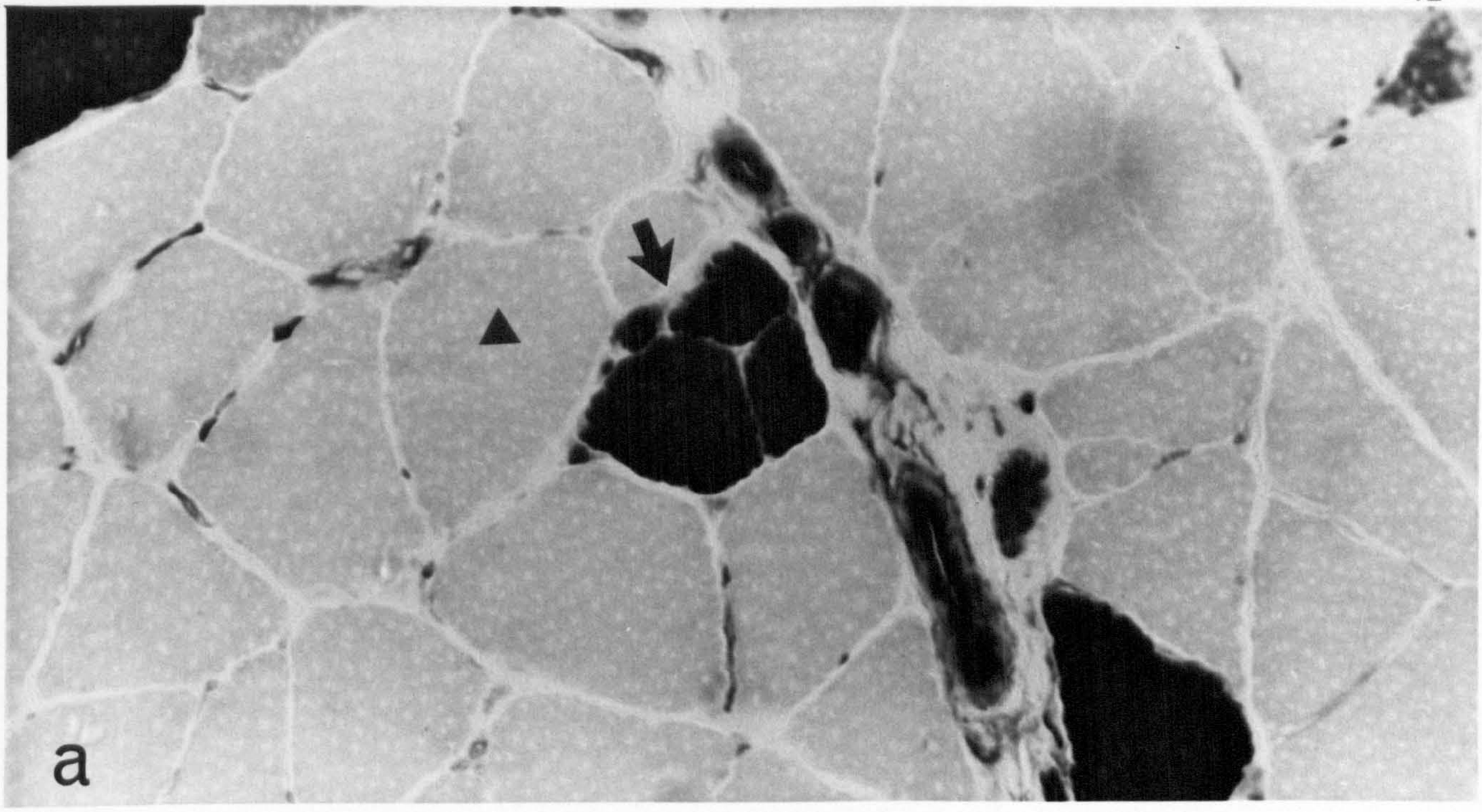


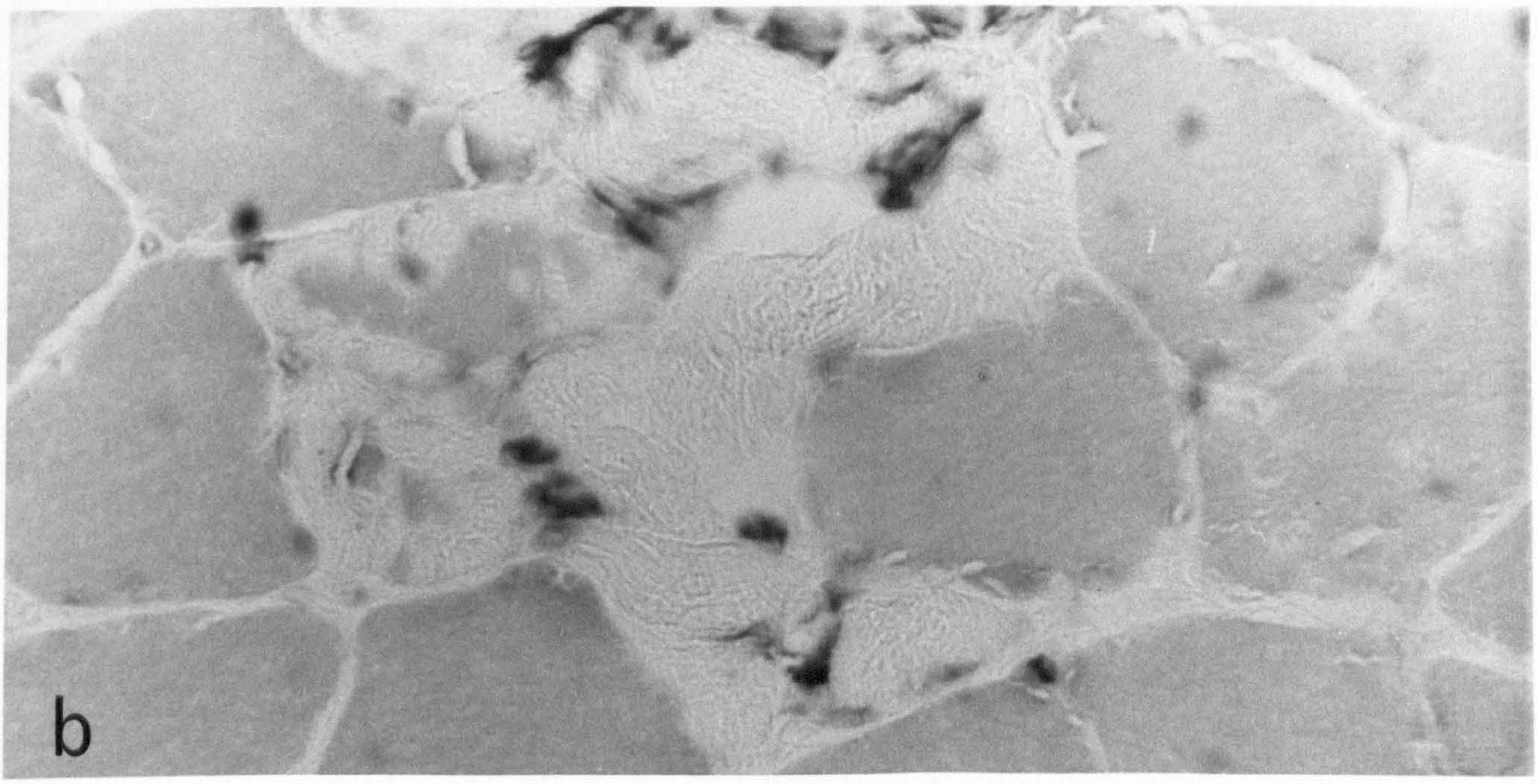
a

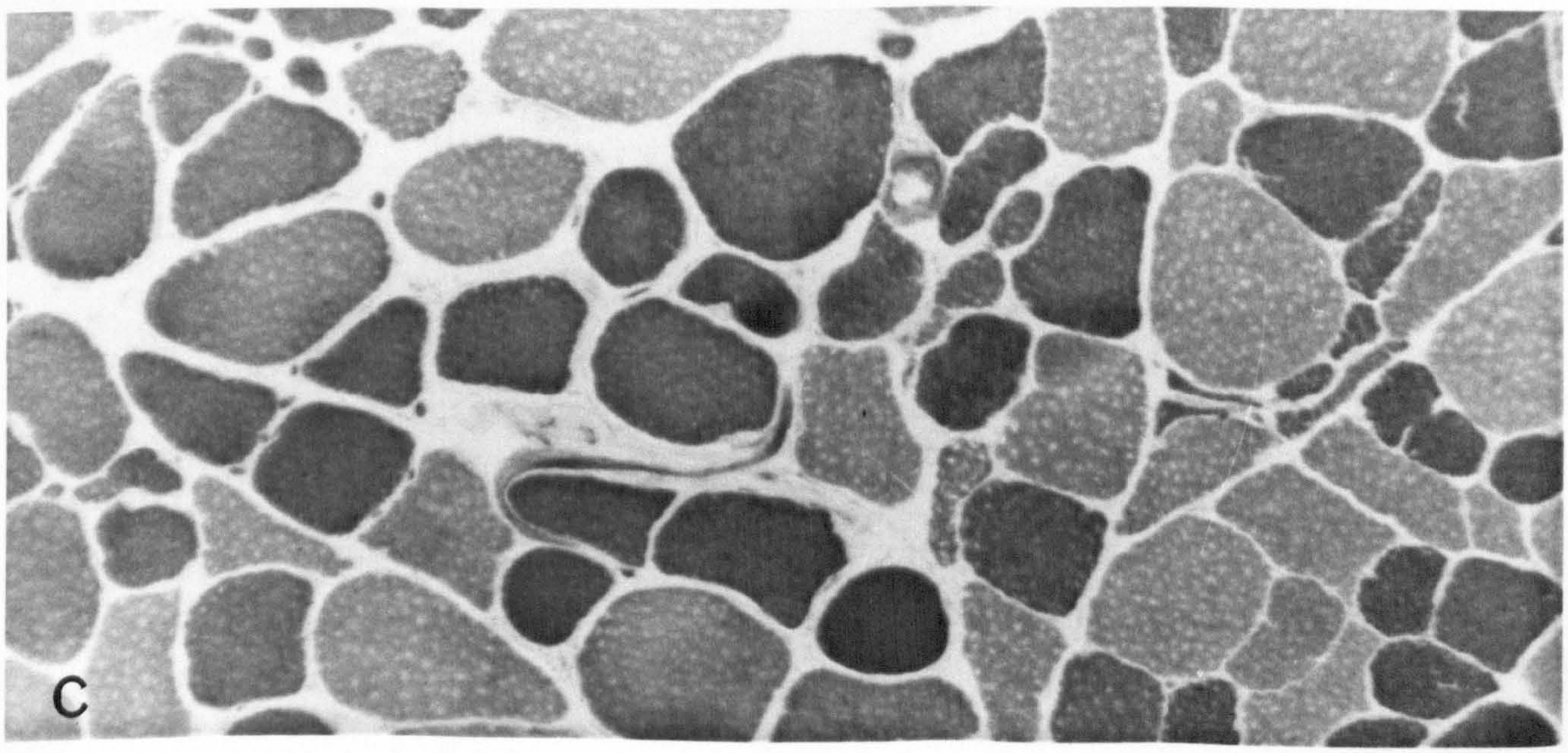


b

Plate 3.2 Senile muscles exhibit a variety of abnormal features. Longitudinal splitting occurred more frequently in senile muscles (a) so 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 (magnification a and b x300 c x135).


a


b


C

when identifying muscle fibre types.

In general the reactivity of senile muscles with the various stains was poorer than those of younger animals.

Controls incubated for ATPase did not stain when ATP was excluded or replaced with ADP. Sections incubated for succinic dehydrogenase in the absence of sodium succinate did not stain.

4. Muscle fibre size

The nested analysis of variance revealed that all three fibre types in the extensor digitorum longus under went age-related changes. A detailed examination of every fibre type confirmed the occurrence of such changes (Table 3.4).

FG fibres continued to increase in diameter from weaning up to extreme age (716 days). The increase in diameter was rapid during the 84 days after birth. The rate decreased until no further significant increase beyond 188 days could be seen. FG fibres appeared to outgrow the two other fibre types, reaching a mean fibre diameter of 75.9μm at 716 days, compared with 41.6μm and 48.3μm for SO and FOG fibres respectively. The maximum FOG diameter occurred at an intermediate age of 508 days. FOG fibres showed an increase in diameter between the ages of 21 and 188 days. Fibre diameter thereafter remained unchanged except in senile animals where there was a slight decrease in diameter.

Slow oxidative fibre diameters increased rapidly over the 84 days post partum ($P<0.001$) and then at a reduced rate between the ages of 84 and 188 days. A further significant increase ($P<0.05$) occurred after 508 days.

In the soleus muscle the two fibre populations grew in diameter at a fast rate during the first 84 days. The SO fibres reached a peak diameter by 299 days (Table 3.5).

Table 3.4 Diameter of FOG, FG and SO fibres in the extensor digitorum longus of the developing and ageing rat

Age (Days)	No. of Muscles	FOG Diameter (μm)			FG Diameter (μm)			SO Diameter (μm)		
		Diameter	± S.D.	Pr. <	Diameter	± S.D.	Pr. <	Diameter	± S.D.	Pr. <
21	4	19.0	1.0	0.001	26.9	1.3	0.001	20.7	0.8	0.001
84	5	42.8	0.9	0.002	60.1	1.8	0.001	35.6	1.3	0.05
188	5	50.6	1.7	n.s.	69.9	3.1	n.s.	38.3	1.7	n.s.
299 ± 1	5	48.1	4.8	n.s.	71.4	3.1	n.s.	38.2	2.1	n.s.
508	5	51.7	8.7	n.s.	73.7	8.5	n.s.	40.3	3.0	0.05
716-745	8	48.3	5.2		75.9	7.2		43.0	2.3	

Table 3.5 Diameters of FOG and SO fibres in the soleus of the developing and ageing rat

Age (Days)	No. of Muscles	FOG Diameters (μm)			SO Diameters (μm)		
		Mean Fibre Diameter	± S.D.	Pr. <	Mean Fibre Diameter	± S.D.	Pr. <
21	5	25.0	2.3	0.001	32.2	2.8	0.001
84	5	56.3	2.8	0.001	65.6	2.0	n.s.
188	5	65.4	2.4	0.001	68.5	3.1	0.001
299 ± 1	4	74.5	3.9	n.s.	79.8	3.6	0.05
508	4	74.1	3.3	0.001	74.6	2.5	n.s.
716-745	5	47.6	6.6		73.6	4.4	

However, the a priori comparison revealed a significant decrease in their diameter ($P < 0.05$) after the age of 299 days. FOG fibres demonstrated an increase in mean fibre diameter from birth to 299 days of age thereafter the mean diameter remained almost unchanged up to 508 days, this was followed by a very considerable (35.8%) reduction in mean fibre diameter in senile animals aged 716 days ($P < 0.001$).

5. Other structural changes

Longitudinal splitting of muscle fibres occurred more frequently in senile muscles (Plate 3.2). The percentage of split fibres was difficult to quantify, because this would have entailed the examination of many serial sections. Nevertheless the number of splitting fibres observed in a few sections confirmed that older muscles were very susceptible to this condition. The extensor digitorum longus and soleus were alike in showing splitting and fibre degeneration (Plate 3.2). Again no attempt was made to quantify the extent of degeneration and hypertrophied fibres (Plate 3.2) in either muscle. Because of the localisation of these it was almost impossible to devise a suitable sampling technique. However, when young muscles were compared with senile ones the differences were obvious.

6. Muscle fibre diameter frequency analysis

An examination of fibre diameter distribution within the extensor digitorum longus and the soleus revealed that every fibre type usually occupied a well-defined range of diameters. The size distribution of each type approximated to a normal distribution.

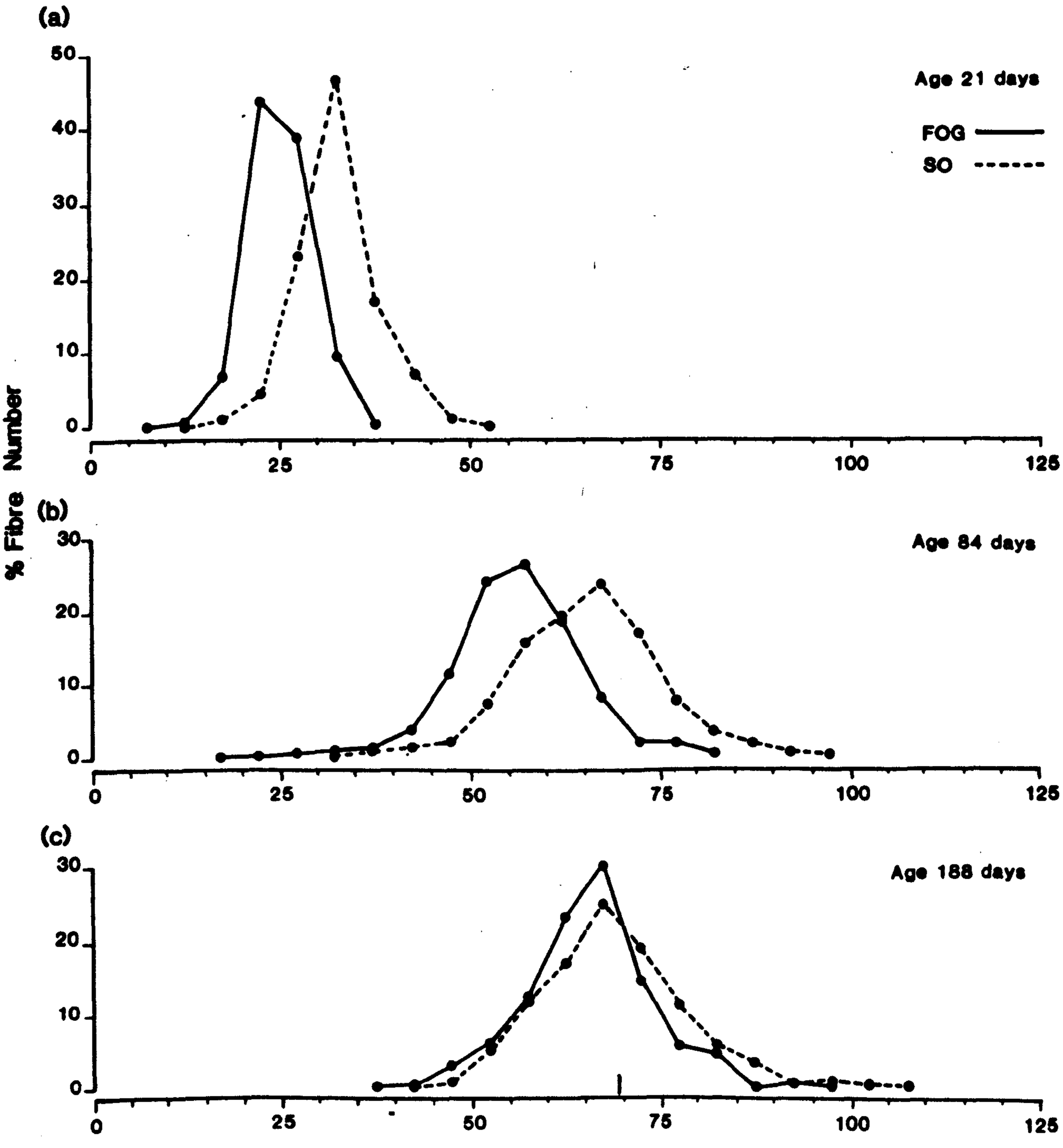
The results of fibre distribution of FOG and SO fibres within the soleus are displayed as polygons (Fig. 3.1a). Although slow and fast fibres overlap in their distribution, both had distinct modes with the

slow fibre population initially possessing the highest modal diameter. But because of an uneven rate of growth of the two fibre types peaks changed in respect to each other. A complete overlay of the two populations was evident by 188 days of age as the fast fibres had caught up (Fig. 3.1c). Slow fibres continued to show a shift towards larger diameters (Fig. 3.1d), this trend was reversed in old (508 days) and senile (716 days) animals (Fig. 3.1e,f). The slow fibres were less affected by the ageing process in later life. Indeed the FOG population in senile animals showed a totally disrupted distribution with no really distinct single peak. The tails of fast and slow populations in elderly and senile animals were exceedingly flat and covered a wide range of diameters (Fig. 3.1f). The tails no doubt correspond to hypertrophied fibres at one end and splitting fibres on the other end. The distribution therefore reflects the earlier qualitative descriptions of the senile soleus.

In the extensor digitorum longus all three fibre populations showed a normal distribution during the first 188 days post partum. In the youngest muscles examined the three different fibre types had mode diameter values in the order of FOG, SO and FG the latter being of the largest diameters (Fig. 3.2a). This sequence changed by 84 days to SO, FOG and FG (Fig. 3.2b,c). The distribution of all populations shifted towards increased diamters with age. Peak frequencies of FOG and FG fibres remained unchanged from 299 days of age to senility (Fig. 3.2d). However, the SO peak frequency continued to shift towards larger diameters.

As the animals aged the FG and particularly FOG distributions deviated markedly from a normal distribution giving rise to assymetrical tails. This situation was most obvious in 508 and 716 day old animals (Fig. 3.2e,f). Fibre diameter ranges in senile and old animals were exceedingly wide, this once again probably reflects the presence of hypertrophied and degenerating fibres.

Fig. 3.1 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.


(a)
Age 21 days
FOG
SO
% Fibre Number
50
40
30
20
10
0
0
25
50
75
100
125
(b)
Age 84 days
30
20
10
0
0
25
50
75
100
125
(c)
Age 188 days
30
20
10
0
0
25
50
75
100
125
Fibre Diameter (µm)

cont.

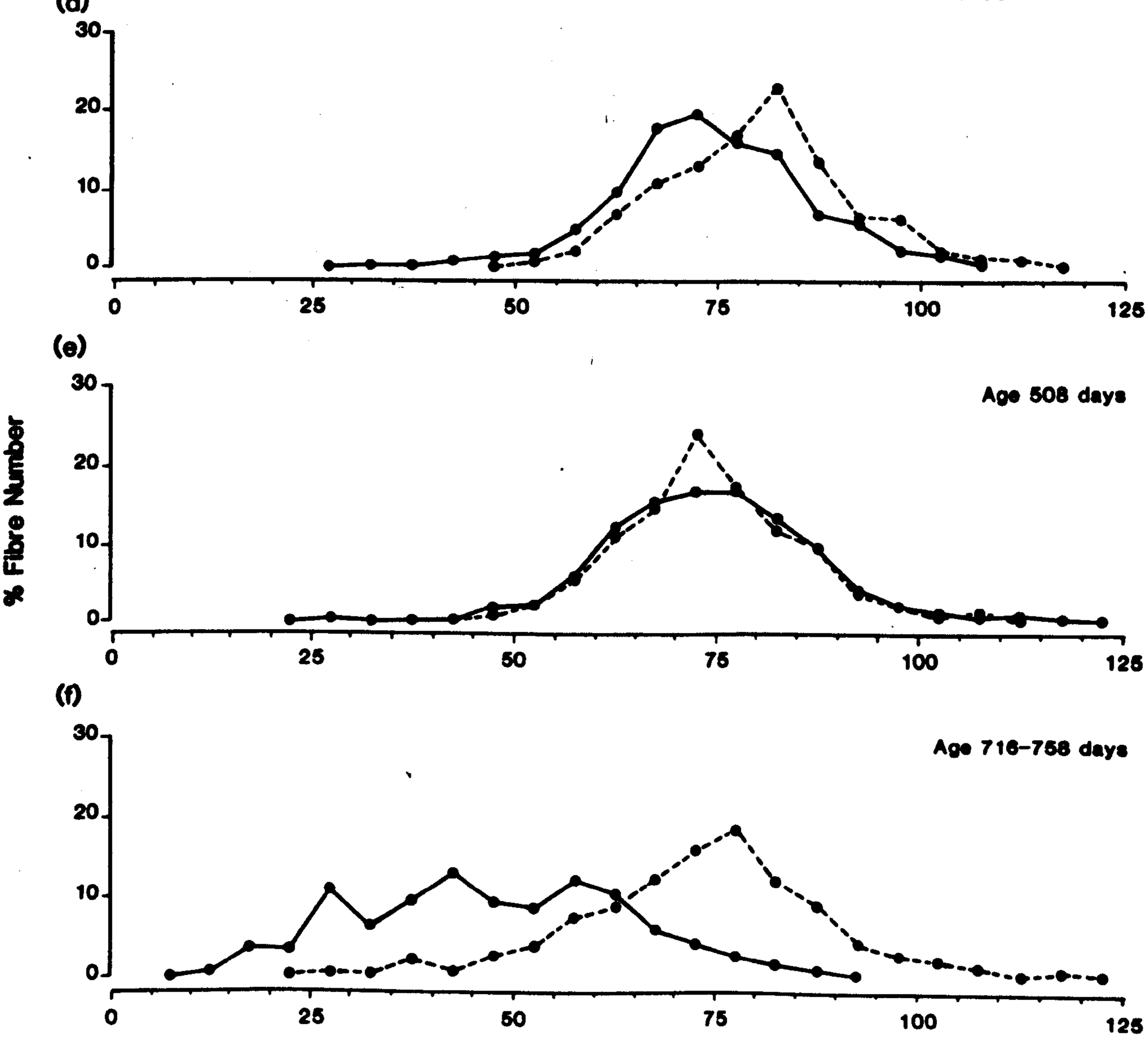
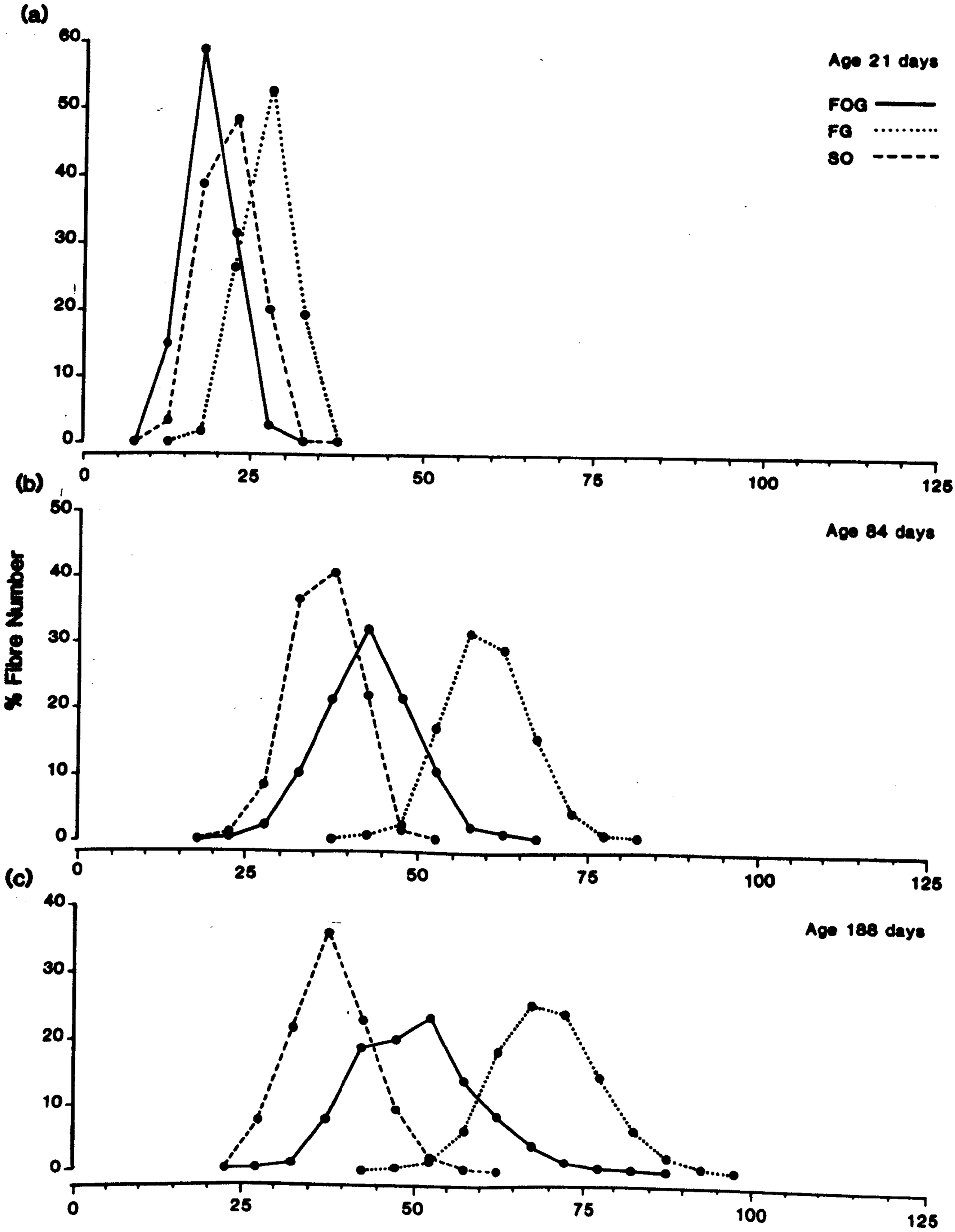
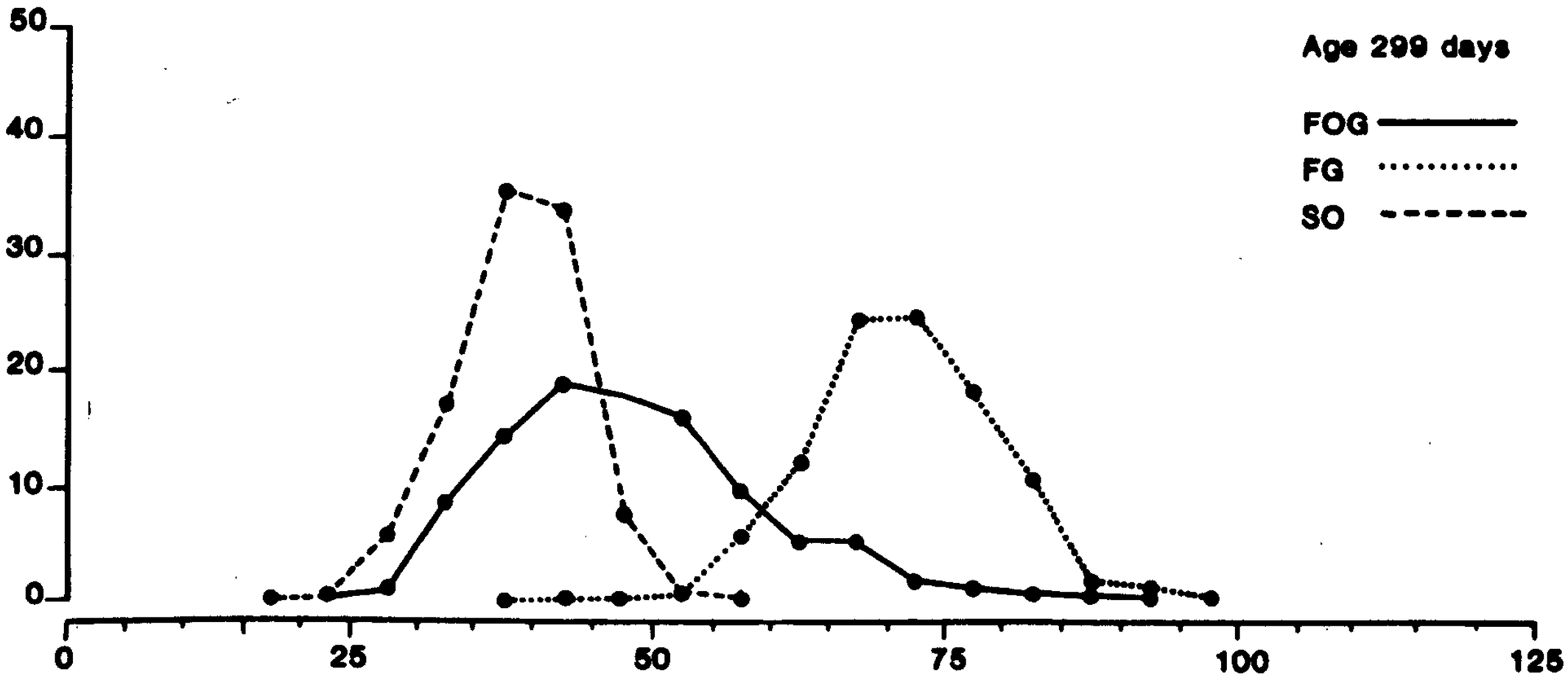

Age 299 days
FOG
SO
(d)
30
20
10
0
0
25
50
75
100
125
(e)
Age 508 days
% Fibre Number
30
20
10
0
0
25
50
75
100
125
(f)
Age 716–758 days
30
20
10
0
0
25
50
75
100
125
Fibre Diameter (µm)

Fig. 3.2 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.

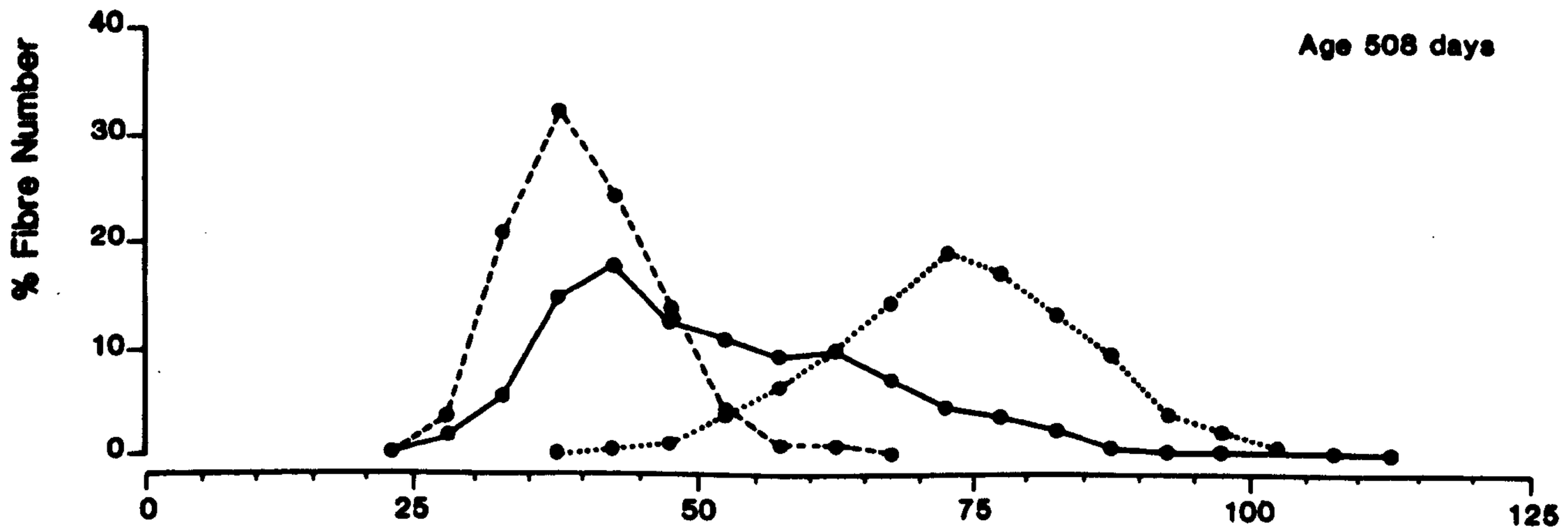

(a)
Age 21 days
FOG
FG
SO
60
50
40
30
20
10
0
0
25
50
75
100
125
(b)
Age 84 days
% Fibre Number
50
40
30
20
10
0
0
25
50
75
100
125
(c)
Age 188 days
40
30
20
10
0
0
25
50
75
100
125
Fibre Diameter (µm)

cont.

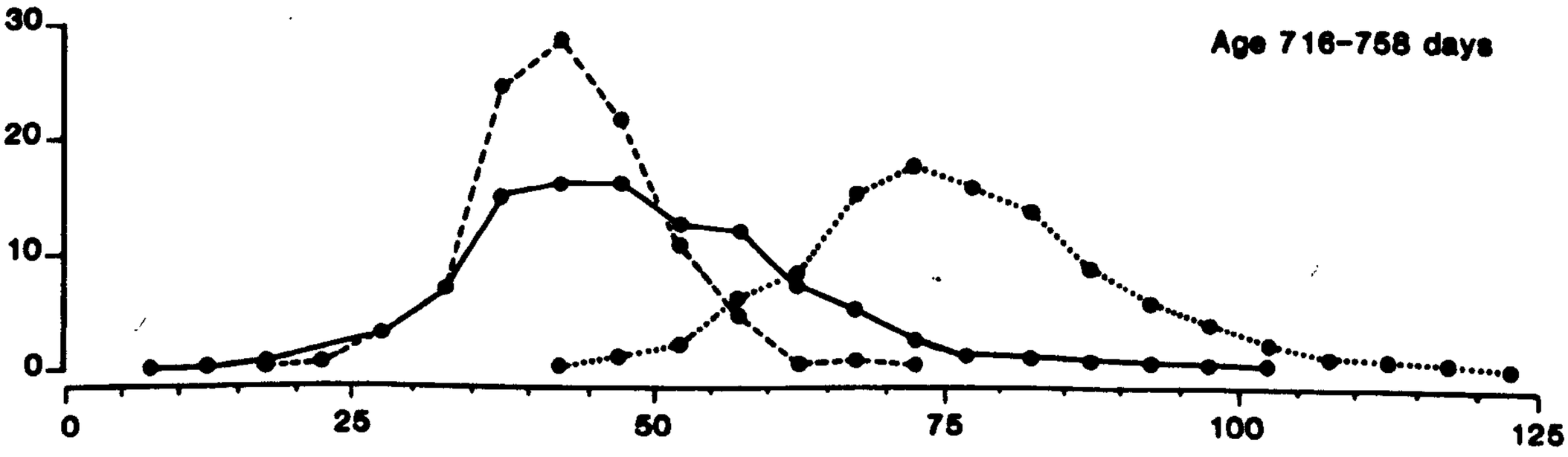
(d)



(e)



(f)



Fibre Diameter (µm)

The differences between fibre populations within pooled age groups was tested statistically with a Kolmogorov-Smirnov Two Sample Test. The test confirmed the existance of different fibre type populations within both the soleus and the extensor digitorum longus. Of course, when the distributions of the different fibre populations coincided the test failed to detect separate populations.

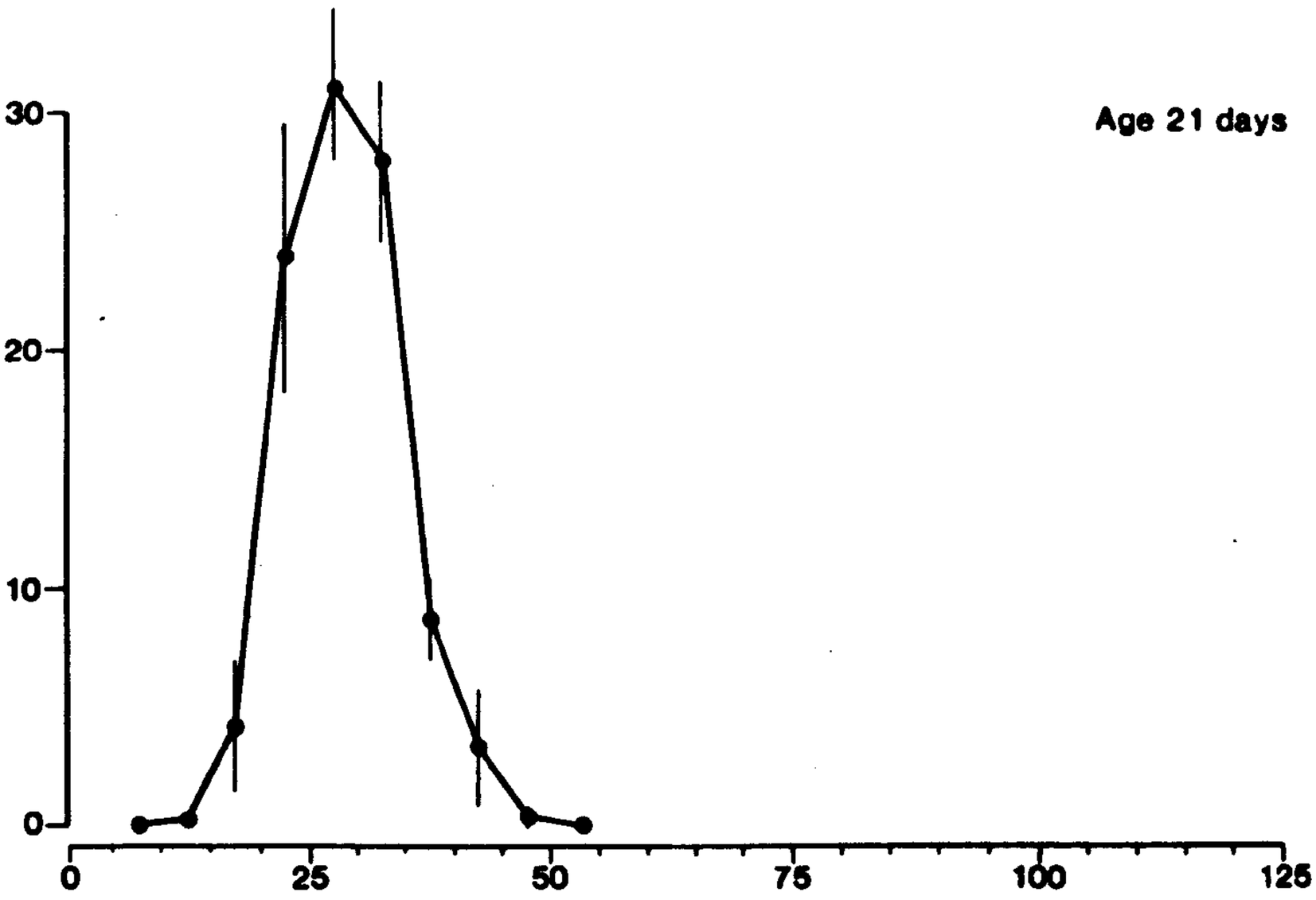
Composite polygons

Composite polygons were constructed from fibre type ratios and frequency distribution for the soleus and the extensor digitorum longus. The reconstructions estimated 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. 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. 3.3a,b,c,d and f).

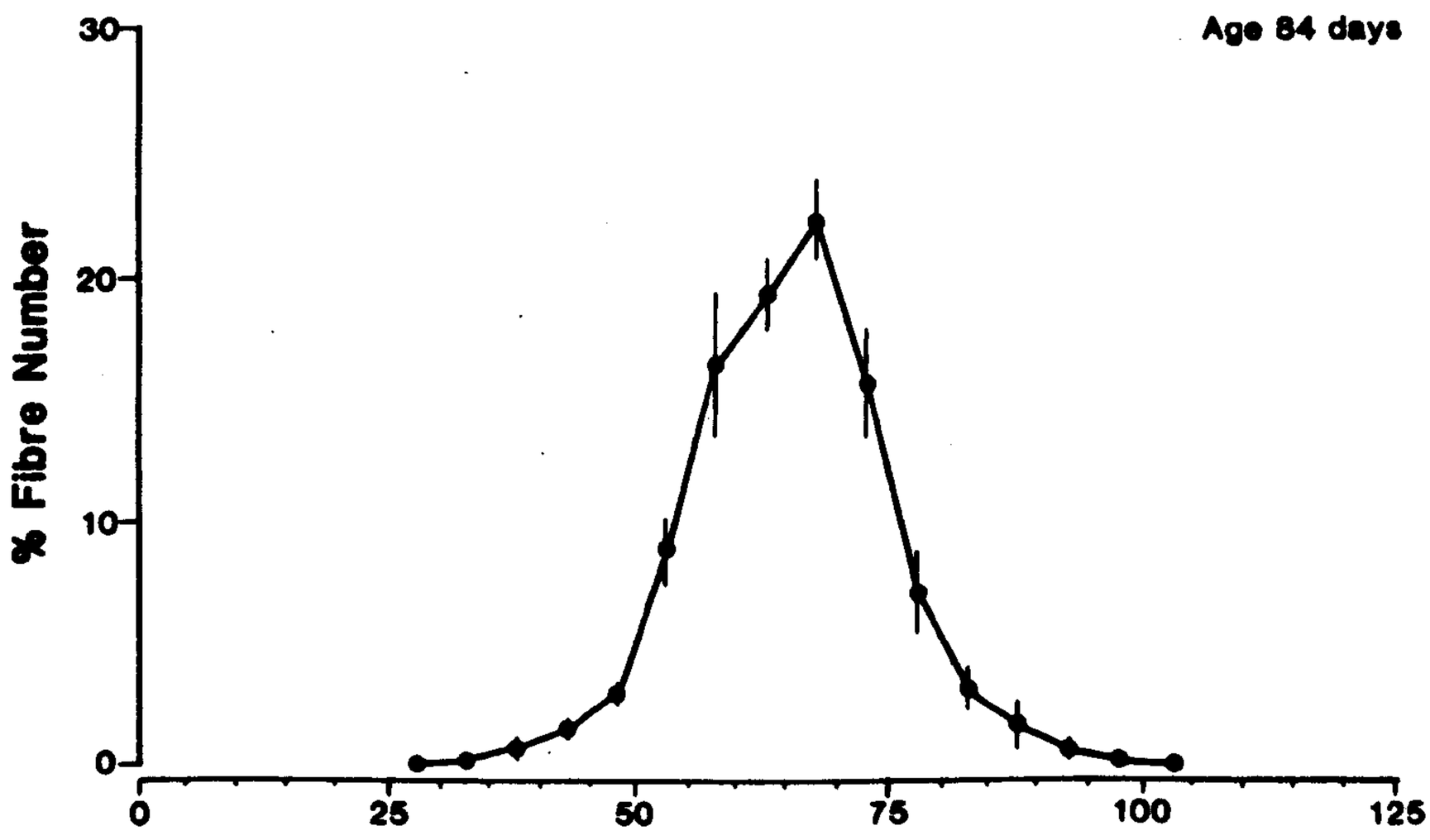
The reconstruction of extensor digitorum longus polygons however, revealed interesting distributions. In younger animals the distribution approximated to a normal one because of the close proximity of the modes of the constituent fibre populations. As the differences between the mean fibre diameter increased the normal distribution gradually transformed into a bimodal one (Fig. 3.4a,b,c,d). Animals aged 299 days demonstrated a statistically significant bimodal distribution ($P < 0.05$). Two peaks corresponding to the positions of the FOG and FG peak frequencies was readily identifiable (Fig. 3.4d). In older animals (508 days) and senile animals (716 days) the bimodality was progressively eroded until a skewed distribution with a peak and plateau was left in 716 days old animals (Fig. 3.4d,e and f). This was due mainly to the increased variability of FOG fibre diameters (Fig. 3.2e,f).

Fig. 3.3 Composite frequency distribution of fibre diameters in the soleus of developing and ageing rats. The distribution allows for the ratio of the two fibre populations occurring in the soleus.

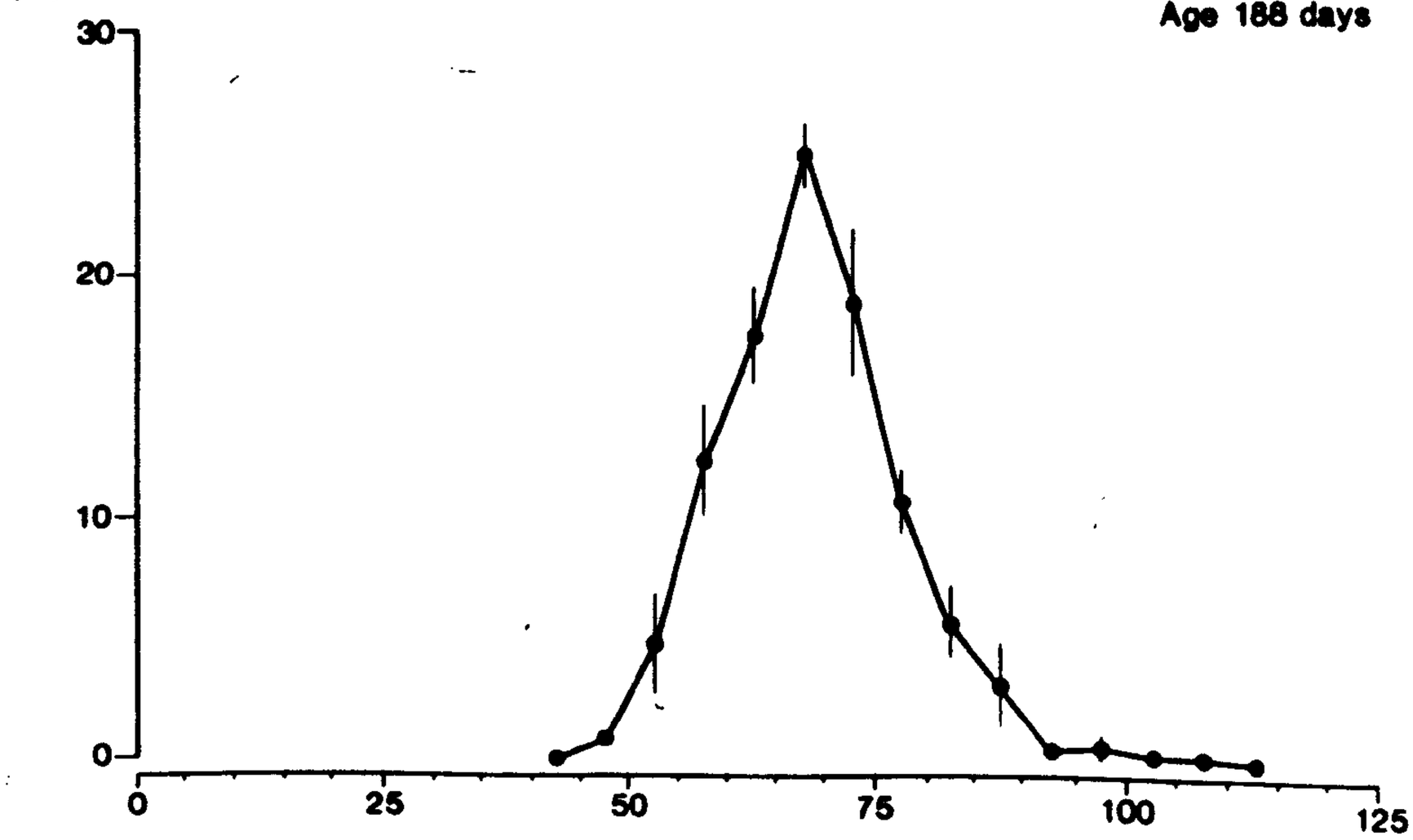
(a)


Age 21 days
30
20
10
0
0
25
50
75
100
125

(b)

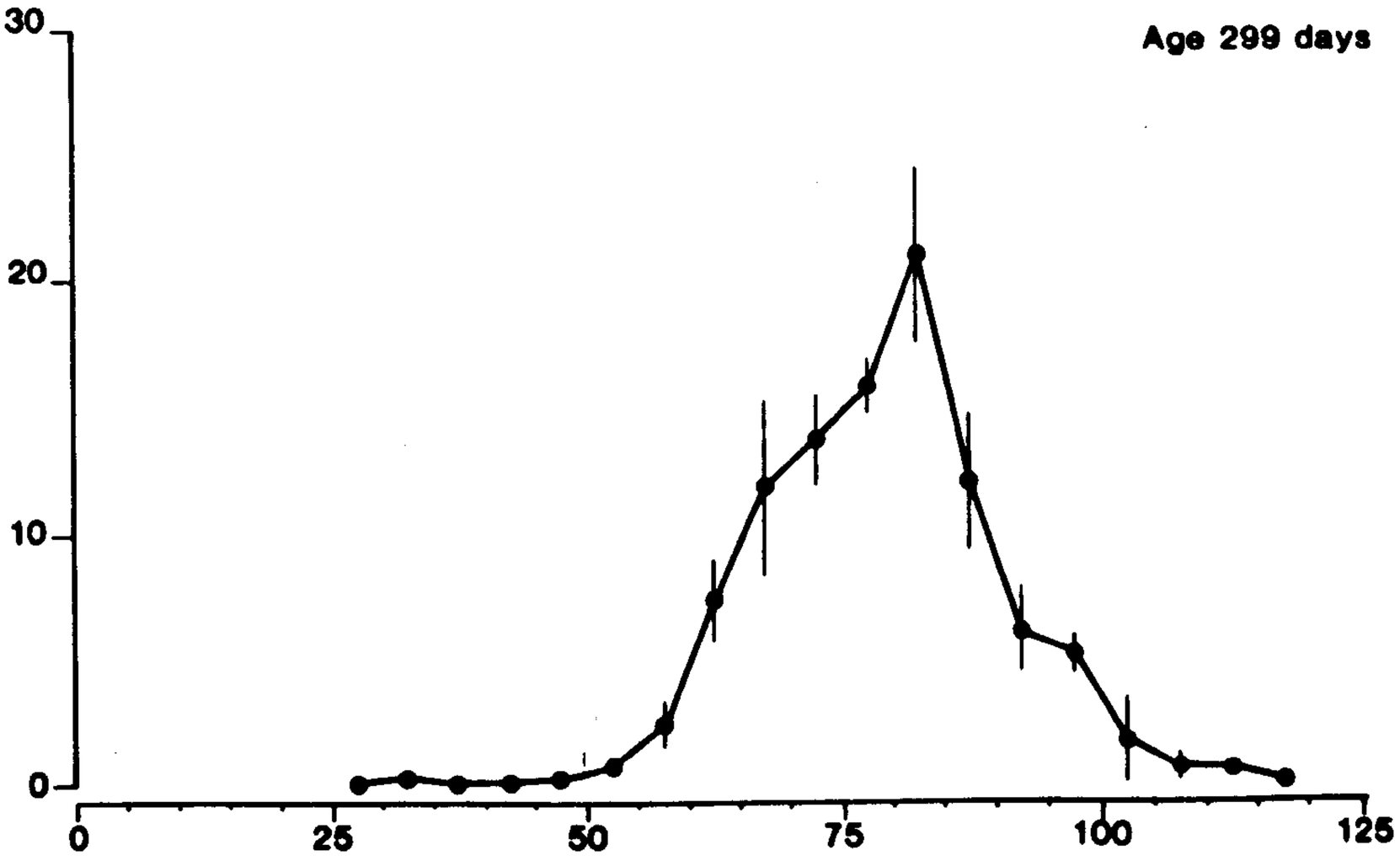

Age 84 days
% Fibre Number
30
20
10
0
0
25
50
75
100
125

(c)

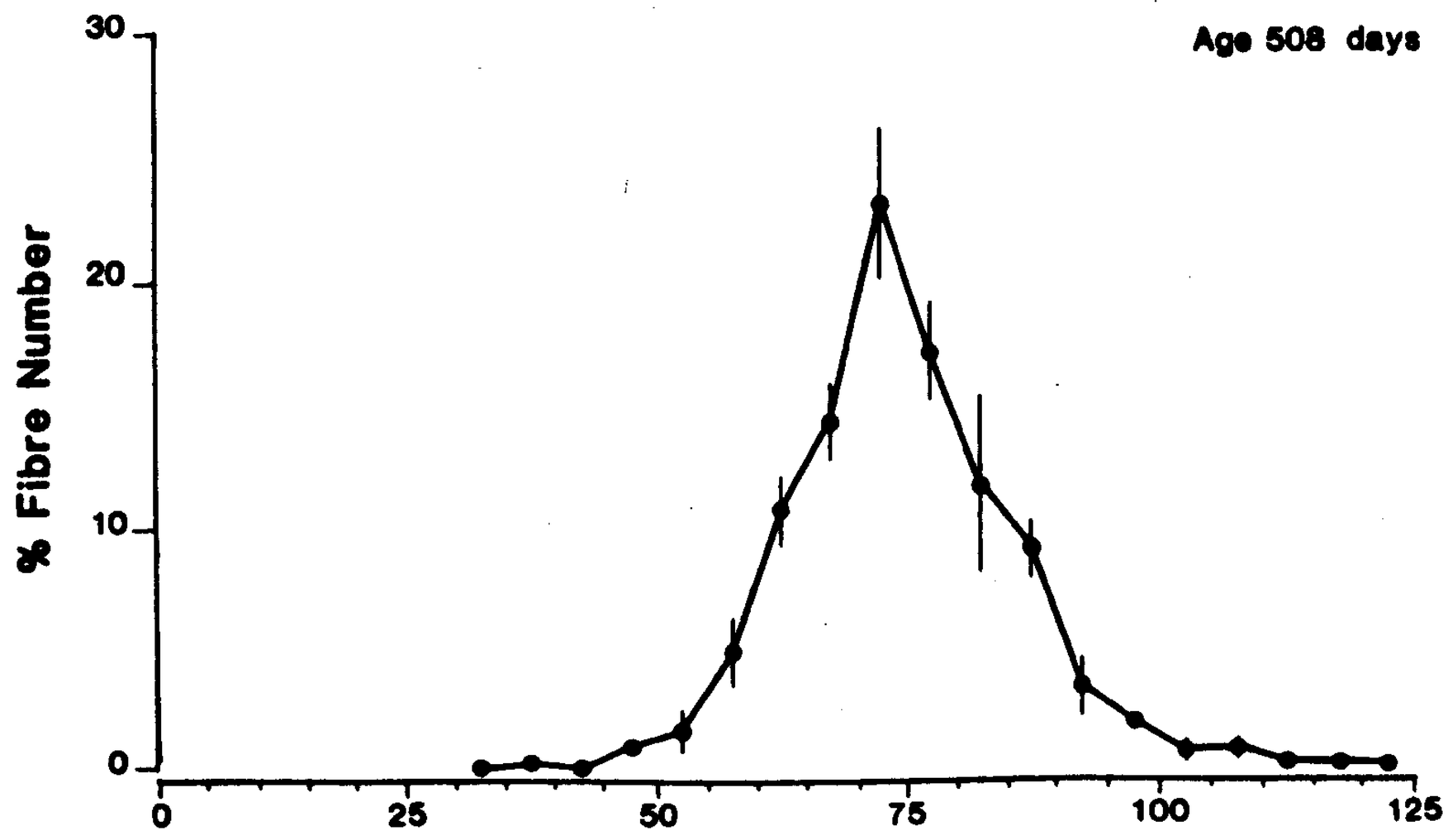

Age 188 days
30
20
10
0
0
25
50
75
100
125
Fibre Diameter (µm)

cont.

(d)


Age 299 days
30
20
10
0
0
25
50
75
100
125

(e)


Age 508 days
% Fibre Number
30
20
10
0
0
25
50
75
100
125

(f)

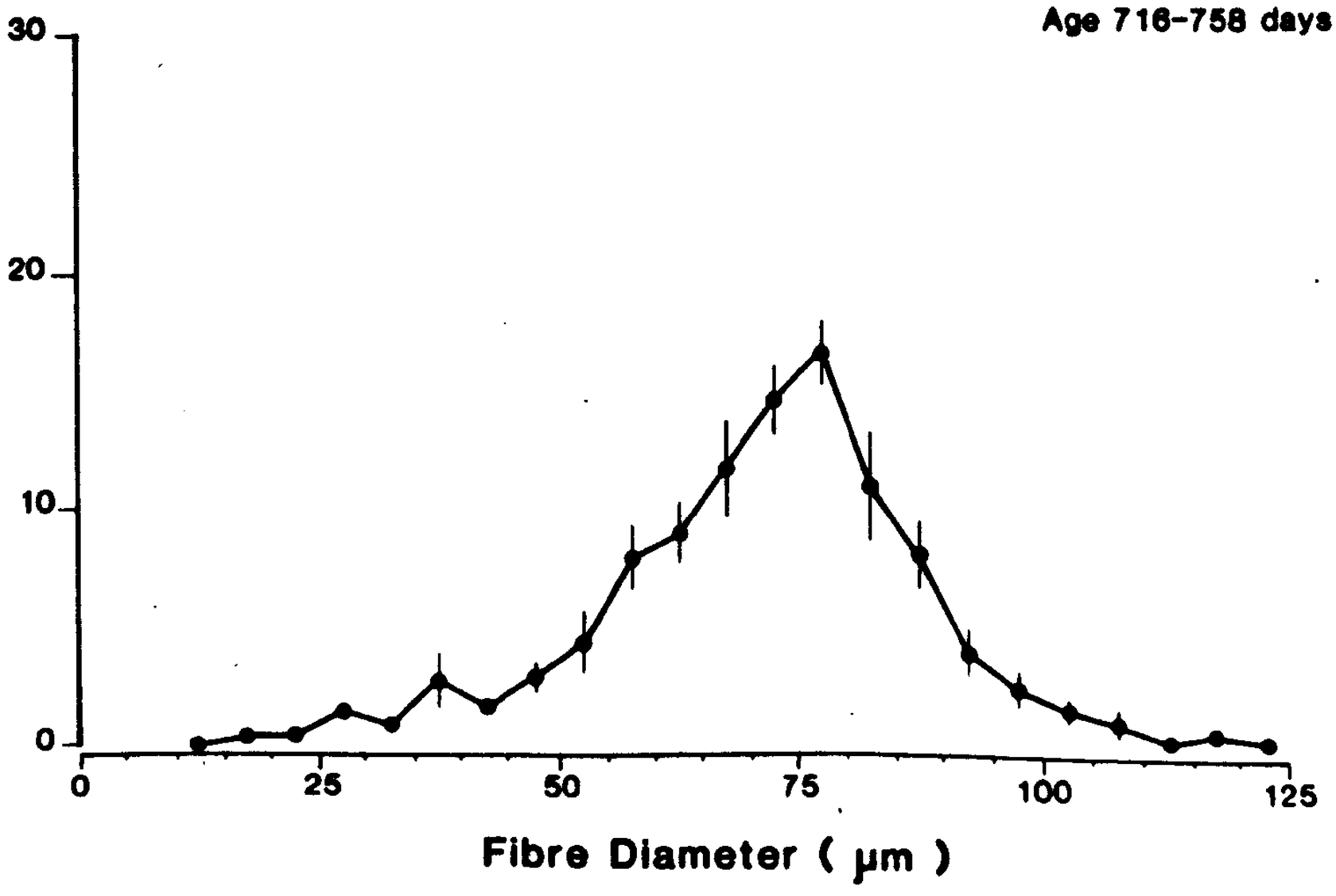
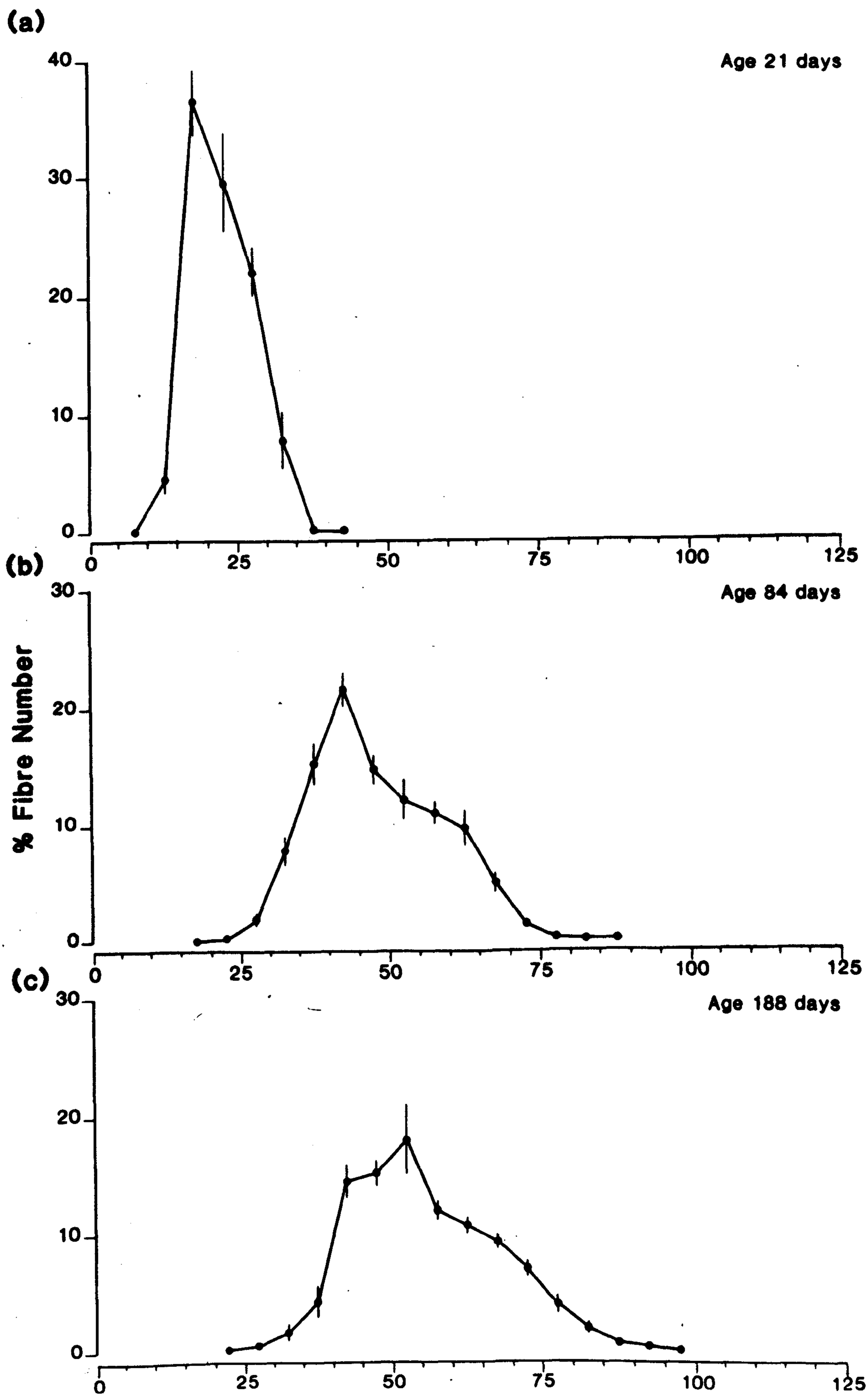
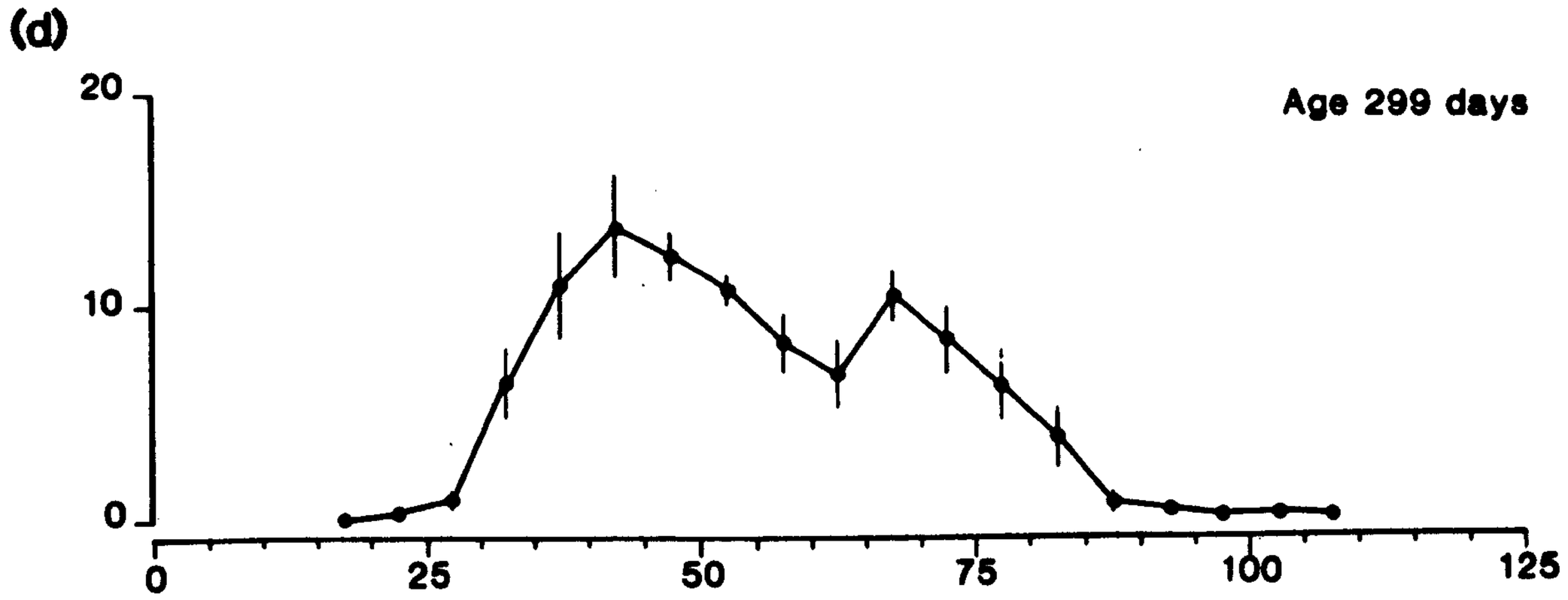
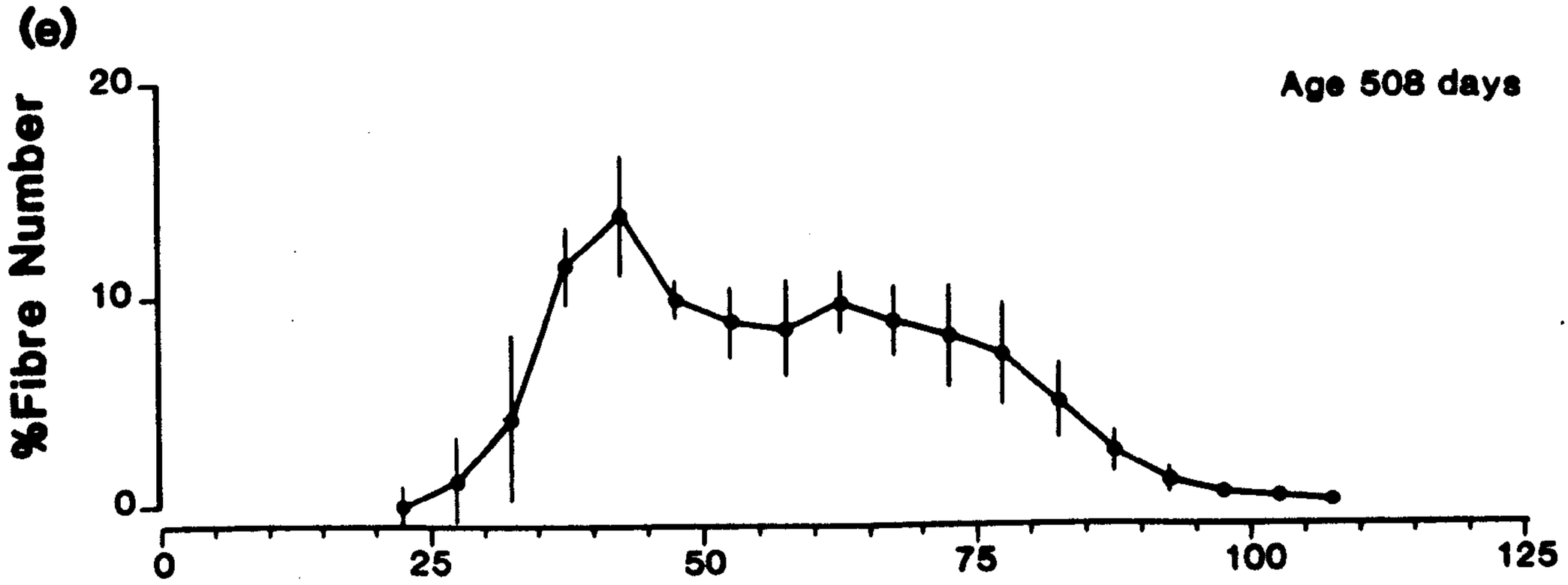

Age 716-758 days
30
20
10
0
0
25
50
75
100
125
Fibre Diameter (µm)

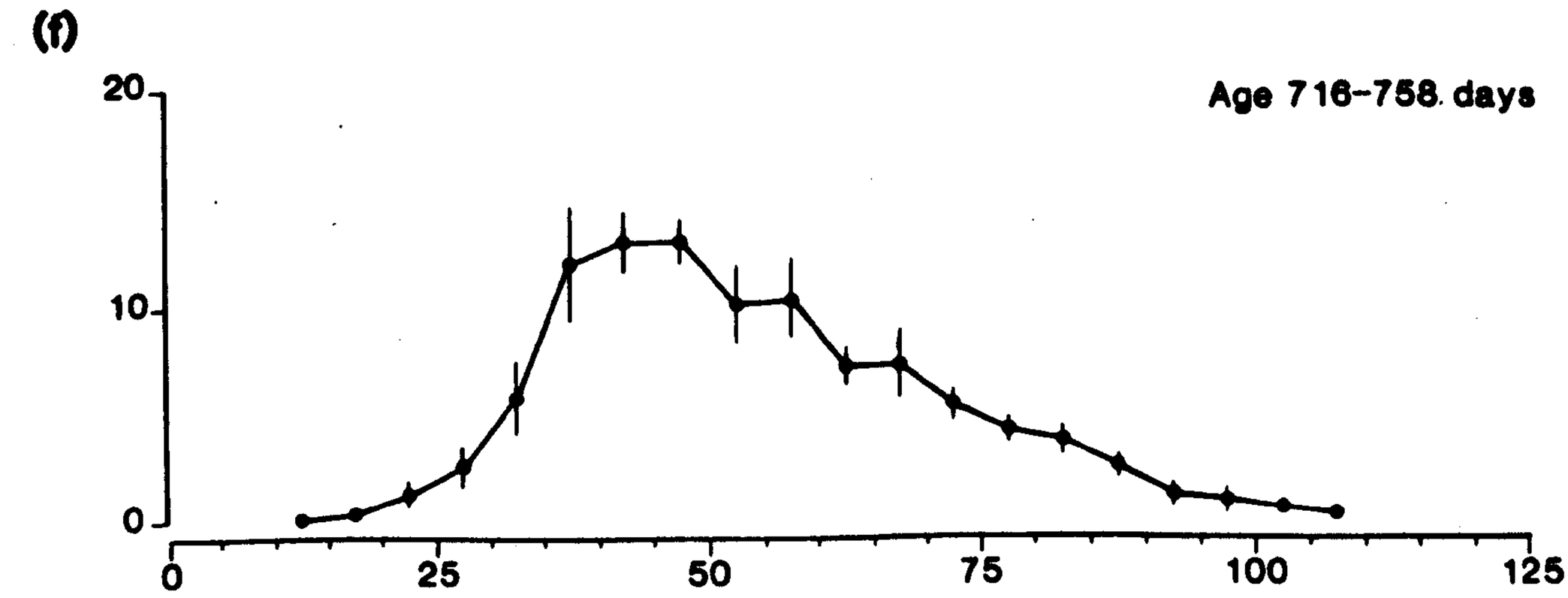
Fig. 3.4 Composite frequency distribution of fibre diameters in the extensor digitorum longus of developing and ageing rats. The distribution allows for the ratio of the three fibre populations occurring in the extensor digitorum longus. Note the slow development of a bimodal distribution which becomes clear at 299 days and is later modified in senile animals.


(a)
Age 21 days
40
30
20
10
0
0
25
50
75
100
125
(b)
Age 84 days
% Fibre Number
30
20
10
0
0
25
50
75
100
125
(c)
Age 188 days
30
20
10
0
0
25
50
75
100
125
Fibre Diameter (µm)

cont.


(d)
Age 299 days
20
10
0
0
25
50
75
100
125


(e)
Age 508 days
%Fibre Number
20
10
0
0
25
50
75
100
125


(f)
Age 716-758 days
20
10
0
0
25
50
75
100
125
Fibre Diameter (µm)

7. Muscle fibre number

The analysis of variance failed to detect any significant difference in the total fibre number in the soleus with age. Nevertheless there seemed to be a trend towards a reduced mean fibre number in senile animals (Table 3.6).

The underlying number of SO and FOG fibres showed a rapid transformation from an almost equal population of both types of fibres (Table 3.6) to a muscle predominately made up of SO fibres; the change was complete by 84 days post partum. Thereafter FOG fibres showed a small but insignificant increase in their number up to 299 days and then remained virtually unchanged up to at least 716 days. SO fibres on the other hand were reduced in number significantly over the period between 188 to 299 days ($P < 0.05$), but did not demonstrate any significant alteration in their number in older animals. The earlier decrease in SO fibres numbers was not detected in the total fibre number because of a corresponding increase in FOG fibre numbers over the same period.

Total fibre number in the extensor digitorum longus decreased dramatically between the ages of 21 and 188 days ($P < 0.001$). The remaining fibres (Table 3.7), represented 54.7% of the original population. The small changes in fibre number between remaining age groups were not statistically significant. Both FOG and FG fibres in the extensor digitorum longus decreased significantly ($P < 0.001$) during the 188 days after birth. SO fibres, which represent only a very small percentage of the total population tended to decrease in number over the same period. The increase in FOG fibre number at 716 days appears to correspond to a decrease in FG fibres. However the changes in the numbers of the three fibre types between the ages of 188 to 716 days were not statistically significant.

Although the number of FG fibres was usually smaller than FOG fibres the cross-sectional area that the FG fibres occupied was 37.9 and 54.7% of the

Table 3.6 Total fibre number and the number of individual fibre types in the developing and ageing soleus

Age (Days)	No. of Muscles	FOG fibre number			SO fibre number			Total fibre number		
		Mean	± S.D.	Pr. <	Mean	± S.D.	Pr. <	Mean	± S.D.	Pr. <
21	5	1345	169	0.001	1395	107	0.001	2740	245	n.s.
84	5	261	67	n.s.	2628	97	n.s.	2889	97	n.s.
188	5	166	94	n.s.	2587	258	0.05	2753	272	n.s.
299 ± 1	4	341	150	n.s.	2164	419	n.s.	2505	276	n.s.
508	4	323	131	n.s.	2237	204	n.s.	2561	265	n.s.
716-745	7	299	169		2226	277		2525	234	

Table 3.7 Total fibre number and the number of individual fibre types in the developing and ageing extensor digitorum longus

Age (Days)	No. of Muscles	FOG fibre number			FG fibre number			SO fibre number			Total fibre number		
		Mean	± S.D.	Pr. <	Mean	± S.D.	Pr. <	Mean	± S.D.	Pr. <	Mean	± S.D.	Pr. <
21	4	3057	647	0.001	1905	181	0.001	112	47	n.s.	5074	762	0.001
84	5	2490	200	0.05	1194	115	0.001	125	62	n.s.	3808	313	0.001
188	5	2027	156	n.s.	664	159	n.s.	84	22	n.s.	2775	126	n.s.
299 ± 1	5	1988	181	n.s.	916	304	n.s.	70	17	n.s.	2974	232	n.s.
508	5	2096	279	n.s.	687	221	n.s.	81	24	n.s.	2864	182	n.s.
716-745	9	2215	234		626	172		69	28		2990	333	

Table 3.8 Contribution of the three main fibre types to the cross-sectional area of the developing and ageing extensor digitorum longus

Age (Days)	FOG fibres		FG fibres		SO fibres	
	x.s. Area mm^2	% Total x.s.Area	x.s. Area mm^2	% Total x.s.Area	x.s. Area mm^2	% Total x.s.Area
21	0.8632	43.44	1.0861	54.66	0.0376	1.89
84	3.5685	50.39	3.3895	47.86	0.1237	1.75
188	4.0825	60.66	2.5510	37.90	0.0971	1.44
299 $\pm$ 1	3.6090	49.08	3.6639	49.82	0.0806	1.10
508	4.4043	59.20	2.9325	39.42	0.1029	1.38
716-745	4.0551	58.04	2.8312	40.52	0.1002	1.43

total muscle area (Table 3.8). The reason for this was the larger fibre diameter of FG fibres as compared to the FOG fibres.

8. Nuclear DNA content

Total DNA content in the soleus increased significantly (372%) between the ages of 21 and 90 days (Table 3.9). However, when DNA content was related to protein the trend was reversed. Protein content increased at a higher rate than DNA between the ages of 21 and 90 days giving a lower DNA/protein ratio in older animals.

Total DNA content in the extensor digitorum longus increased significantly ($P < 0.001$) between the ages of 21 and 90 days (Table 3.9) then dropped sharply by 244 days of age ($P < 0.005$). The relative quantity of DNA to protein decreased continuously up to 244 days. Only the initial decrease in ratio between the ages of 21 to 90 days was significant ($P < 0.001$).

Although the total DNA content in the EDL was less than that in the soleus, the ratio of DNA to protein in both muscles was the same at 21 days of age. Interestingly during later life the ratio was lower in the extensor digitorum longus as compared to the soleus of similar ages.

9. Nerve fibre number

The number of nerve fibres in the nerve supplying the extensor digitorum longus and the soleus did not change significantly with age (Table 3.10). The slight differences between the young animals and mature ones are probably a result of the extremely small axon size in young animals and the limited resolution of the light microscope (Plate 3.3).

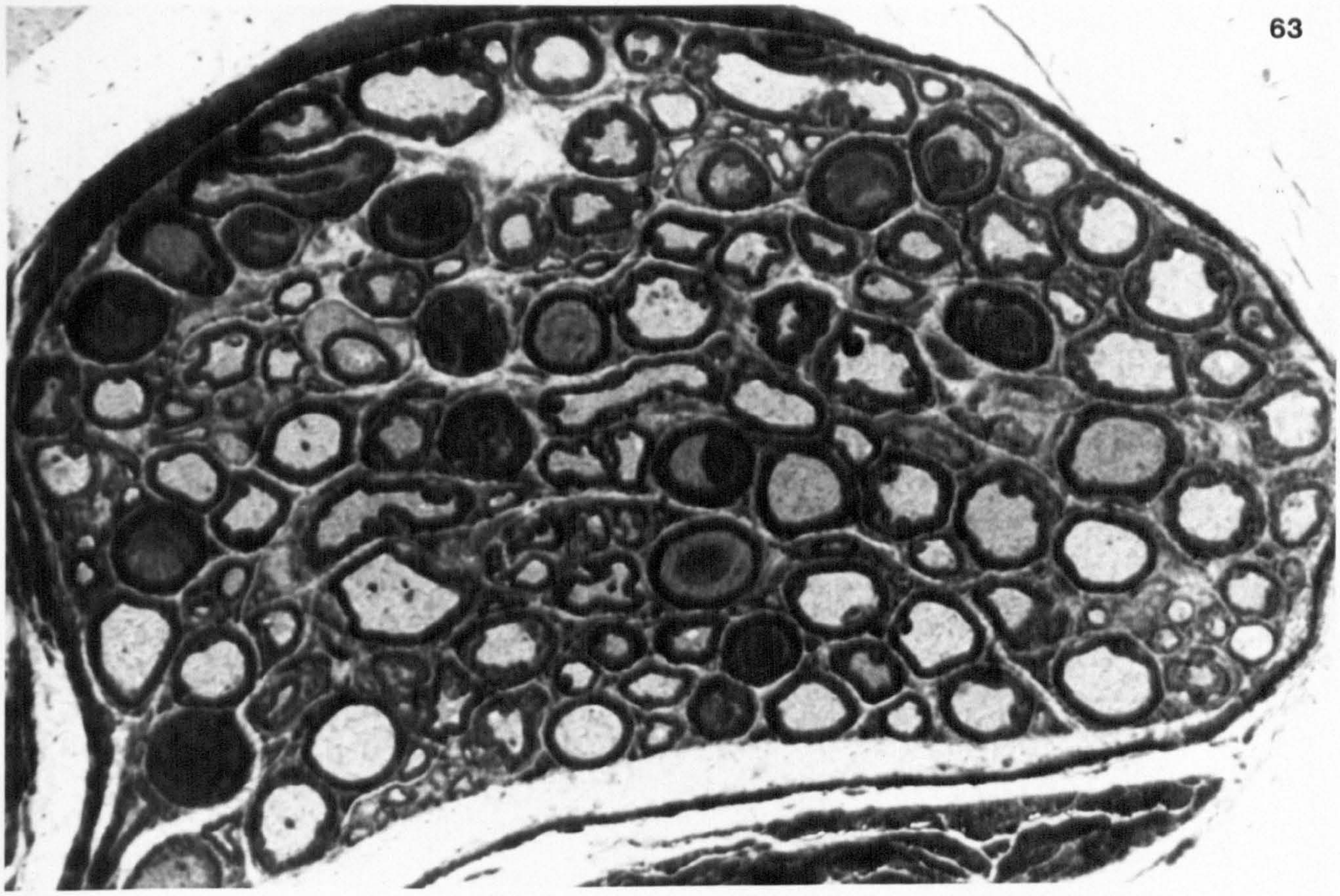
DISCUSSION

A continuous increase in body weight of aged rats up to 716 days in this study was not unexpected. The number of animals housed in the same cage was found to influence the rate at which body weight changes with age. Individually housed CFY rats have been shown to continue increasing in weight reaching a maximum value around 700 days after which they start wasting rapidly (Merry and Holehan 1981). The high mortality rate in

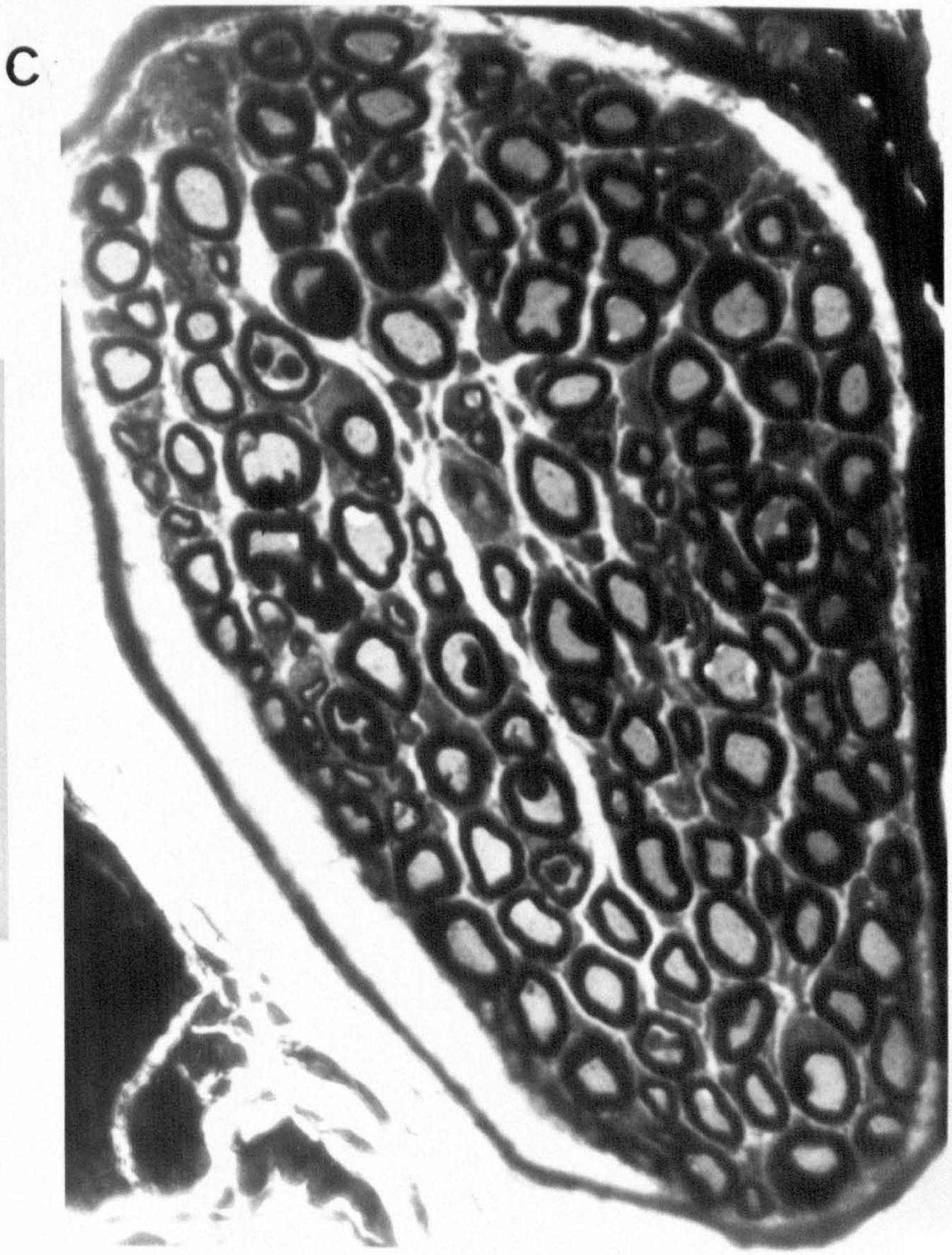
Table 3.9 Absolute and relative DNA content of the extensor digitorum longus and soleus

Age (Days)	No. of Muscles	extensor digitorum longus				soleus			
		Total DNA µg ± S.D.	Pr. <	µg DNA/mg Protein ± S.D.	Pr. <	Total DNA µg ± S.D.	Pr. <	µg DNA/mg Protein ± S.D.	Pr. <
21	5	30.90 ± 4.88	0.001	12.32 ± 1.31	0.001	80.40 ± 6.58	0.001	12.19 ± 0.86	0.001
90	5	170.40 ± 20.66	0.005	2.73 ± 0.32	n.s.	298.80 ± 31.23	n.s.	4.96 ± 0.62	n.s.
244	5	126.00 ± 26.88		2.00 ± 0.49		277.80 ± 23.18		4.46 ± 0.54	

Plate 3.3 The number of nerve fibres supplying the extensor digitorum longus and the soleus did not change significantly with age The distinction between myelinated and non-myelinated nerves in young animals (21 days) was not always possible (a). The differences were clearer in older animals; 388 days (b) and 714 days (c) magnification x580.



b



c

a

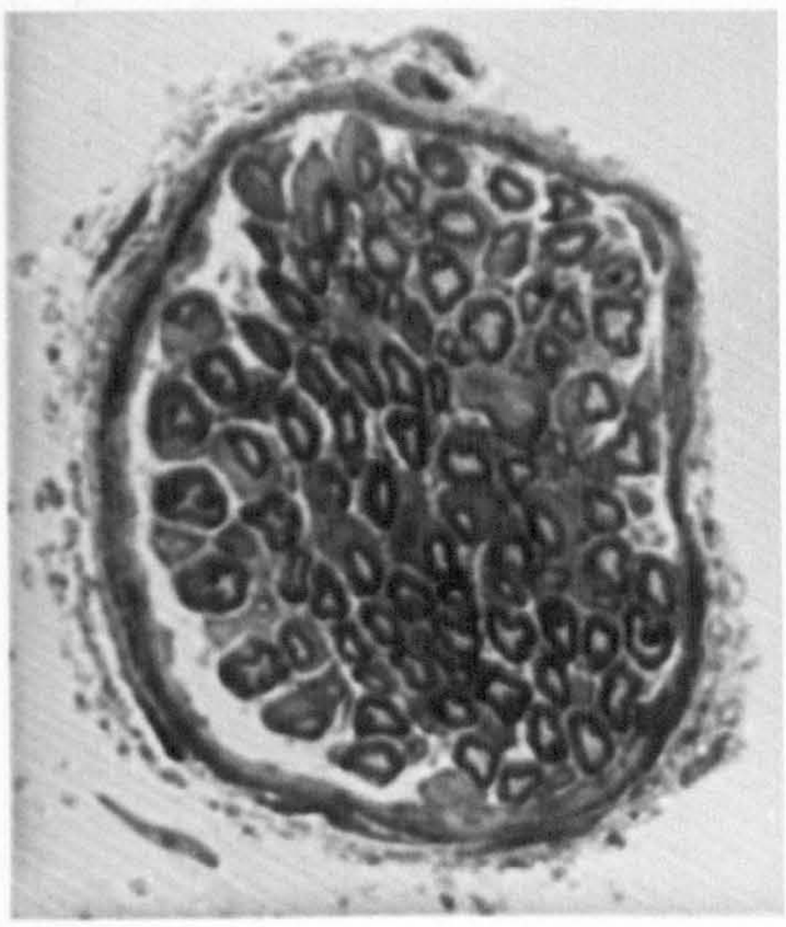


Table 3.10 Number of axons within nerves supplying the extensor digitorum longus and soleus

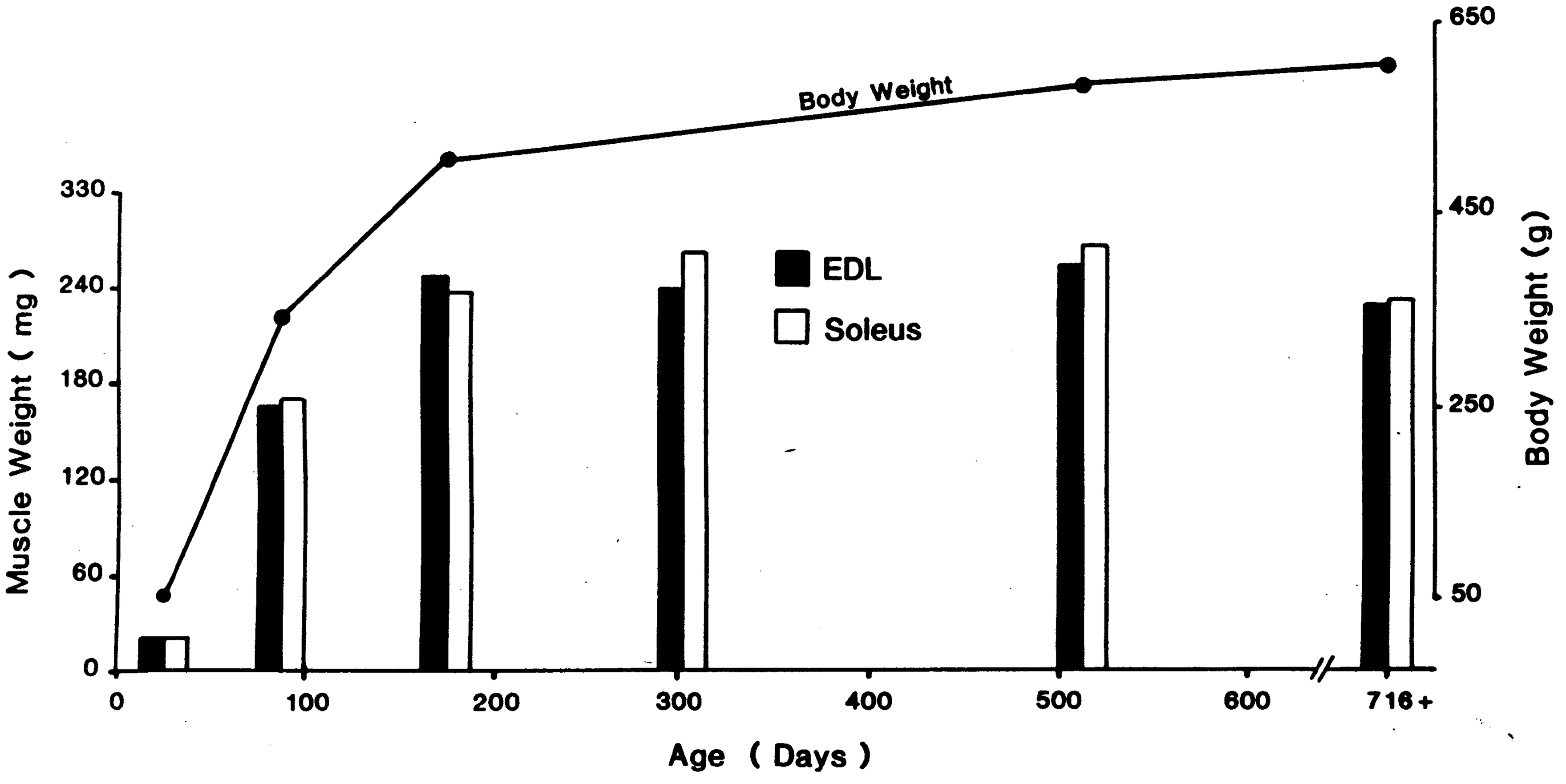
Age (Days)	No. of Nerves	Extensor digitorum longus			Soleus		
		Mean fibre no.	± S.D.	Pr.<	Mean fibre no.	± S.D.	Pr. <
21	5	87	6	n.s.	144	6	n.s.
388±2	5	97	9	n.s.	151	9	n.s.
714±6	6	92	8		139	15	

older animals introduced variability in the number of animals in any given cage, thus a mixture of solitary and grouped animals was unavoidable especially at later ages. Skeletal muscles and the heart followed similar patterns in young animals, increasing by several hundred percent over a relatively short period of time. A similar trend has been reported for a variety of muscles in other strains of laboratory rat (Enesco and Puddy 1964, McCafferty and Edington 1974, Sands et al 1979). However the trend changed somewhat in senile animals in that only the heart continued to increase in weight at a rate higher than that of body weight, this is in accord with earlier findings by McCafferty and Edington (1974). The weight of skeletal muscles investigated responded differently to age. Except for the extensor hallucis proprius, all the muscles examined decreased in weight but the magnitude of the reduction differed (Fig. 3.5). Skeletal muscles have been reported to waste at different rates (Rubinstein 1960 , Gutmann and Hanzlikova 1976) the rate being modified by their activity (Muravov 1969) which in older animals was rather limited. The bulkier muscles in this study responded more markedly to extreme age, the anterior tibialis losing on average 24.2% of its normal adult weight.

Changes in weight and cross-sectional area may be influenced by a change in the number of muscle fibres within the muscle or a change in individual fibre diameters or a combination of the two factors. Increases in muscle's cross-sectional area during growth appears to result mainly from massive increases in individual fibre diameters. The soleus showed no significant changes in fibre number throughout the ages examined, yet muscle weight increased considerably during development. The increase was accounted for by increases in the diameters of the two fibre populations making up the soleus.

The extensor digitorum longus showed a marked reduction in fibre number, however fibre diameter increased very considerably in this muscle

Fig. 3.5 Extensor digitorum longus and soleus weights of the developing and ageing rats and their body weights.


Body Weight
EDL
Soleus
Muscle Weight (mg)
330
240
180
120
60
0
650
450
250
50
Body Weight (g)
0
100
200
300
400
500
600
716+
Age (Days)

and in this way appeared to compensate for the muscle fibres lost during early development and the net effect was to increase muscle weight and cross-sectional area. The loss of muscle fibres in early life in some muscles have been reported by several workers (Ihemelandu 1980, Layman et al 1980). The nature of this change will be discussed later.

Decrease in cross-sectional area and weight of old and senile animals followed a complex pattern. Changes in fibre number and fibre diameter combined to influence the total cross-sectional area. Added complications were the varying degree of atrophy and compensatory hypertrophy of different fibre types , and the occurrence of splitting fibres which artificially increased the total fibre number. In the soleus for example, the number of FOG and SO fibres in this muscle decreased slightly in senile animals. The mean fibre diameter of FOG fibres decreased by 35.8% and this was due mainly to splitting, SO fibres on the other hand decreased marginally over the same period. More pronounced changes in SO mean fibre diameter was probably masked by 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 when compared with younger animals (Fig. 3.1f). So in a given population of fibres the net effect of atrophy, splitting and hypertrophy determines the degree of loss of cross-sectional area with age.

The extensor digitorum longus presented a different model. The two fibre types that decreased in number in senile animals, namely SO and FG fibres,continued to increase in mean fibre diameter. FOG fibres decreased in diameter but showed an increase in number. Whether the increase in mean fibre diameter in SO and FG fibres was due to functional hypertrophy caused by the increased work load on a declining fibre population is difficult to judge. Different types of muscle fibres have been shown to hypertrophy in response to increased functional load (Goldspink and Ward 1979). The response of ageing muscles to exercise

appears to be limited, with no significant increase in muscle weight (McCafferty and Edington 1974), nevertheless increases in mean fibre diameter presumably reflect compensatory hypertrophy (Rubinstein 1960, Rowe 1969). The combined total cross-sectional area of FG and SO fibres (Table 3.8) however decreased in senile animals, an indication of a faster rate of fibre loss than could be compensated for by hypertrophy.

The ratio of the cross-sectional area of the three fibre types in the extensor digitorum longus appears to shift towards one of higher oxidative capacity. The most affected were the FG fibres (Table 3.8) losing as much as 22.7% of their cross-sectional area between the adult (299 days) and senile (716 days) animals. FOG fibres increased their cross-sectional area by 12.4% over the same period while SO fibres increased by 24.3%. The only real contribution was that of the FOG fibres, since SO fibres occupy a very small percentage of the whole muscle.

The soleus which acquired a high oxidative activity by the time the animal was weaned, showed a continuous increase of 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 fast oxidative glycolytic fibres into slow oxidative ones. Caccia et al (1979) reported the presence of a transitional fibre type 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 increased activity by selectively increasing one or more fibre types (Leiberman et al 1972). Similar transformations were attainable in cross-innervated muscles (Dubowitz 1967, Brooke et al 1971).

Frequency distribution analysis of fibre diameters in young, growing and ageing muscles revealed that young fibre populations in general had symmetrically distributed populations around a distinct well-defined peak (Fig. 3.1a). As the population continued to grow the distribution polygons

mouse muscles which show bimodality even more clearly and how the biomodal distribution could be explained by the size difference of the two main fibre populations.

The soleus which consisted practically of one fibre type did not exhibit any bimodality. If either rigor or fixative penetration was responsible for bimodality both the homogenous soleus and the heterogenous extensor digitorum longus would have showed similar bimodalities within their fibre distributions.

Total DNA content in the extensor digitorum longus and the soleus increased after weaning and was then reduced before adulthood. Although the decrease was only significant for the extensor digitorum longus, the implications are difficult to define. Muscle fibre nuclei contribute between 50% and 70% of the total population of nuclei within a muscle (Enesco and Puddy 1964, Ontell 1974). The remaining nuclei belong to endomysial and perimysial cells. DNA estimates are therefore influenced by the relative presence of each component. Muscles have been shown to proportionally increase their DNA content during early growth (Enesco and Puddy 1964) and exercise (Bailey et al 1973). The increase in muscle DNA content is thought to arise from satellite cells being incorporated in existing muscle fibres (Moss and Leblond 1970, 1971) as no mitoses are observed within fibres. The increase in DNA content has been shown to level out or increase at a reduced rate as the animals further mature (Bailey et al 1973, Sands et al 1979). From the above mentioned studies a relation between DNA content and fibre nuclei number can be deduced. A reduction in total DNA content can thus be viewed as an indication of a reduction in the number of fibre nuclei, i.e. the extensor digitorum longus in showing such reductions is probably reflecting a reduced nuclei population.

The ratio of DNA to protein decreased continuously with age in both muscles, the ratio being lowest in adult animals (Table 3.9). The similar ratios of protein to DNA in the two young muscles indicate a consistent

relation between the two components. However, the greater reduction in DNA to protein ratio in the extensor digitorum longus may be associated in part, to the loss of fibres which occurs earlier in life in this muscle. It is also of interest to note that the mature fast contracting muscle has a lower overall DNA content than the mature slow contracting muscle.

The dividing line between senile atrophy and denervation atrophy is a slim one. Some of the changes associated with denervation atrophy have been observed in senile animals. This was a consequence of degeneration and malfunction of the nerve supply of senile muscles (P. 14). The number of axons in the nerves supplying the extensor digitorum longus and the soleus did not change significantly in number in senile animals (Table 3.10). The general increase in axon diameter early in life was in line with the normal growth rate of both muscles. Certainly it seems that the considerable loss of muscle fibres in the extensor digitorum longus early in life cannot be explained by neuron loss. Nevertheless visible denervation changes, in the form of muscle fibre denervation, were observed in the senile extensor digitorum longus and to a lesser extent in the soleus. The fibres which degenerated may have been those which failed to become innervated by lateral branches. Alternatively innervation may take place but the axonal branch may fail to function. Also in the senile animals functional denervation may occur, i.e. individual branches or the motor-neurons may fail to function.

It has been suggested that functional denervation rather than anatomical denervation may take place in senile muscle thus resulting in muscle fibre degeneration (McComas 1978).

CHAPTER IV

PHYSIOLOGICAL CHANGES ASSOCIATED WITH DEVELOPING AND AGEING SKELETAL MUSCLES

The development of skeletal muscles may be divided into three physiological phases from birth to death. The first phase is a continuation of embryonic differentatiation. This involves the maturation of myotubes, which is accompanied by marked proliferation of myofibrils thus the contractile power of the muscle is increased. This proliferation is accompanied by development of the sarcoplasmic reticulum and by a slow transformation of myosin from a predominantly foetal type to the representative adult forms (Gauthier et al 1978, Kelly and Rubinestein 1980). The net result of these transformations is a change in the speed of contraction and relaxation of the muscle (Buller et al 1960, Close 1964, Gutmann and Melichna1972). At the end of this phase the muscles differentiate into either slow or fast contracting muscles. During the next phase the muscle matures further. The fibres enlarge and the muscle produces peak performance, as far as force is concerned (Gutmann and Melichna 1972, Caccia et al 1979). The number and size of motor units remained unaltered (Caccia et al 1979). Phase three is associated with ageing; a slow but marked deterioration in function becomes apparent. The rate of change is dependent amongst other things, on the type of

muscle and its functional activity. In general, muscles appear to slow down with variable prolongation of latent periods, contraction times and relaxation times and also a fall in twitch tension.

The isometric and dynamic strength of the knee extensor muscles were reported to decrease in old people, as was the maximum extension velocity (Larsson 1978, Larsson and Karlsson 1978). The extensor digitorum brevis in healthy human subjects showed reduced twitch tensions and in a few cases longer latent periods (Campbell et al 1973). The levator ani of the rat had prolonged contraction times, latent periods, and half relaxation times and maximum rate of tension development (Gutmann et al 1971). Although the latter muscle is androgen sensitive it behaved in a similar way to fast muscles. The diaphragm is also an unusual muscle as it also showed an increase in the contraction time, latent period and half relaxation time of its twitch responses (Gutmann and Syrovy 1974).

In vitro measurement of extensor digitorum longus parameters - a muscle made up predominantly of fast motor units (Close 1967) - exhibited an increase in contraction time, latent period and half relaxation time (Syrovy and Gutmann 1970, Gutmann et al 1971, Gutmann and Syrovy 1974). There are certain discrepancies in the literature; Syrovy and Gutmann (1970) reported an unchanged latent period and an increased contraction time in the senile soleus. Gutmann and Syrovy (1974) later reported a prolongation of the latent period and half relaxation time and a shortening of contraction time of the same muscle. In vivo experiments on the senile mouse soleus also indicated an increase in contraction time (Caccia et al 1979).

In the present study the disturbance of the muscle was minimal when compared with in vitro procedures employed by other investigators (Syrovy and Gutmann 1970). The advantages of the procedures adopted here were that all body systems were operational, i.e. both blood supply and temperature regulatory mechanisms were intact, this is important since the

size of mature and aged muscles was considerable and mass stimulation of in vitro muscle does not allow effective diffusion of metabolites or close temperature control.

The investigation included measurements of single twitch, latency, fatigability, rate of tetanus development and maximum tetanic forces produced by the developing and ageing muscle.

MATERIALS AND METHODS

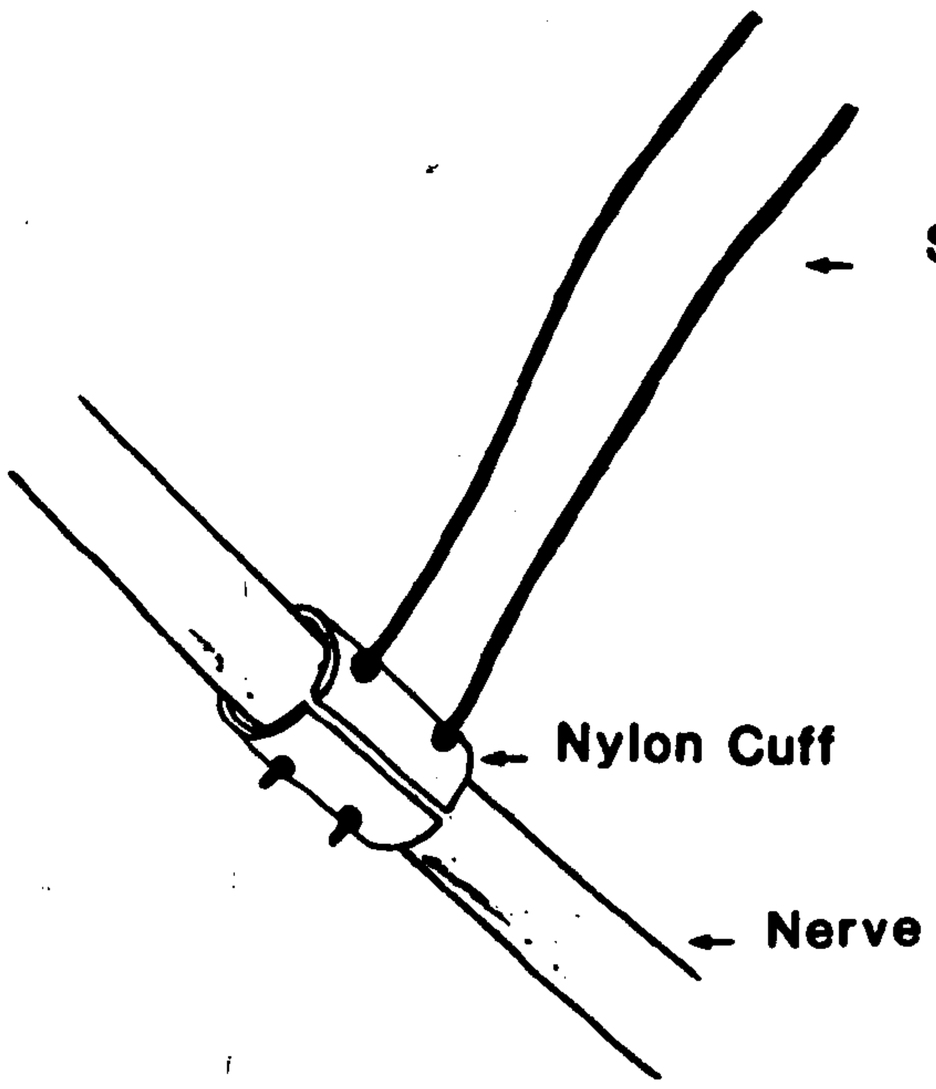
I The System

The recording system was built around the Devices UF1 isometric force-displacement transducer and transducer amplifier 3559. Slow events in the order of seconds were recorded on a Devices multichannel thermal recorder type MX412 (Devices Ltd., Hertfordshire, England). Faster events were stored on a Telequipment storage osciloscope DM53A with a type B differential amplifier. Permanent records were obtained using a Polaroid camera. Events were timed with a programmable Digitimer D100 (Devices Ltd.) which also served as a trigger and synchronization control. Stimulation was carried out with an SD9 Grass stimulator (Grass Instruments, Quincy, Massachusetts, U.S.A.) and a cuff type stainless steel or silver stimulation electrodes (Fig. 4.1).

The temperature of the immediate environment was controlled with a thermostatically governed infra-red lamp. The muscle was continually bathed with a physiological solution of 137mM NaCl, 4mM KCl, 1mM $MgCl_2$, 1mM KH_2PO_4, 12mM $NaHCO_3$ and 2mM $CaCl_2$. This solution was gassed with a mixture of 95% O_2 and 5% CO_2. The temperature of the solution was regulated to maintain 37.5 $\pm$ 0.5^{o}C by passing through a thermostatically controlled water jacket.

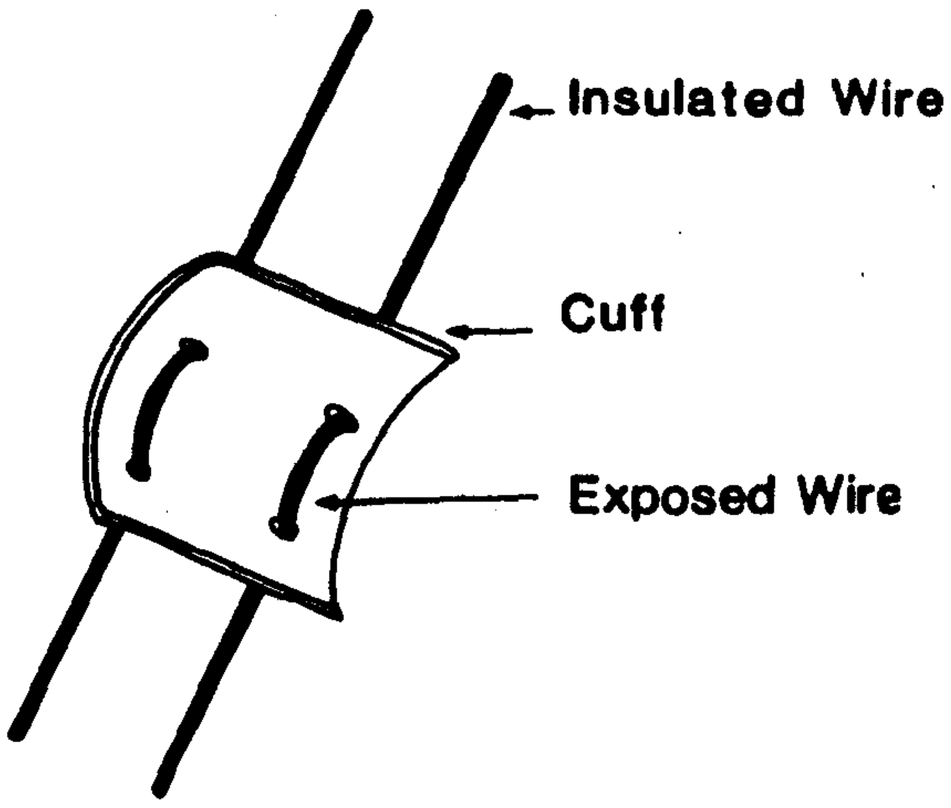
Rigidity of the preparation was accomplished by fixing the limb at both the knee joint and the ankle with steel pins. Cross members were used to secure the pins to the remainder of the framework. The muscles were tied to the transducer with a short length of EP3 surgical silk (Davis

Fig. 4.1 Stimulation electrodes fitted around the nerve(a) and opened to show contact area(b). The arrangement ensures good contact and minimum stimulation voltage.



* Stainless Steel ϕ 0.003 inch
(SS-3T,Clark Electromedical
Instruments, Reading, England)

(a)



(b)

and Geck, Cyanamid of G.B. Ltd., Gosport, Hampshire). The measured compliance of the system was 4.71×10^{-5} cm/g $\pm$ 0.65×10^{-5} for the 2 ounce transducer and 3.79×10^{-5} cm/g $\pm$ 0.27×10^{-5} for the 8 ounce transducer. The transducer was coupled to the main framework through a multidirection rack and pinion and a micrometer. This arrangement allowed the correct alignment of the transducer and the muscle, it also permitted coarse and fine adjustment of muscle length and position.

II Animals and Anaesthesia

Animals were selected from the inbred colony (Page 18) at the ages of 21, 387 $\pm$ 2 and 714 $\pm$ 6 days. Only healthy animals were used in these experiments in an effort to minimise fatalities arising from surgical and anaesthetic traumas in senile animals. Older obese animals were highly sensitive to anaesthetics. A combination of low barbiturate levels and chloroform was found to be most suitable. Sagatal (Pentobarbitone Sodium, May and Baker Ltd., Dagenham, England) was diluted in a mixture of 2ml propylene glycol, 1ml absolute alcohol and 7ml distilled water at a ratio of one part mixture to one part Sagatal stock solution (60mg/ml). An initial dose of 20mg/kg was administered intraperitoneally to senile animals followed by small dosesof 5mg/kg of Sagatal until surgical anaesthesia was reached not exceeding 50mg/kg total dose. Young animals required a total dose of 60mg/kg administered in a similar fashion. Maintenance of anaesthesia was assured by regular application of chloroform through a mask dispenser.

III Surgical and Stimulation Procedures

(a) The extensor digitorum longus

A skin incision on the inner side of the right leg extending from the ankle to the knee was made. A smaller incision through the fascia and parallel to the fibula was cut to expose the proximal tendon of the extensor digitorum longus. Excessive connective tissue interfering with the free movement of the muscle was carefully parted and a thread was secured

to the lower tendon. The blood and nervous supply to the muscle was kept intact. Stimulation electrodes of the cuff type were placed around the nerve supplying the extensor digitorum longus. The in situ length of the muscle at its resting position was determined using a pair of calipers. The tendon was then cut and the preparation was rigidly secured in the framework described earlier in such a way that, the animal was in the supine position. The extensor digitorum longus and the transducer arm were carefully aligned. Stimulation commenced after allowing the muscle to recover for 10 minutes whilst being bathed in physiological solution.

(b) The soleus

The skin was removed from the right limb and the Achilles tendon and the soleus were exposed. The muscle was freed of connective tissue taking care not to damage the nerve and blood supply. The proximal tendon was tied to EP3 surgical silk. Cuff type stimulation electrodes were placed around the nerve supply. The resting length of the muscle was measured and the tendon was cut free from the bone. The animal was placed in the prone position and the tendon was secured to the force transducer. Minimum lengths of thread were used in securing the muscle to minimize compliance. The transducer was manipulated to obtain the best position for the recording and the whole preparation was firmly clamped. Warm Ringer (37.5^{o}C) was slowly dripped over the muscle for 10 minutes and the muscle was then stimulated.

(c) Active length - tension measurements

The muscle was stimulated at a rate not exceeding two stimuli per minute. The muscle length was increased by turning the micrometer after each stimulation until optimum length was reached. This continued until the muscle length reached 105% optimum length for the extensor digitorum longus and 110% optimum length for the soleus. Stimulation pulses were between 3 and 12 volts and 0.5 milliseconds in duration. Isometric

contractions were recorded on both the oscilloscope and the chart recorder.

(d) Isometric contraction at optimum length

A record of isometric contraction was obtained at optimum length and maximal voltage using a 0.5 ms pulse. The event was recorded on the oscilloscope at several scan rates to improve the accuracy of latent period and twitch parameter meaurements. Contraction parameters were calculated from Polaroid photographs.

(e) Tetanic contraction measurements

Tetanic contractionswere obtained at optimum length and maximal voltage. The stimulation frequencies were 80 and 100 pulses per second for the extensor digitorum longus, and 40 and 60 pulses per second for the soleus. The recording was carried out in two phases:

1. A one second stimulation to obtain a high speed record of the rate of tetanus development.
2. Continuous stimulation until half maximum isometric tetanic tension was reached.

As a matter of course the lower stimulation rate was carried out first followed by the higher stimulation rate. A suitable rest period was allowed between the various recordings to allow the muscle to regain normal contractions.

IV Definition of Terms

Optimum length: The optimum length is the length at which the muscle developed maximum twitch tension in response to a supramaximal stimulus.

Maximal voltage: The voltage just in excess of that required to elicit maximal twitch tension.

Maximum isometric twitch tension (Pt): The tension produced in response to a maximal voltage at the muscle's optimal length.

Latent period: The time from the initiation of stimulation to the beginning of the muscle's mechanical response.

Contraction time: The time from the end of the latent period to

peak isometric tension at optimal length and maximal voltage applied to the muscle's nerve.

Half relaxation time: The time from the peak of isometric tension to one half of the peak tension.

Maximum isometric tetanic tension (Po): The maximum isometric tension developed by the muscle at optimum length in response to a stimulation rate high enough to produce fused twitches. (The optimum frequency varies according to muscle type and age, therefore, a compromise optimum frequency producing fused twitches at all ages investigated was used).

Rate of tetanus development: The rate at which isometric tension developed in the muscle in response to tetanic stimulation at optimum length and maximal voltage. The rate was expressed as gram tension per ms and gram tension per mm^2 cross-sectional area per ms.

Half fatigue time: The time from the peak of maximum isometric tetanic tension to one half of that peak tension.

V Post-recording Procedure

Immediately the recordings were finished, the animals were sacrificed by cervical dislocation. The extensor digitorum longus and the soleus from both legs were dissected out. Re-evaluation of the resting muscles length was carried out to confirm earlier measurements. The muscles were cleaned of excess tissue, weighed and then frozen in super-cooled iso-pentane (see Page 28). Sections were then cut in a cryostat at 10μm and were used to examine the histology and histochemistry of the muscles.

Stained sections were projected using a Leitz projection microscope at a suitable magnification, and the outline of the muscles was traced. Using a Planimeter (Allbrit Planimeter, U.K.) the total cross-sectional area of each muscle was obtained and used in later calculations. The planimeter method was found to be marginally better than cutting and weighing paper traces of the muscles, mainly because of its repeatability and simplicity.

The recording system was electronically calibrated before and after individual records were obtained using the built-in facilities on the amplifier. Signals of a known magnitude were injected into the amplifier to obtain full scale deflections. Back-off voltage was employed to remove imbalance errors in the bridge-type force-displacement transducers. More elaborate calibration was usually carried out at regular intervals using mechanical and physical procedures. Weights were hung from the force-displacement transducers to calibrate them at different ranges. In all cases the relation between force and transducer response was linear. The compliance of the system was checked regularly by attaching the transducer and the accompanying manipulators through an EP3 surgical thread to a rigid low compliance frame. This procedure simulated the experimental compliance minus the muscle.

VI Statistical Analysis

An analysis of variance of a single level (Model I) was used to examine changes arising from age changes amongst the three groups. The analysis was invariably followed by a serial group comparison and an F test as described on Page 21.

Regression fitting and analysis was used to examine the effect of muscle length on tension production. The force produced at a given muscle length, expressed as a percentage of the maximum force produced at the optimum length, was regressed on the muscle length at that point expressed as a percentage of the optimum muscle length. The points were fitted to a straight line of the form:

$$y = a + bx$$

where y = % force produced and x = % length

The independent variable x was restricted to values $<95\%$ in the animal groups aged 387 and 714 days, and to values $<98\%$ in the youngest age group. This was necessary because of the exponential nature of the relationship (Fig. 4.2c). The restriction allowed a simple comparison of

the linear segment of the relationship. The slope of the fitted line was used as an indicator of the rate at which contraction forces increased in response to muscle length changes. In all cases the correlation coefficient was better than 0.97 with 0.99 being not uncommon.

The non-linear relation existing approximately 5% around the optimum length, was found to be of an extremely complex nature and no satisfactory statistical technique was considered to be a suitable representation of the model. The nature of this curvilinear relationship is discussed later.

RESULTS

I The extensor digitorum longus

(a) Length:Tension relationship

As expected all muscles responded positively to increases in initial muscle length. The response was reasonably linear until a few length increments before optimum length was reached. Thereafter the response of the muscle to further length increases was to produce lower tension increments causing the resulting graph to flatten out at 100% (optimum length). This general relationship held true for young 21 day animals (Fig. 4.2a) as well as 387 day adult animals (Fig. 4.2b) and 714 day senile ones (Fig. 4.2c).

The fitted regression lines and the associated analysis indicated a very good linear relationship between muscle length and tension. This relationship extended to lengths of 98% optimum length in the 21 day old group and 95% optimum length in the remaining two groups. By excluding the non-linear segment of the relation a comparison between the slopes of the fitted lines showed an increase in the steepness of the response of the extensor digitorum longus to increases in muscle length with age (Table 4.1). The adult muscles (387 days) showed a steeper slope, that is to say they showed a greater length dependance for force development as compared with the 21 day old animals ($P < 0.05$). The senile muscles had even steeper slopes than the adult (387 day) muscles ($P < 0.01$).

Fig. 4.2 Examples of length : tension relationships in representative extensor digitorum longus muscles aged (a) 21 days (b) 387 days and (c) 714 days. Tension is expressed as a percentage of maximum twitch tension produced at optimum length. r = correlation coefficient, b = regression coefficient.

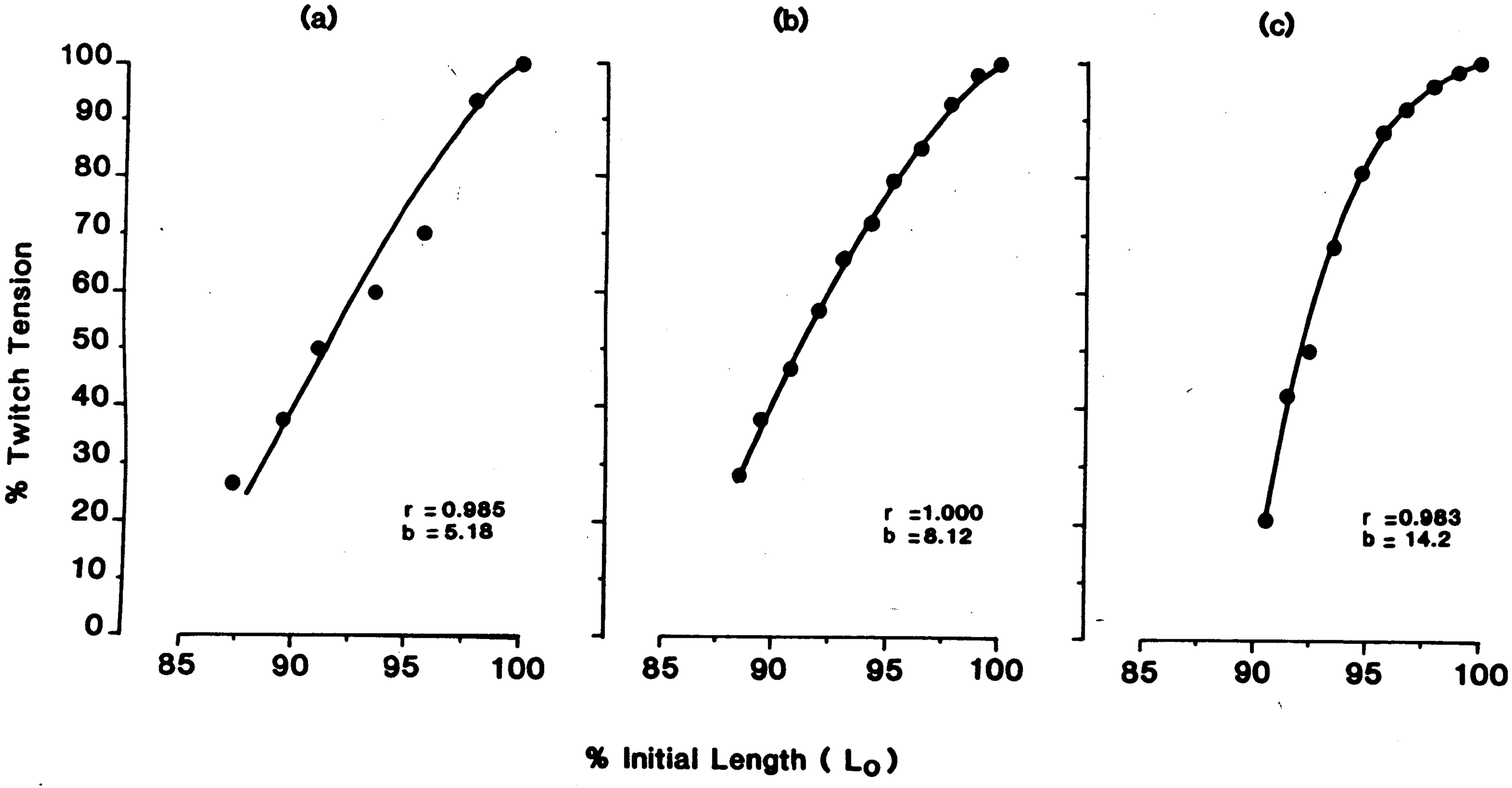

(a)
(b)
(c)
% Twitch Tension
100
90
80
70
60
50
40
30
20
10
0
r = 0.985
b = 5.18
r =1.000
b =8.12
r =0.983
b = 14.2
85
90
95
100
% Initial Length (L_o)

Table 4.1 Length:Isometric Twitch Tension relation in the extensor digitorum longus of the rat

Age (Days)	Animal No.	Regression Coefficient	Correlation Coefficient	Mean Regression Coefficient ± S.D.	Probability <
21					
	1	5.83	0.995		
	2	5.54	0.981		
	3	7.90	0.993		
	4	5.18	0.999		
	5	5.18	0.985		
				5.93 ± 1.13	
387					
	1	9.85	0.999		
	2	6.76	0.990		
	3	6.62	0.976		
	4	8.12	1.000		
	5	10.64	0.997		
				8.40 ± 1.80	0.05
714					
	1	14.20	0.983		
	2	13.88	0.995		
	3	10.30	0.988		
	4	12.17	0.998		
	5	10.19	0.992		
				12.15 ± 1.90	0.01

Qualitatively the top, non-linear segment of the relation was of a more curved nature in the senile group as compared to the adult (387 day) group or the youngest group (Fig. 4.2).

(b) Single isometric twitches

Single twitch tension increased significantly with age ($P < 0.001$). The isometric twitch tension of the adult muscle increased by 327% when compared with the 21 day weanlings (Table 4.2). The isometric twitch tension in senile animals showed a tendency to decrease as compared to the 387 day adults but this was not statistically significant.

The analysis of variance of timed events associated with isometric twitch contractions showed significant changes in the latent period and contraction time (Table 4.2). The latent period of young muscles decreased significantly with age ($P < 0.01$) reaching its minimum at adulthood. Senile animals exhibited an 8% increase in latent period as compared to adult animals ($P < 0.01$).

Contraction times, measured as the time to peak contraction, showed no significant change between 21 and 387 day old animals. Senile animals on the other hand showed a significant prolongation of contraction time ($P < 0.01$). The increase in contraction time was in the order of 21% as compared to adult contraction times.

Half relaxation times did not change significantly over the ages investigated.

(c) Isometric tetanic tension

Maximum tetanic tension produced by the extensor digitorum longus increased five-fold between weaning (21 days) and the 387 days old adults ($P < 0.001$). This tension was significantly lower in senile animals ($P < 0.01$) declining by approximately 68% of the original adult tension.

The rate of tetanic tension development in adult animals was significantly higher than young 21 day old animals ($P < 0.001$). In senile animals, tetanic tension developed at a slightly lower rate (24%) which

Table 4.2 Isometric twitch parameters of the young and ageing extensor digitorum longus

Age (Days)	No.	Latent period ± S.D. ms	Pr. <	Time to peak tension ± S.D. ms	Pr. <	Half relaxation time ± S.D. ms	Pr. <	Twitch tension ± S.D. g/mm^2	Pr. <
21	5	2.50 ± 0.08	0.001	13.40 ± 1.52	n.s.	14.90 ± 2.75	n.s.	1.38 ± 0.26	0.001
387	5	2.10 ± 0.06	0.01	13.00 ± 0.35	0.01	13.90 ± 2.0	n.s.	4.52 ± 0.39	n.s.
714	6	2.27 ± 0.10		15.75 ± 1.17		16.58 ± 3.5		4.26 ± 1.07	

was significantly different from adult animals ($P < 0.05$). This was true even when the cross-sectional area of the muscle was considered (Table 4.3)

Twitch:Tetanus ratios were also determined, however the analysis of variance showed no statistically significant variation between the three age groups, although there was a tendency for it to be higher in young animals decreasing by 33% in adult animals and rising again by 37% in senile animals.

Half fatigue times, obtained as detailed on Page 78 , were found to decrease by 62% between the ages of 21 and 387 days ($P < 0.01$). Senile extensor digitorum longus muscles, although showing a slight increase in fatigue time were not statistically significant. High stimulation rates (100Hz) produced similar results to the 80Hz rate, no statistically significant difference was detected between adult and senile animals.

Although it appears that younger muscles are more resistant to fatigue it is apparent from the data for maximum isometric tension per unit area of muscle that younger muscles were developing far less tension per unit cross-sectional area, therefore if fatiguability was expressed as the ability to maintain a given isometric force for a given time; it is apparent that the mature muscle will perform better than the senile muscle and much better than the young muscle.

II <u>The soleus</u>

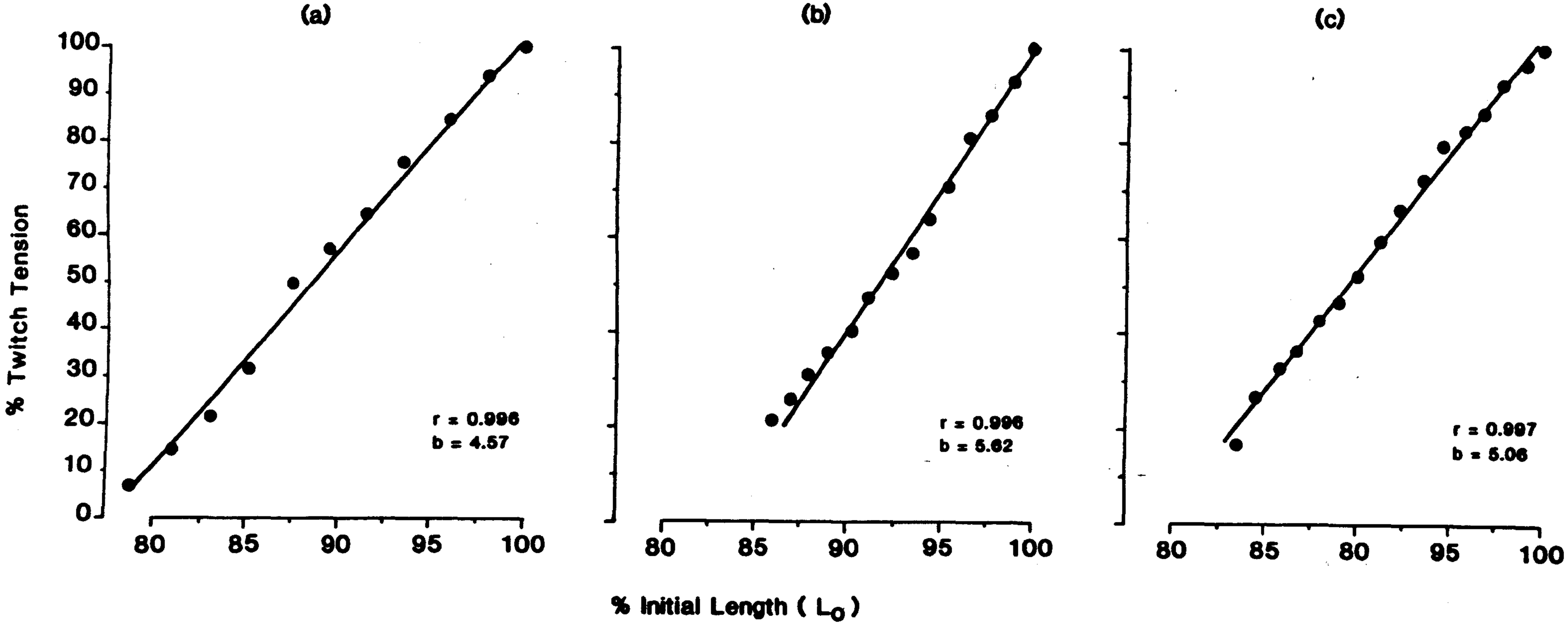
(a) Length:Tension relationship

Isometric twitch tension increased linearly with increased muscle length. The exponential trend observed in the extensor digitorum longus was only encountered in senile solei and was of a less curvilinear nature (Fig. 4.3). This allowed the examination and the fitting of all recorded points up to and including optimum length and maximum tension to the straight line formula described on Page 80 . The fitted regression lines and the associated analysis of regression confirmed a good relationship between the tension produced and the various muscle lengths.

Table 4.3 Isometric tetanic contraction parameters of the young and ageing extensor digitorum longus

Age (Days)	No.	Maximum Tetanic Tension ± S.D. g/mm^2	Pr. <	Rate of tetanus development ± S.D. g/ms	Pr. <	Rate of tetanus development ± S.D. $g\ mm^{-2}/ms$	Pr. <	Ratio of twitch to tetanus Pt/Po ± S.D.	Pr. <	Half fatigue time ± S.D. (s)		Pr. <
										80 Hz	100 Hz	
21	5	4.58 ± 1.74	0.001	17.90 ± 9.36	0.001	112.40 ± 37.53	0.001	0.3482 ± 0.1845	n.s.	17.7 ± 6.2	-	0.001
387	5	21.20 ± 2.19	0.001	304.44 ± 82.04	0.05	459.88 ± 47.75	0.05	0.2160 ± 0.0373	n.s.	6.8 ± 1.4	5.3 ± 1.5	n.s.
714	6	14.34 ± 3.04		232.42 ± 19.13		379.83 ± 71.94		0.2970 ± 0.0313		9.8 ± 1.9	6.6 ± 1.3	

Fig. 4.3 Examples of the length : tension relationship in representative soleus muscles aged (a) 21 days (b) 387 days and (c) 714 days. Tension is expressed as a percentage of maximum twitch tension at optimum length. r = correlation coefficient, b = regression coefficient.


(a)
(b)
(c)
% Twitch Tension
100
90
80
70
60
50
40
30
20
10
0
80
85
90
95
100
r = 0.996
b = 4.57
r = 0.996
b = 5.62
r = 0.997
b = 5.06
% Initial Length (L_o)

An examination of the slopes of the fitted lines of the three age groups showed no significant changes of the rate at which tension increased with increased muscle length (Table 4.4). The 387 day old animals showed a slight increase in the steepness of the slope as compared with the youngest age group (21 days). The steepness of the slope was reduced in the senile group. The relationship between soleus length and its tension was much less dependent on age than was the case for the extensor digitorum longus.

(b) Single isometric twitches

Analysis of variance indicated a significant change in the single twitch isometric tension between the three age groups investigated (Table 4.5). An increase of 58% in twitch tension was observed between 21 and 387 days and a further 36% between 387 and 714 days of age ($P < 0.05$). As is mentioned in the discussion these findings contrasted with the results obtained for the extensor digitorum longus.

The latent period of the soleus did not show any significant change with age, although there was a tendency for the adult soleus to have a shorter latent period as compared to either young or senile animals.

Time to peak contraction of single twitches of the soleus did not show any statistically significant change with age when all three age groups were tested individually. A comparison of a combination of the two groups aged 21 and 387 days versus the senile group revealed a 12% increase in contraction time with old age ($P < 0.05$).

The results of half relaxation times of the soleus were not statistically significant, however, a trend towards an increased relaxation time in senile animals was observed.

(c) Isometric tetanic tension

The magnitude of the isometric tetanic tension resulting from a 40Hz stimulation rate increased by 250% between 21 and 387 days of age ($P < 0.01$). The increase tended to continue into old age (Table 4.6) but was not

Table 4.4 Length:Isometric Twitch Tension relation in the soleus of the rat

Age (Days)	Animal No.	Regression Coefficient	Correlation Coefficient	Mean Regression Coefficient ± S.D.	Probability <
21	1	7.59	0.981		
	2	4.57	0.996		
	3	4.79	0.986		
				5.65±1.69	
387	1	7.16	0.972		
	2	6.26	0.980		
	3	6.30	0.993		n.s.
	4	5.62	0.996		
				6.34±0.63	
714	1	8.13	0.980		
	2	5.33	0.997		
	3	5.35	0.986		n.s.
	4	5.20	0.996		
	5	5.06	0.997		
				5.81±1.30	

Table 4.5 Isometric twitch parameters of the young and ageing soleus

Age (Days)	No.	Latent period ± S.D. ms	Pr.	Time to Peak tension ± S.D. ms	Pr.	Half relaxation time ± S.D. ms	Pr.	Twitch tension ± S.D. g/mm^2	Pr.
21	4	3.20 ± 0.27	n.s.	29.00 ± 2.74	n.s.	32.50 ± 3.11	n.s.	1.58 ± 0.55	0.05
387	4	2.97 ± 0.05	n.s.	30.37 ± 1.11	n.s.	32.12 ± 4.37	n.s.	2.50 ± 0.28	0.05
714	5	3.14 ± 0.17		33.30 ± 2.68	0.05	38.50 ± 10.44		3.39 ± 0.65	

Table 4.6 Isometric tetanic contraction parameters of the young and ageing soleus

Age (Days)	No.	Maximum Tetanic Tension ± S.D. g/mm^2	Pr. <	Rate of tetanus development ± S.D. g/ms	Pr. <	Rate of tetanus development ± S.D. $g\ mm^{-2}/ms$	Pr. <	Ratio of twitch to tetanus Pt/Po ± S.D.	Pr. <	Half fatigue time ± S.D. (s)			
										40Hz	Pr. <	60Hz	Pr. <
21	4	5.34 ± 2.11	0.05	11.50 ± 4.56	0.01	80.05 ± 29.64	n.s.	0.3162 ± 0.1019	0.05	55.0 ± 12.6	0.05	39.2 16.2	n.s.
387	4	13.37 ± 2.92	n.s.	82.25 ± 18.45	n.s.	97.55 ± 15.49	n.s.	0.1927 ± 0.040	n.s.	40.7 ± 6.1	n.s.	26.2 ± 5.4	n.s.
714	5	14.96 ± 4.85	n.s.	86.60 ± 35.32	n.s.	139.68 ± 47.34	n.s. 0.05	0.2446 ± 0.0794	n.s.	38.7 ± 4.9	n.s.	24.8 ± 5.8	n.s.

statistically significant.

The rate of isometric tetanic tension development in the adult soleus was sevenfold higher than freshly weaned 21 day old animals ($P<0.01$). The rate of tetanus development in senile animals was slightly higher but not significant when compared with adult animals.

Twitch:Tetanus ratios (Pt/Po) for the soleus was 39% lower in adult animals as compared to young ones ($P<0.05$). This ratio tended towards higher values in senile animals, but was not statistically significant (Table 4.6).

Half fatigue times for solei stimulated at 40Hz were higher in animals aged 21 days as compared to older animals ($P<0.05$). Stimulation at a higher pulse rate (60Hz) revealed similar trends, but the statistical confidence was considerably reduced making the changes amongst the age groups "non-significant".

When comparing muscles of different ages it appeared that the youngest soleus muscles were more fatigue resistant, however, it must be remembered that the maximum tension output of these muscles is considerably less than for adult muscles. Therefore in absolute terms the young muscles are probably less fatigue resistant than the older muscles.

DISCUSSION

Length:Tension relationship: The effect of varying the length of muscle is to alter the overlap between the thin and thick filaments. An optimum overlap is obtained when the highest number of crossbridges can interact with the thin actin filaments. The optimum for the muscle as a whole is obtained when a high proportion of sarcomeres exhibit optimum overlap. At this length maximum force is developed. At either side of the optima the force declines (Close 1972, A. F. Huxley 1974, for reviews). Experimental optimum sarcomere length appears to vary slightly from one muscle to another ranging from as low as 2.7μm for the flexor helicalis longus of the cat to 3.1μm for the soleus of the same animal under tetanic

isometric conditions (Buller and Lewis 1965). Calculated optimal values for the soleus and the extensor digitorum longus sarcomeres under isometric twitch conditions were 2.55μm and 2.50μm respectively (Close 1972). Since the optimal sarcomere length was virtually identical, the expected behaviour of both muscles might have been expected to be the same. This was true for young muscles from 21 day old animals (Tables 4.1 and 4.4) but the behaviour altered significantly in older extensor digitorum longus muscles. The soleus was consistent in showing an increase in tension proportional to increases in length. The extensor digitorum longus on the other hand showed proportionally higher tensions for a proportionally similar increase in length producing slopes that are significantly steeper in older animals. This implies that the optimum sarcomere length was reached faster in aged extensor digitorum longus muscles than in younger ones. This might be due to differences in the series and parallel elastic component and hence a better correspondence of fibre length changes with sarcomere length changes. Because of the poor development of the connective tissue in the young muscle it is conceivable that the tendons and sarcolemma can be stretched without the sarcomeres being pulled out to the same extent.

The above explanation requires the maintenance of two conditions; firstly an unchanged sarcomere length with age and secondly an unchanged number of sarcomeres in series. There is some uncertainty concerning sarcomere length changes with age because of the difficulty of fixing muscle at a given length and under comparable conditions. Sarcomere number is however known to change considerably (Williams and Goldspink 1978). These changes are apparently a response to the use and disuse of the muscles. A decrease in sarcomere number in old age due to disuse (reduced excursion of the limbs) would have the effect of increasing the load on the series elastic component, thus enhancing the steepness of the tension:length relation.

It is clear that further investigation of this point is required to establish whether sarcomere number does change in senility.

The overall increase in isometric twitch tension during early development reflects changes at the muscle fibre level. Muscle fibres increase in cross-sectional area considerably during normal growth (Rowe and Goldspink 1969). This increase was associated with a proliferation of contractile material within those fibres (Goldspink 1970) thus effectively increasing the ability of the mature muscles to develop more tension per muscle unit area. Both the extensor digitorum longus and the soleus exhibited significant increases in their contractile tension as seen in Tables 4.2 and 4.3 during early life. Senile muscles on the other hand behaved inconsistently. The extensor digitorum longus exhibited a trend towards lower twitch tension whilst the soleus continued to produce even higher tensions. The inability of senile muscles to develop tension comparable to younger subjects has been reported by several other investigators (Campbell et al 1973, Larsson and Karlsson 1978, see Page 6).

The tendency towards a decreased latent period around maturity indicated a higher efficiency in transmitting and depolarising the adult muscle. The increased myelination of terminal axons in the developing muscle as well as the maturation of the end-plate region in the muscle act together to reduce the latent period.

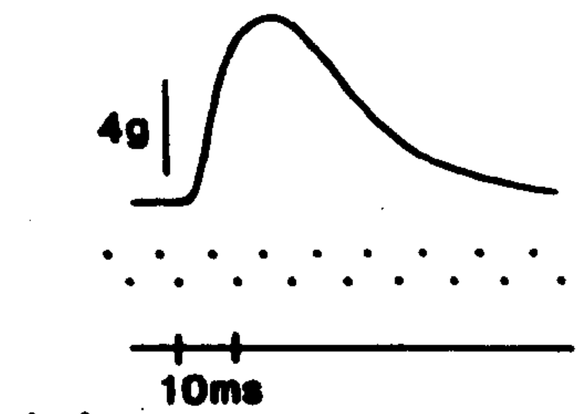
Changes in the latent period with age were difficult to explain. In senile animals these may reflect a large number of changes. Conduction velocities in aged nerves were usually reduced (Campbell et al 1973). A general degeneration and shrinkage of end-plates (Fujisawa 1976) and innervation irregularities (Gutmann and Hanzlikova 1965) have been reported in aged neuromuscular junctions. The neuromuscular

junction appears to suffer from mild and sometimes severe abnormalities which by nature affect the normal functioning of the junctions (Fujisawa 1976). Loss of axons contribute to the general disorganisation of nerve muscle relations (Caccia et al 1979). In addition, lateral sprouting is believed to occur and this will result in unmyelinated axon branches which will have lower conduction velocities.

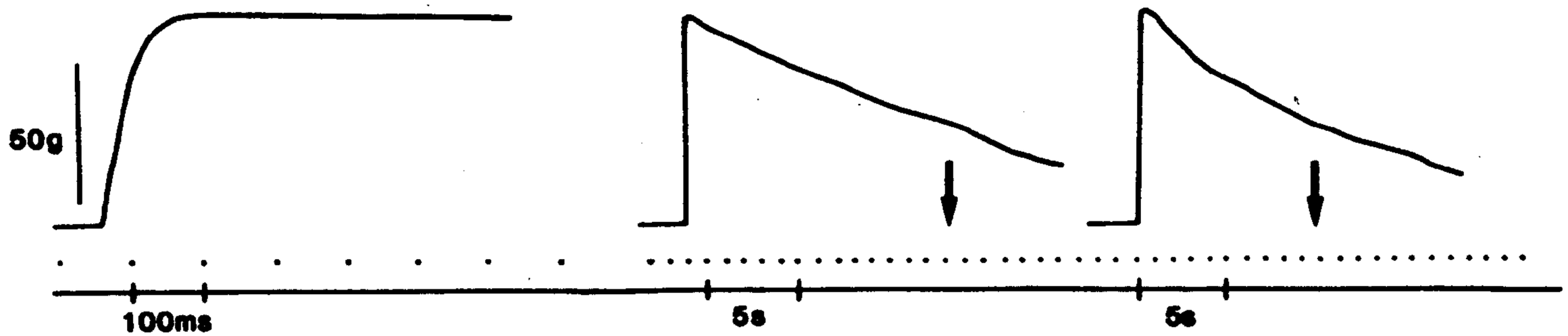
In accord with the general slowing down of muscle processes with old age, contraction times (time to peak) of both the extensor digitorum longus and the soleus increased with age. This was in agreement with earlier reports by Syrovy and Gutmann (1970) but not in agreement with later reports by the same authors (Gutmann and Syrovy 1974). Non-destructive measurements of muscle performance showed increases in contraction times of several muscles with age (Campbell et al 1973, Larsson and Karlsson 1978). Animal experiments confirmed this trend in the soleus of the mouse (Caccia et al 1979) and the extensor digitorum longus, diaphragm and levator ani of the rat (Gutmann and Melichna 1972). In this work both the slowed rate of isometric force development and the prolongation of the single twitch time to half relaxation indicated a slowing down of the muscle with age.

Skeletal muscles are known to be slow at birth and reach their characteristic contraction parameters at maturity (Close 1967, Gutmann and Melichna 1972). Early in life these muscles contained an embryonic myosin form (Whalen et al 1979) which was later replaced by various combinations of slow or/and fast myosin (Gauthier et al 1978, Hoh and Yeoh 1979). The coexistence of isoenzymes within the same fibre as suggested by the work of Lutz et al (1979) might explain the transformation of both physiological and histochemical behaviour during growth, enforced activity (Salmons and Vrobora 1969, Salmons and Sreter 1976, Kuelberg 1976) and cross-innervation (Dubowitz 1967, Luff 1975). It appears that an individual fibre can synthesise different types of myosin although slow fibres produce

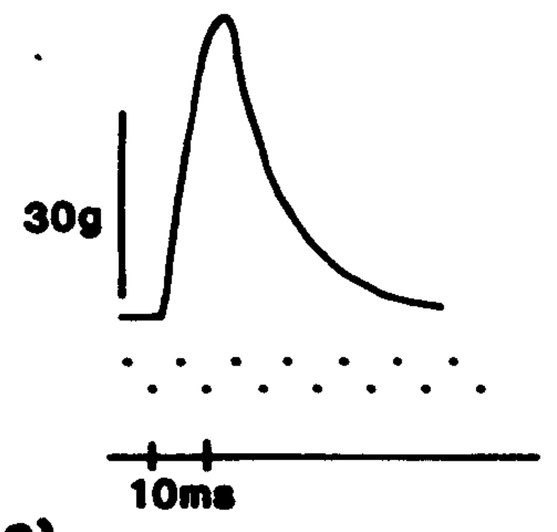
Fig. 4.4 Representative records of twitch and tetanic isometric contractions of the extensor digitorum longus at ages of (1) 21 days (2) 387 days and (3) 714 days. Each series showes from left to right (a) single isometric twitch at optimum length (b) rate of tetanus development at 80Hz (c) half fatigue time at 80Hz (d) and at 100Hz. The arrows indicate time to 50% maximum tetanic tension.



(1) a.



b. c. d.



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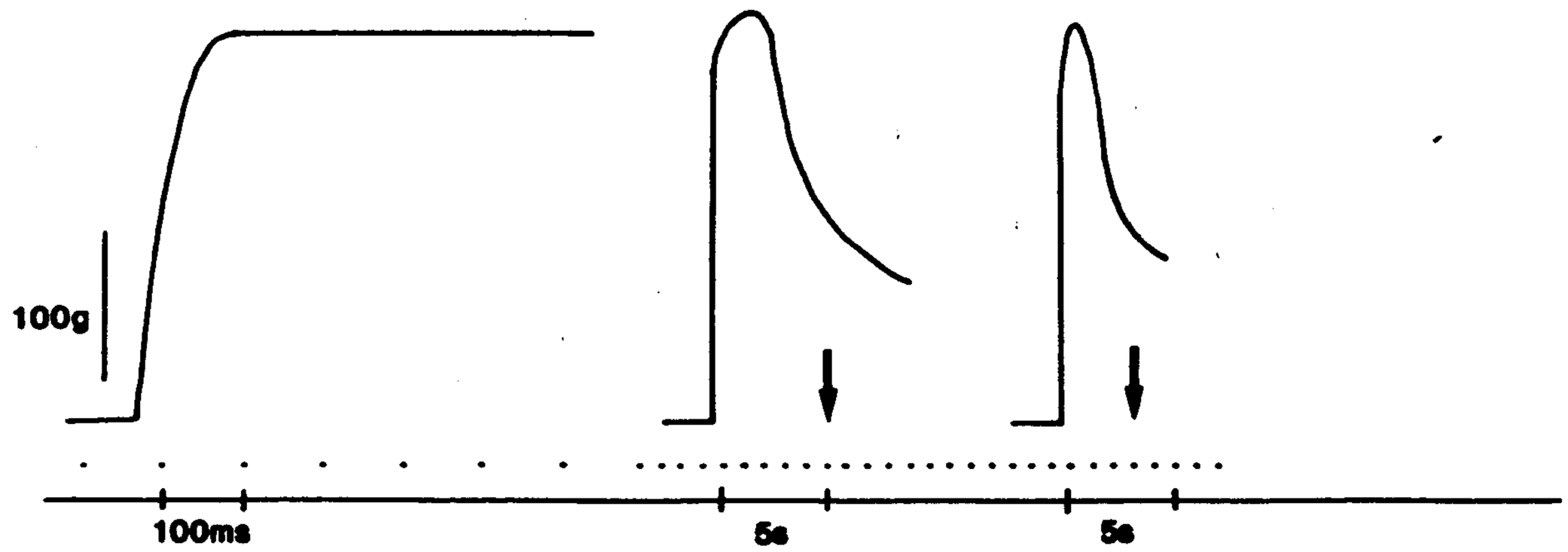
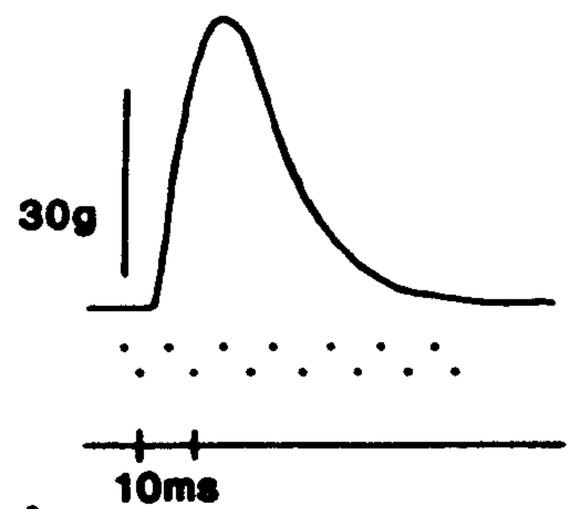
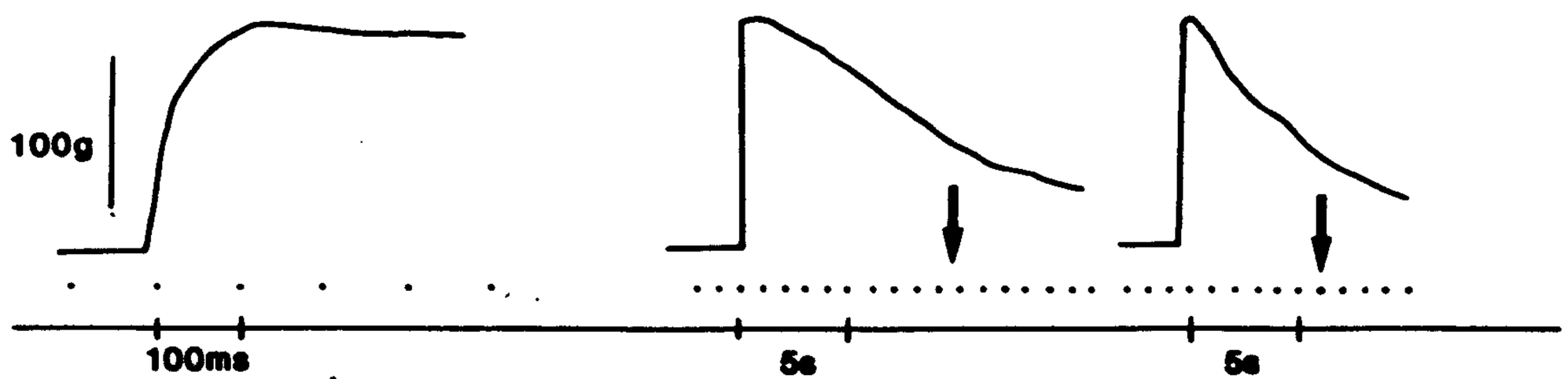


b. c. d.

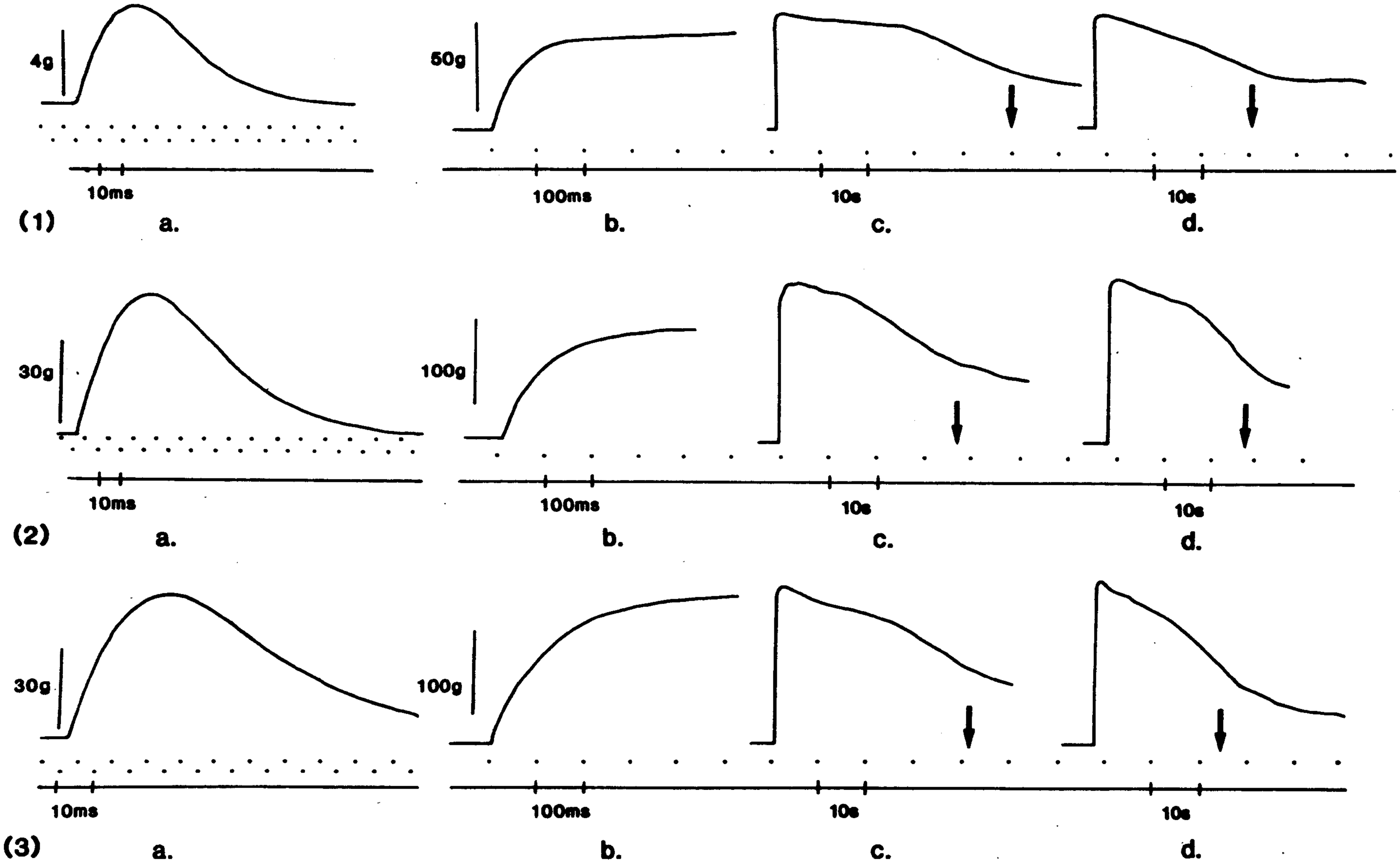


(3) a.



b. c. d.

Fig. 4.5 Representative records of twitch and tetanic isometric contractions of the soleus at ages of (1) 21 days (2) 387 days and (3) 714 days. Each series showes from left to right (a) single isometric twitch at optimum length (b) rate of tetanus development at 40Hz (c) half fatigue time at 40Hz (d) and at 60Hz. The arrows indicate time to 50% maximum tetanic tension.


4g
10ms
(1)
a.
50g
100ms
b.
10s
c.
10s
d.
30g
10ms
(2)
a.
100g
100ms
b.
10s
c.
10s
d.
30g
10ms
(3)
a.
100g
100ms
b.
10s
c.
10s
d.

predominantely slow myosin and fast fibres produce predominantly fast myosin. However the possibility exists that the balance between the synthesis of these different myosins may change with age within individual fibres. Hence a slow fibre in a senile animal may have slightly different properties to a slow fibre in mature or young animals. In other words the slow fibre may become even slower.

Although the time for half relaxation in both the extensor digitorum longus and the soleus showed a trend towards prolongation, they were not statistically significant. The half relaxation time supposedly reflects the deactivation of the coupling of myosin and actin. The calcium ion concentration which is elevated during the activation state is reduced again by the pumping of calcium to the terminal cisternae of the S.R. This is believed to deactivate the actin filament. Relaxation apart from the pumping of calcium is believed to be a mechanically passive process, i.e. no active pushing apart of the contractile filaments; it is most likely modified by other factors such as elasticity of the muscle and its ability to return to its initial length under the influence of extrafibrillar connective tissue. Living single fibres stripped of their sarcolemma apparently refuse to relax spontaneously although they can be easily pulled back to their original length once the activating Ca^{++} is pumped away (Bendall 1969).

Maximum tetanic tension can be used as an indicator of the real capacity of a muscle to produce tension. A tetanic contraction at a high enough stimulation rate will reduce the contribution of any elastic component to a miniumum by the virtue of summating consecutive twitches against their natural elasticity at such a rate so as to prevent any relaxation induced by either the series or parallel elastic components. The inability of senile extensor digitorum longus muscles to produce maximum tetanic tension comparable to those of younger animals indicates either a loss of contractile material or the muscle's inability to

activite the contractile material. The senile soleus on the other hand produced tetanic tensions at levels slightly higher than those of younger animals, indicating that the muscle is léss affected by the ageing process as borne out by the histological and histochemical data presented in the preceding section.

At stimulation rates producing fused tetanic contractions the soleus was by far the more fatigue resistant than the extensor digitorum longus (Fig. 4.4 and 4.5). The relation of fatigue resistance to fibre type and motor unit characteristics has been demonstrated by Kugelburg and Edstrom (1968, Kugelburg 1973, Burke et al 1973, Burke and Tsairis 1974). It has been suggested by Kugelburg and Edstrom (1968) that motor units are more resistant to fatigue if they are stimulated at frequencies approaching and matching their end-plate acetylcholine transmission levels. Therefore stimulation frequencies must be matched as close as possible to in vivo stimulation patterns.

The fatigue resistance fundamentally reflects the enzyme systems supporting the contraction, the rate at which ATP is used and supplied. In the soleus fast motor units are predominately type II fibres (FOG), while slow motor units are predominantely type I fibres (SO) (Kugelburg 1973). The slow more resistant units which are the major contributor in the soleus appear to retain their ability to resist fatigue even at old age.

The higher relative fatigue resistance of the young extensor digitorum longus and the soleus muscles is mainly due to the lower maximum tetanic tension produced by the young muscles. Older muscles are probably able to sustain equivalent tension for longer periods of time as demonstrated on human subjects by Larsson and Karlsson (1978). The problems arising of comparing young and ageing muscles in this respect are the inability to match tensions and still obtain fused tetanic contractions.

The higher fatigue resistance of the young extensor digitorum longus

and soleus muscles may also be due to the better blood supply to the younger muscles (Sillau and Banchero 1978) and their small physical dimensions.

The prolongation of time to half fatigue of the senile extensor digitorum longus when compared to the adult muscle indicates an improved ability to supply energy for longer periods of time. Such a change would presumably involve enzymatic alterations. The ability of senile muscles to sustain isometric exercise has been reported by Larsson (1978) for the extensor muscles of the leg. These changes were accompanied with changes in the histochemical profiles towards a more oxidative capacity.

This work has shown that there are very definite changes associated with ageing in muscle, however, further work is required to understand the changes at cellular level.

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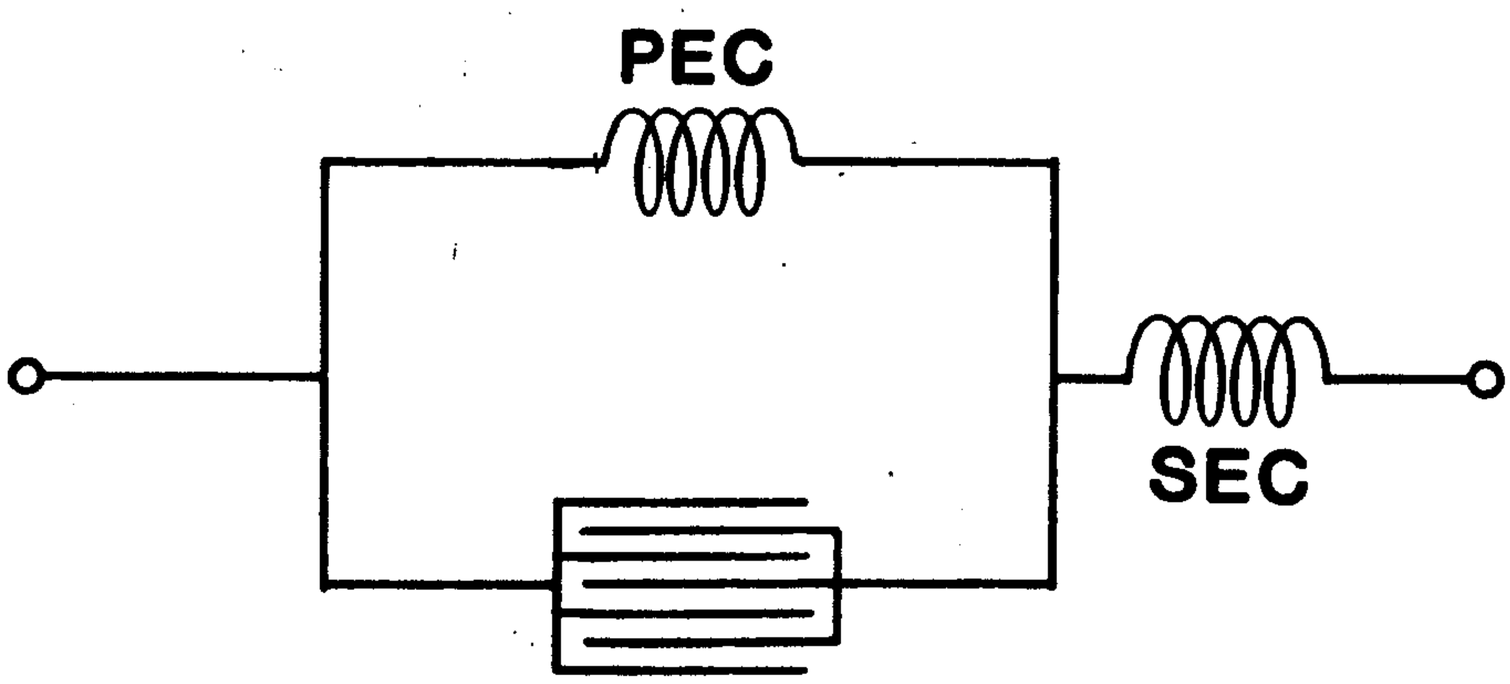
CHAPTER V

CONNECTIVE TISSUE IN THE DEVELOPING AND AGEING MUSCLE

Connective tissue plays an important role in transmitting the forces generated by the contractile elements and in binding the tissue together. Individual muscle fibres are surrounded by a network of connective tissue called the endomysium which meshes with thicker layers of connective tissue called the perimysium which surrounds muscle fibre bundles. Around the whole muscle is a third layer of connective tissue called the epimysium which is attached to the tendons. All three components inter-connect in a three dimensional framework that helps distribute loads within the muscle and prevent over-stretching. The main component of muscle connective tissue is collagen; a fibrous protein with high tensile strength and high longitudinal stiffness. Muscle tendons which are made up mainly of collagen can only stretch elastically by 4% before reaching irreversible lengths and later break under stress (Woodhead-Galloway 1980). Low stretchibility and strength are necessary qualities if precise muscular contractions are to be translated into flawless motion.

The interaction of the contractile component and elastic component have been described in a model suggested by Starling and Evans (1968). The model distinguishes between two sources of elasticity (Fig. 5.1), a parallel

Fig. 5.1 A mechanical representation of the contractile and elastic components in muscle, CC = contractile component, PEC = parallel elastic component and SEC = series elastic component.


PEC
SEC
CC

elastic component and a series elastic component. The former represents elements that are mechanically parallel to the contractile elements of which the main component is interstitial connective tissue, that is to say the endomysium, perimysium and epimysium. The series elastic component on the other hand is in series with the contractile component and under its direct influence. This component is made up from the elasticity of both the tendon and the protein between consecutive sarcomeres. The latter element is believed to occur in series at the Z line of the sarcomere. This elastic protein is known as connectin and is thought to be responsible for the continuity of myofibrils (Fujii and Kurosu 1979). The interaction of all components under tension conditionsproduces a passive tension curve that exhibits an exponential relationship. Initial loads cause the components to extend gradually at a slow rate, but as the load increases the components become less and less extensible and the relation becomes steeper as the components become stiffer showing smaller length changes. At muscle lengths where both the parallel elastic component and the series elastic component are engaged the passive tension is proportional to the amount and distribution of connective tissue in the muscle and the changes in the elements cementing sarcomeres end to end.

Connective tissue has been noted to increase in diseased dystrophic muscle (Lowry et al 1942, Dreyfus et al 1954) denervated muscle (Tomanek and Lund 1973) and immobilized muscle (Williams and Goldspink 1981). In most cases such changes are associated with degenerative processes and a general loss of muscle tissue. Histological examination of developing muscle revealed conflicting evidence on the status of connective tissue during this period of life. Chiakulas and Pauly reported a decrease in the ratio of connective tissue to muscle of three muscles in the rat over the 24 weeks after birth (1965). Inokuchi et al (1975) on the other hand reported an increase in connective tissue content in abdominal muscles of human subjects up to 50 years of age in both males and females. Biochemical

estimates of collagen in several muscles showed a decrease in connective tissue early in life followed by a rise continuing well into adulthood (Schaub 1963).

Senile muscles appear to show considerable variability in this respect. Decreases in connective tissue ratios to muscle tissue using histological techniques have been reported in the rectus abdominis of humans in extreme age (Inokuchi et al 1975). This was not in agreement with findings in a variety of ageing rat muscles using biochemical techniques (Schaub 1963, Mohan and Radha 1980). The discrepancy appears to be in part due to the differences in collagen content of individual animal muscles and partly to the differences in the distribution of collagen amongst different muscles. Abdominal muscles of the rat for example have a higher collagen content than either extensor or adductor muscles of the hind limb (Schaub 1963). In addition the experimental approach may make results from different studies incompatible; the study of Chiakulas and Pauly for example produced estimates of connective tissue based on the area not occupied by muscle divided by the total cross-sectional area (1965). Another histological study produced dissimilar estimates based on wave patterns produced by connective tissue after being stained with Hematoxylin and Eosin (Inokuchi et al 1975). The first technique was susceptible to shrinkage and area sampling errors.

The passive mechanical behaviour of connective tissue is affected by both qualitative and quantitative changes. The latter are simply due to the increased presence of collagen and the accompanying stiffness arising from the larger collagen bundles. Qualitative changes on the other hand in the structure of the molecule achieves similar results by changing the organisation and shape of collagen molecules and therefore the mechanical properties of individual molecules or group of molecules as is the case in cross-linking.

Verzar (1963) reported higher tensile strength and lower elasticity

of collagen from old rats compared to young animals. The changes in mechanical properties reflect the structural qualitative alterations in the protein makeup. Cross-linking starts at single molecule level linking the three strands of the helix with ester side bonds and progresses to link a number of molecules (Verzar 1963, Woodhead-Galloway 1980). This change has the effect of increasing the stiffness of the molecule.

The ageing of the collagen molecule appears to progress at a predictable and reproducible rate. Tail collagen from aged mice had higher resistance to the combined effect of urea and temperature (Harrison and Archer 1978). "Break-time" under load was higher in senile animals; this appears to confirm earlier findings on the effect of cross-linking on making the molecule of a more stable and resistant configuration.

Cross-linking nevertheless has been reported to cease near or just past maturity in bovine tendons (Davison 1978). Whether this trend holds true for interstitial collagen and for other species of animals is difficult to assess. An alternative mechanism to explain the absence of detectable cross-links in aged animals has been proposed and reported by other workers (Mechanic et al 1974); if cross-links are stabilized by an in vivo oxidation process, then the cross-links that are routinely detected by tritiated borohydride reduction cannot proceed with the reaction. This would falsely give the impression that reducible linkages are absent, yet the reducible links were merely converted to a form that cannot be detected using the borohydride technique. This form of stabilization would still create cross-links that could decrease the compliance and reduce the degradability of collagen.

The effect of increased collagen and cross-linking in the series elastic component acts in a similar way by decreasing the compliance of the tendon. Similarly connectin, the elastic component that is believed to occur serially in sarcomeres has been shown to change structurally with age (Fujii and Kurosu 1979). The number of reducible links in this protein

decreased with age. This would invariably decrease the elasticity of the protein and the compliance of the series elastic component.

In this study an examination of the mechanical properties of the slow soleus and the fast extensor digitorum longus was attempted in an effort to establish the influence of age on their passive mechanical properties. Collagen content of both tendon free and whole muscles was examined using biochemical techniques. Connective tissue estimates of endomysium and perimysium were determined using a quantitative microphotometric method. The results of all three approaches were interrelated and correlated to the available evidence of muscle performance and qualities during development and old age.

MATERIALS AND METHODS

I Passive Tension

Animals and surgical procedure

Male rats were selected from the ageing colony (Page18) at the ages of 21, 387 $\pm$ 2 and 714 $\pm$ 6 days. The anaesthetic and surgical procedures were the same as those described on Page 76. The muscles were kept under physiological conditions throughout the recording.

Recording procedure and conditions

The recording system was the same as that detailed on Page 74. The Devices UF1 force-displacement transducer was used as a passive tension transducer. After allowing the muscle to recover for 10 minutes the recording was initiated with the muscle at a length less than its resting length. The length was increased stepwise by turning a micrometer to the equivalent of 2.5% the optimum length in the young muscles and 0.75% the optimum length of the adult and senile muscle each time. The stretching was followed by a single stimulation at the parameters described in an earlier section (Page 77). The recordings were made on a slow running chart recorder (Devices MX412, Devices Ltd., Hertfordshire). The time between consecutive stimulations was enough to allow the muscle to settle

down to the new length. The process was continued until the muscle length exceeded the optimum length by a reasonable margin (5% optimum length in the extensor digitorum longus and adult and senile soleus, and up to 8% in young soleus). Optimum length was determined by measuring the amplitude of the response. Recording was terminated when the twitch tension produced by the muscle decreased below that obtained at optimum length. Length changes were related to optimum length.

Statistical analysis

Passive tension in both the extensor digitorum longus and the soleus responded in a close to exponential fashion to increased muscle length (Fig. 5.3,5). Passive tension was expressed as a percentage of single twitch tension and muscle length was expressed as a percentage of optimum muscle length. The exponential relation was fitted to a straight line formula in the form of:

$$\text{Log } y = a + bx$$

where Log y = the logarithm (base 10) of the percentage of the force produced and x = percentage length change of the optimum length.

The independent variable x was restricted to values $>95\% < 105\%$ in all ages of the extensor digitorum longus and to values $>90\% < 105\%$ in the soleus of animal groups aged 387 and 714 days and to values $>90\% < 108\%$ in the young 21 day old soleus. The "minimum value" imposed on the independent variable was to reduce recording errors since at those values the passive tension produced was comparatively low and was associated with a high degree of mechanical interference.

The fitted relationship had a high degree of correlation, the correlation coefficient in both muscles was better than 0.97 except in two cases where it was 0.95 and 0.96.

By comparing the slopes of the fitted lines it was possible to estimate the comparative rate at which muscles resist changes in length.

The steeper the slope (regression coefficient) the higher the resistance.

II Biochemical Estimation of Collagen

Animals and muscle preparation

Developing and ageing male rats were chosen at the ages of 21, 84, 188, 299 $\pm$ 1 and 716 days for this experiment. This represented a good cross-section of the ageing colony 21 days being the weaning age, 300 days the "adult animal" age and 700 + days the "senile" stage. In addition to examining two hind limb muscles; the extensor digitorum longus and the soleus, heart muscle was also examined. This provided a means of assessing a muscle that does not contain any tendon, also the heart is a continuously active muscle.

Animals were sacrificed with a blow on the head followed by the dislocation of cervical vertebrae. The extensor digitorum longus and the soleus of the left limb were dissected out complete with both tendons intact and cut as close as possible to the bone. Excess superficial connective tissue and fat was removed. The belly of the muscle was carefully cut out with extra care being taken not to include any tendon especially in the older animals where a thin sheath extends on both sides of the muscle from each tendon and overlap in the middle. The belly and the remaining two segments were weighed and placed separately in vented P.V.C. vials (No.690, Sarstedt) and were then quenched in liquid nitrogen.

The heart was excised slit open and washed in a ringer solution (Page 74). It was then weighed, placed in a vial and quenched in liquid nitrogen. All muscles were usually excised and frozen within an hour of the animals death. The muscles were then freeze dried for 12-18 hours at 13.33Pa in a freeze dryer (Chem. Lab. Instruments Ltd., London). The muscles were weighed again to obtain dry weight and were stored in a desiccator to await further processing.

Biochemical assay of hydroxyproline

Hydroxyline occurs almost exclusively in collagen and contributes

about 10% of all amino acids in it (Woodhead-Galloway 1980). Since the proportion of hydroxyproline in muscle collagen is fixed, it makes this amino acid ideal for the identification and quantification of collagen.

Freeze-dried muscles were dissolved in 5ml of 6N HCl and sealed in a 20ml glass tube with a screw-on-top lined with Teflon (Kimax, U.S.A.) and autoclaved at 172.5kPa for five hours.

Hydroxyproline content of the hydrolysates was determined by Grant's method (Grant 1964) for autoanalysis of hydroxyproline adapted from Stegemann's method (Stegemann 1958). The analysis was carried out on a Technicon Autoanalyser II (Technico Inst. Co.). Briefly the procedure allows the oxidation of hydroxyproline to a hydroxyproline chromogen in the presence and the influence of chloramine T. The hydroxyproline chromogen was then reacted with p-dimethylaminobenzaldhyde (P.D.A.) to develop a pink colour. The colour density produced was linear to the concentration of hydroxyproline in the range 2-20µg (Grant 1964) therefore using a colorimeter at a wavelength of 550nm an accurate estimate of hydroxyproline was possible.

Reagents

Acetate buffer: 50g citric acid (monohydrate), 12ml glacial acetic acid, 120g sodium acetate ($3H_2O$) and 34g sodium hydroxide made up to a final volume of one litre, pH6.0 and stored in the cold under toluene.

p-Dimethylaminobenzaldehyde (P.D.A.) 5% solution of P.D.A. in n-propanol, stored in the cold.

Perchloric acid: 27ml of 70% perchloric acid to a final volume of 100ml in water

Chloramine T.: 1.41g chloramine T. in 80ml water diluted to a volume of 200ml in ethylglycol monoethyl ether and added to an equal volume of buffer. The solution was made up fresh for each run.

Reaction and Calculations

The reagents were reacted with a diluted sample solution (1ml sample with 1ml 6N sodium hydroxide and 3ml 3N NaCl) at a ratio of 1ml sample: 2ml chloramine T: 1ml perchloric acid: 1ml P.D.A. Samples with high hydroxyproline content were diluted even furthur to allow reading in the most linear and sensitive range of the technique. The results were read against a calibrated curve of hydroxyproline standards. The collagen content was calculated by multiplying the hydroxyproline content of the hydrolysate by 6.94 (Jackson and Clearey 1967).

Statistical Analysis

The results were subjected to a Model I analysis of variance and an a priori comparison conducted serially amongst all groups involved.

III Microchromometric Estimation of Connective Tissue

Animals and Muscle Preparation

Male rats aged 21, 84, 185 $\pm$ 3, 299 $\pm$ 1, 508 and 758 days were used. The extensor digitorum longus and the soleus were used in this investigation. Both muscles were dissected out and treated as described on page 28. The resulting 10µm thick sections of the belly region were stored in a deep freeze until required.

The Stain

Sirius Red F3BA (Colour Index 35780) is an industrial dye. Its application in histological staining was first described by Sweat et al (1964). It is highly stable in solution and possesses excellent acid fastness (4-5 on a scale of 5) and moderate light fastness (4-5 on a scale of 8) the combination of the two characteristics produced stable staining of both paraffin embedded fixed sections and unfixed frozen sections. The stain is specific, staining connective tissue fibres, except elastic membranes, with a bright red colour. The stain requires the presence of aqueous picric acid. The latter prevents the staining of background tissue such as muscle fibres and increases the contrast by staining such tissue

bright yellow. The selectivity and contrast of this stain was manipulated with a system of optical filters and electronic circuits to enhance the contrast and obtain quantitative estimates of the stain intensity and dispersion.

The Image Analysis System (Fig. 5.2)

A Leitz Ortholux microscope was adapted to accept a high resolution monochrome video camera (Link camera type 109A, Link electronics). A Farnell stabilised power supply type B30/10 (Farnell Inst. Ltd., Wetherby, Yorks), was used to derive the microscope's light source. This was essential since unstable light sources affected the response of both the camera and the electronic processing circuits. The optical image generated by the microscope of the section under study was passed through a green filter (wavelength = 530nm). The colour combination of the bright red stain and green filter increased the contrast of the sections dramatically by preventing the red colour from reaching the camera. The video image generated by the camera was further processed electronically to enhance the contrast. A variable threshold level was set to remove noise and grey gradation, so that the new image was a mixture of either black or white points with no greys. The black areas corresponded to the stained connective tissue while the white areas were the picric acid stained background tissue. The final image was displayed on a video monitor (Plate 5.1).

In addition to being able to select the area on the section mechanically by moving the microscope stage, an electronic circuit allowed the blanking of the processed video image so that a small area of the total field of known dimensions could be studied. Once an area has been selected a counter measured the number of black points in any one frame, or in an average of 10 frames. The readings obtained corresponded in practice to the number of "no pulse" on the video monitor. The blanked area did not interfere with the count although it was a timed "no pulse".

Fig. 5.2 Schematic diagram of the image analyser and the associated systems.

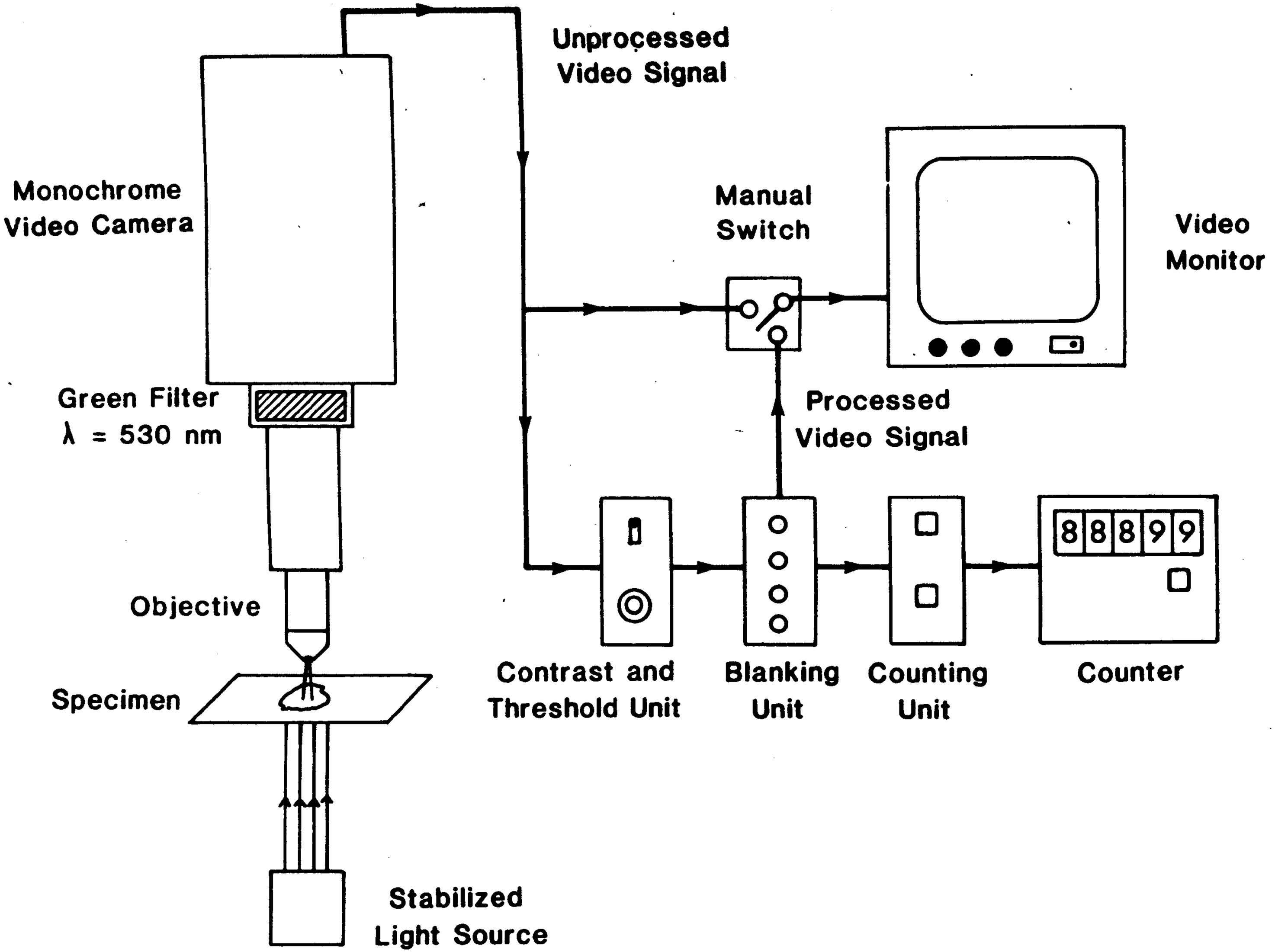
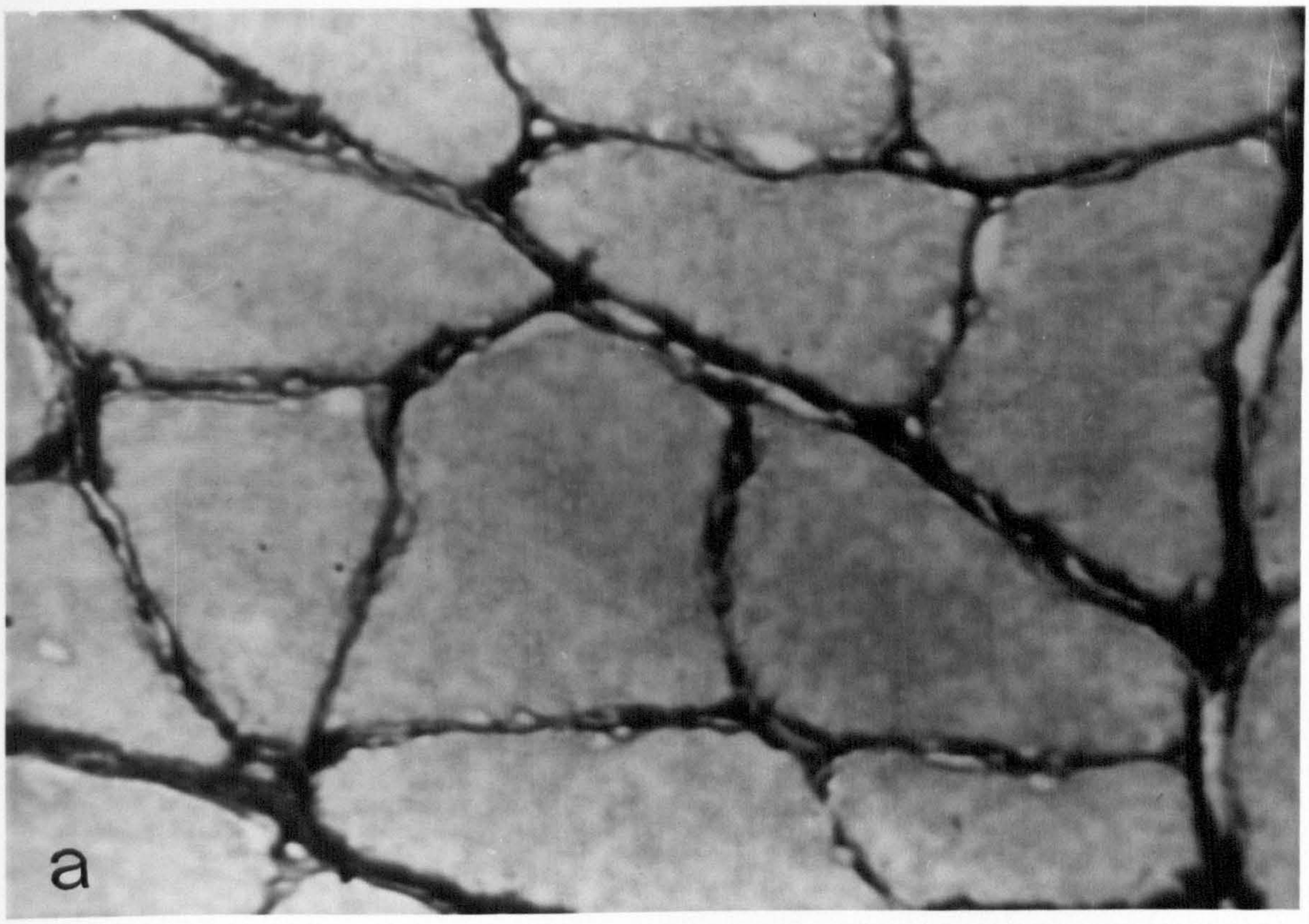

Unprocessed
Video Signal
Monochrome
Video Camera
Manual
Switch
Video
Monitor
Green Filter
λ = 530 nm
Processed
Video Signal
Objective
Specimen
Contrast and
Threshold Unit
Blanking
Unit
Counting
Unit
Counter
Stabilized
Light Source

Plate 5.1 (a) Unprocessed image of a Sirius Red stained section. The dark stain corresponds to connective tissue while grey areas are muscle and background stain. The processed image (b) electronically removes the grey gradation leaving connective tissue as black lines (magnification x485).


a


b

Procedure

The stain was prepared as a 1% solution of Sirius Red F3BA in water. Picro-Sirius red was obtained as a solution of 10% stock solution in saturated aqueous picric acid. Picric acid crystals were added to the freshly prepared solution to ensure the continuous saturation of the stain. The solution was allowed to stand for 24h before use. Frozen sections were rehydrated under running water for at least 10min. This step was necessary to allow the large molecules of Sirius Red to penetrate connective tissue fibres (Sweat et al 1964) and perhaps more important, it assisted the action of the poorly soluble picric acid in preventing the reaction of Sirius Red with background tissue. The sections were then stained in saturated aqueous picric acid for 15min followed by one minute in Picro-Sirius Red. Sections were dehydrated in 3 x 1min changes in absolute alcohol and were then cleared in xylene and mounted in DePeX . The staining time in Picro-Sirius Red was adjusted so that one timing would produce good staining of all age groups and muscles. This acted as a safeguard against over or understaining of different sections. Furthermore all slides were processed in one of 3 batches, each batch containing a mixture of ages and muscles.

The image analyser was set to a threshold that would produce results for both lightly stained young muscles and darker older muscles. This reduced errors arising from incompatible threshold settings. The blanking on the processed image remained constant, hence counts from any two muscles at the same magnification were directly comparable.

Two types of counts were obtained for each muscle; the first at a final magnification of x485 and a second at a final lower magnification of x127. The former readings were obtained using blanked fields covering an area of $0.0144mm^2$. The fields were selected to contain mainly endomysial connective tissue. Between ten and twenty fields were counted and each count was an average of 10 readings. Also the number of whole muscle fibres enclosed within the field and fibres touching two perpendicular

sides of the blanking were counted in each field. At least 5 fields at the lower magnification, each an average of 10 readings, were used to estimate perimysial connective tissue. The combination of low magnification and lower threshold settings produced estimates of the thicker perimysial connective tissue. Perimysium was sampled systematically by sampling fields from the deep to the superficial axes of the muscle, the spacing between any two adjacent scans was wide enough to cover the whole muscle by the time the required number of fields was measured. The fields covered by each reading at the lower magnification was $0.6006mm^2$.

Results were expressed as counts per unit area (Where the unit area was equivalent to the field covered at each magnification i.e. $0.6006mm^2$ and $0.0144mm^2$). Endomysial connective tissue was also expressed as counts per fibre.

Statistical Analysis

The results were subjected to a two level analysis of variance followed by a serial a priori group comparison and an F-test (Page 26).

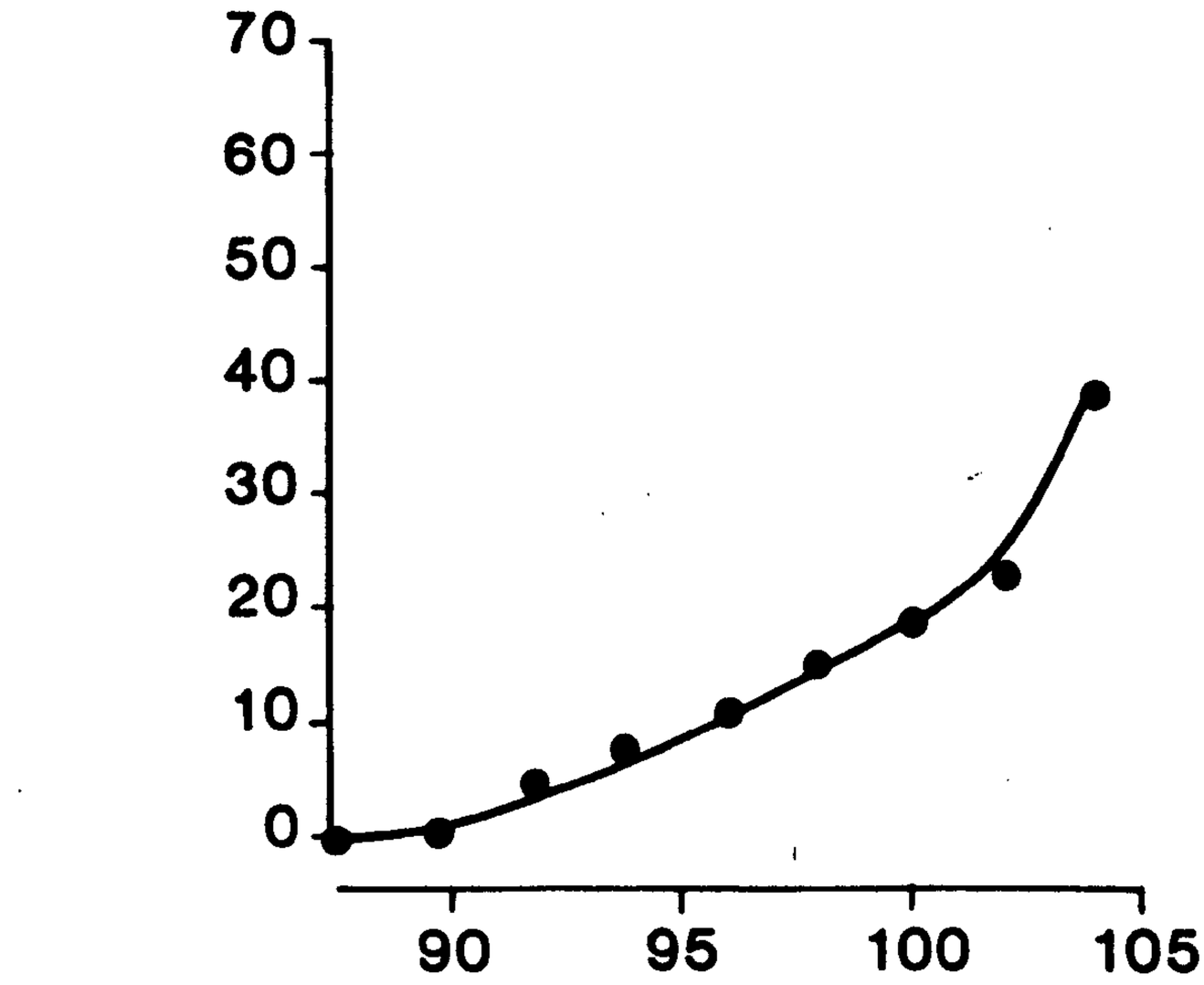
RESULTS

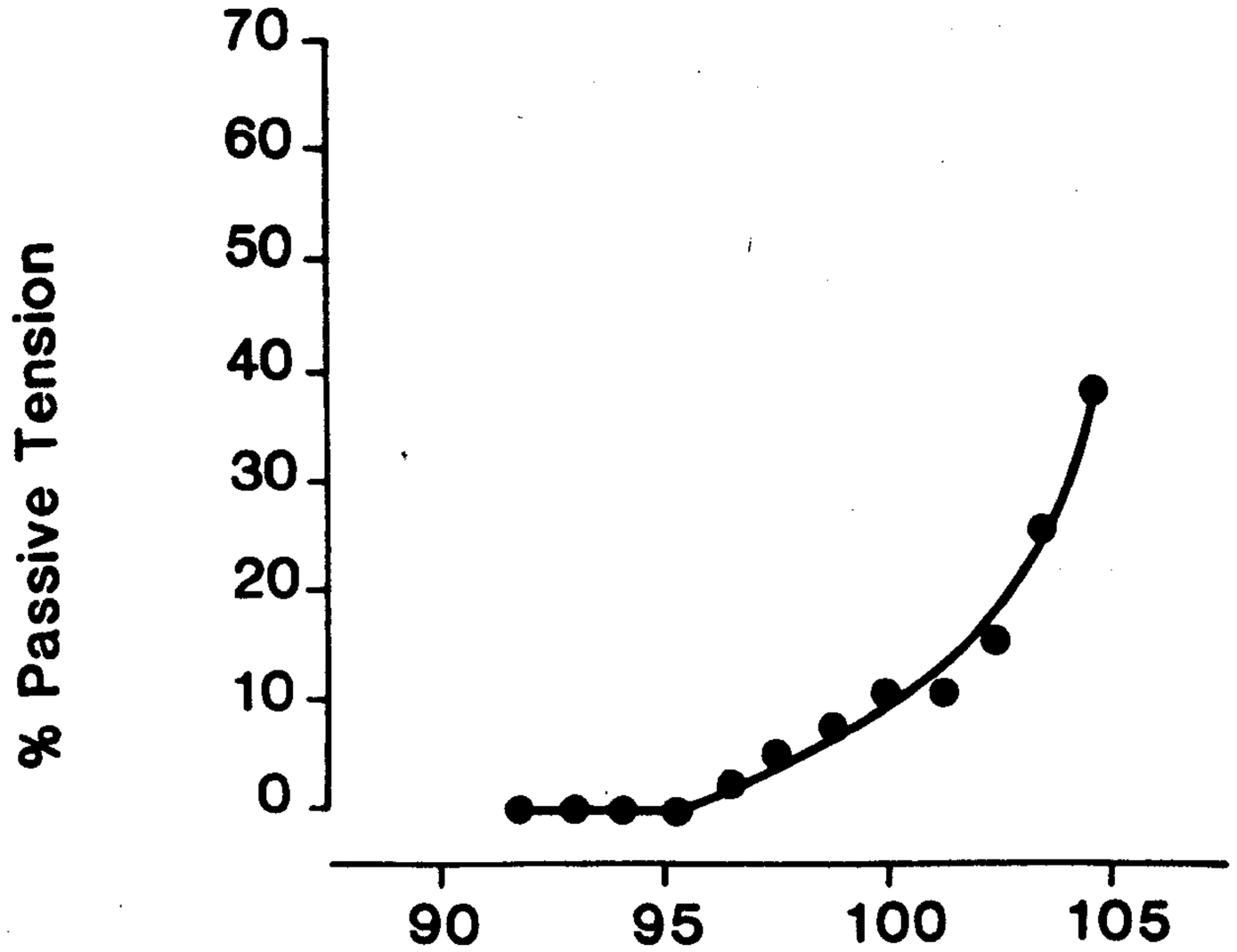
I Passive Tension

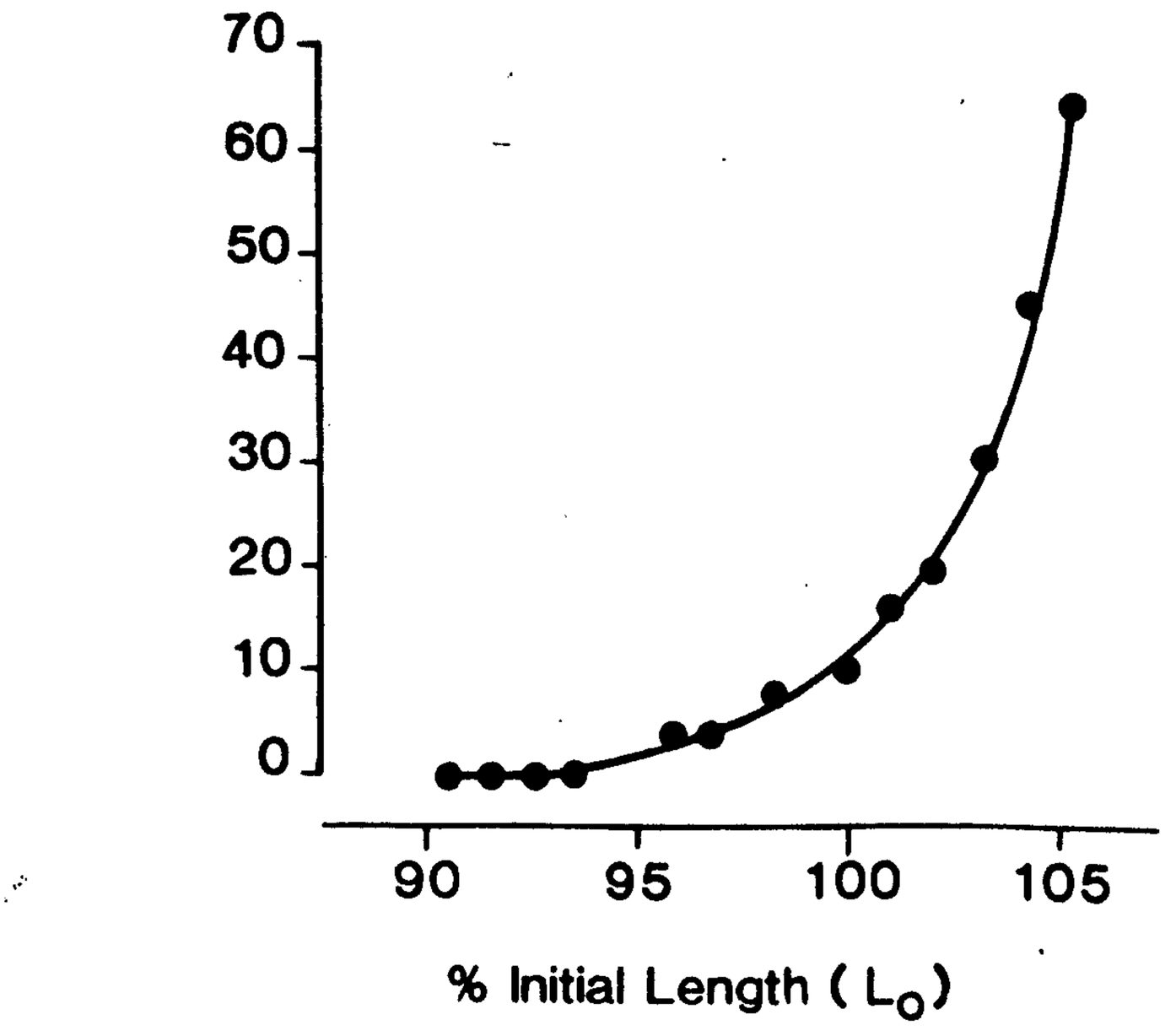
(a) The extensor digitorum longus

The relation between muscle length and passive tension produced within the muscle was of an exponential nature in all age groups investigated. Initial increases in muscle length around the in vivo resting length of the muscle produced small changes in passive tension. As the muscle was stretched passive tensions increased exponentially. The rate of this increase varied with age. The young 21 day old extensor digitorum longus showed a slower rate of passive tension development for every unit increase in length (Fig. 5.3a). The adult muscle developed passive tensions at a higher rate when compared with the young muscle (Fig. 5.3b) and the senile muscles showed the highest increase in tension per unit length (Fig. 5.3c).

Fig. 5.3 An example of passive tension : length relationship in representative extensor digitorum longus muscles aged (a) 21 days (b) 387 days and (c) 714 days. Tension is expressed as a percentage of maximum twitch tension at optimum length.


70
60
50
40
30
20
10
0
90
95
100
105
(a)


% Passive Tension
70
60
50
40
30
20
10
0
90
95
100
105
(b)


70
60
50
40
30
20
10
0
90
95
100
105
(c)
% Initial Length (L_o)

The fitted regression to the relation as described on Page 108 produced a linear relationship (Fig. 5.4). An examination of the slopes in the three age groups studied (Table 5.1) showed a trend towards an increased slope with age. The variation in the regression coefficient was higher between age groups than between individual muscles. Individual variation of the length at which maximum twitch tension occurred made direct comparison of complete straight line formulae difficult and not representative. The use of the log transformation and regression coefficient proved to be more suitable means of comparison. The analysis of regression in all muscles produced a positive correlation of the regression parameters.

(b) The soleus

A similar relationship between muscle length and passive tension was observed in the soleus. The young soleus demonstrated a slow but positive increase in passive tension with muscle length changes (Fig. 5.5a). The rate was considerably lower than either of the two older groups (Fig. 5.5b,c). The adult and senile soleus muscles demonstrated very similar increases in tension. An examination of the logarithmic relationship showed a good fit in all age groups (Table 5.2). An examination of the slopes of the fitted lines showed a large increase in slope steepness between the 21 day old and the adult muscles (Fig. 5.6). In contrast with the situation for the extensor digitorum longus the senile soleus did not differ appreciably from the adult muscles.

A comparison of the extensor digitorum longus and the soleus at the same ages showed similarities in the two young muscles. The behaviour at later ages was completely different; the extensor digitorum longus progressed slowly reaching maximum steepness in old age while the soleus increased the tension developed rapidly early in life and remained unchanged in old age.

II <u>Biochemical Estimate of Collagen</u>

(a) The extensor digitorum longus

Fig. 5.4 The log passive tension : length relationship in the extensor digitorum longus muscles aged (a) 21 days (b) 387 days and (c) 714 days allowed direct comparisons of the "slopes" of the relationship. Passive tension is expressed as log the percentage of maximum twitch tension produced at optimum length.

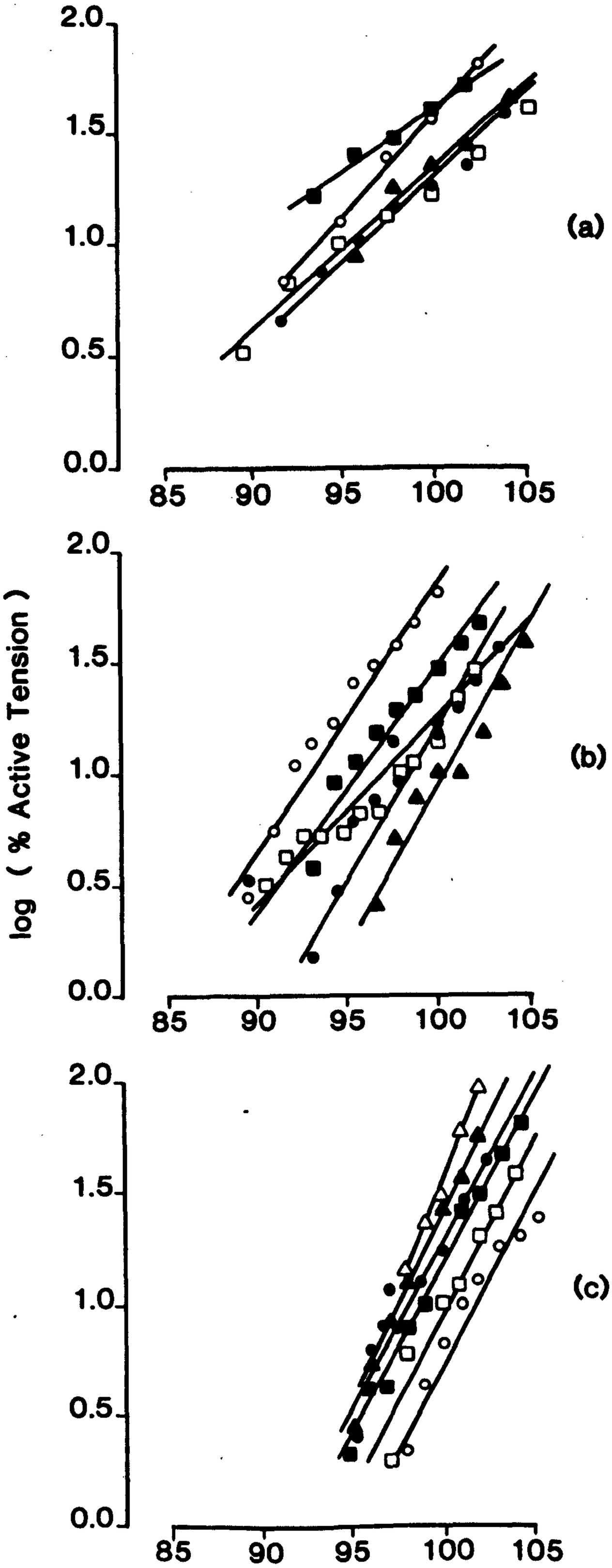
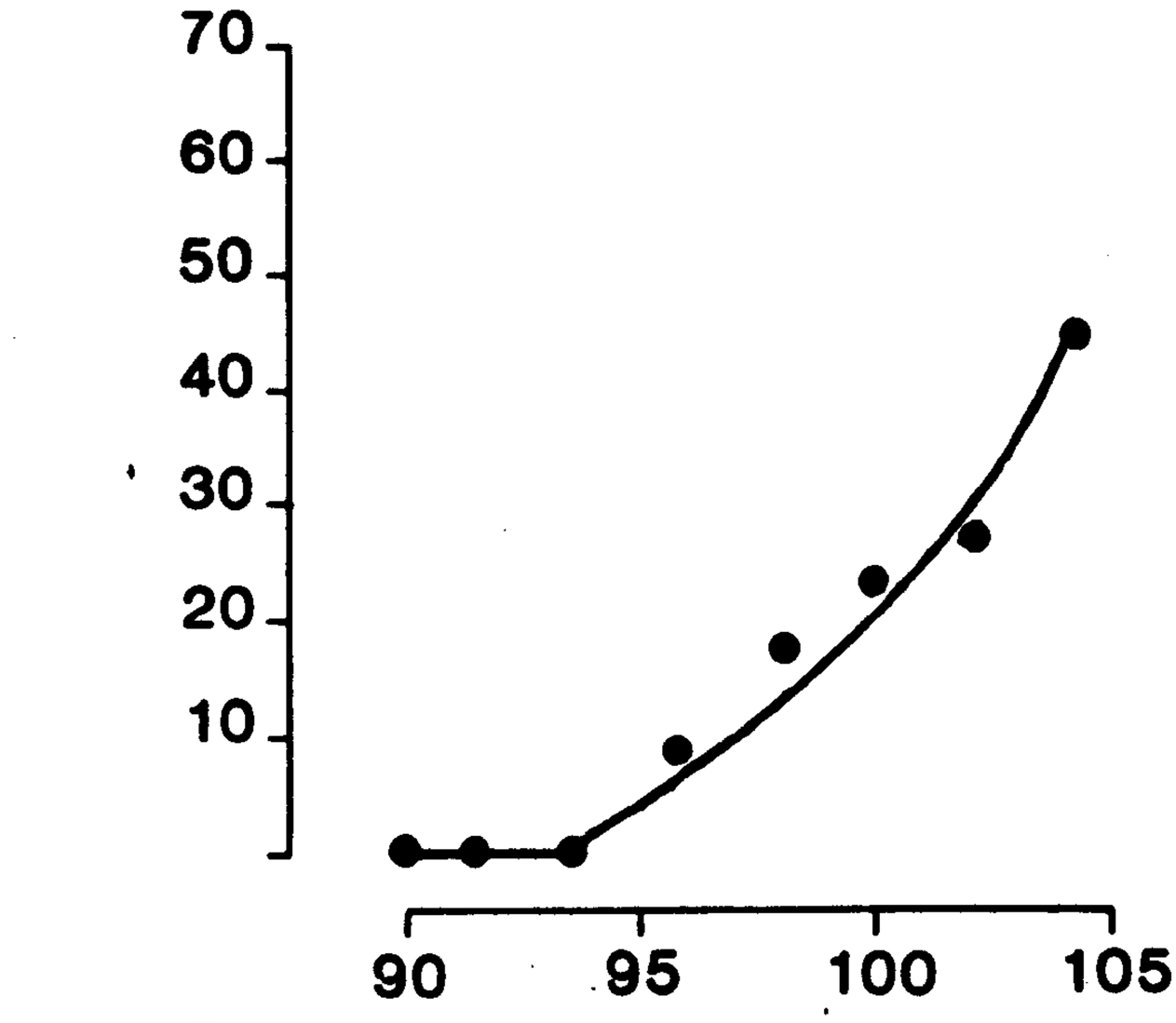

2.0
1.5
1.0
0.5
0.0
85
90
95
100
105
(a)
(b)
(c)
log (% Active Tension)
% Initial Length (L_o)

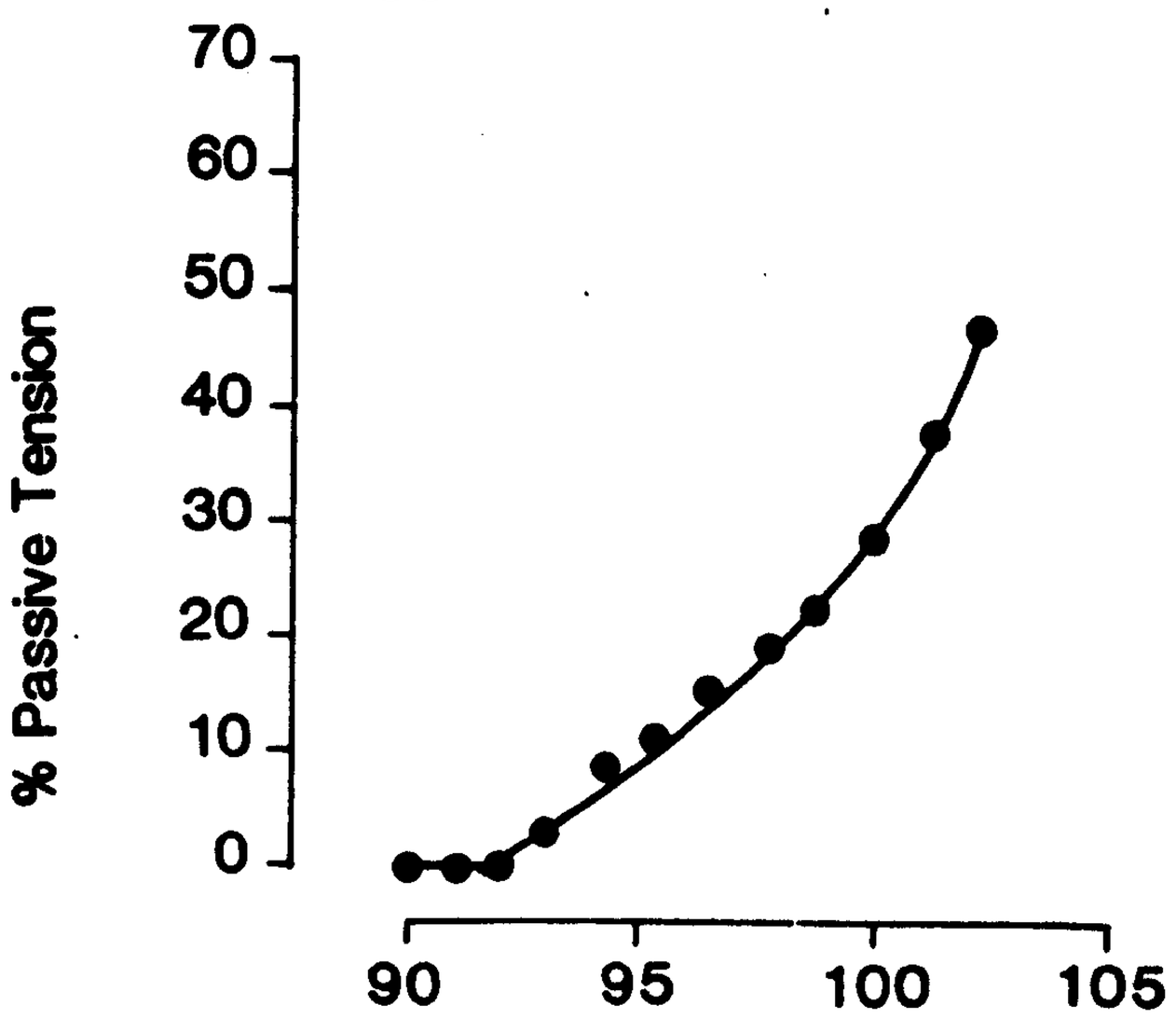
Table 5.1 Length:passive tension relation in the Extensor digitorum longus

Age (Days)	Animal No.	Regression Coefficient	Correlation Coefficient	Mean Regression Coefficient ± S.D.	Probability <
21	1	0.059	0.978		
	2	0.074	0.972		
	3	0.091	0.994		
	4	0.048	0.996		
	5	0.056	0.990		
				0.066±0.017	
387	1	0.094	0.946		
	2	0.125	0.982		
	3	0.082	0.995		n.s.
	4	0.086	0.999		
	5	0.105	0.982		
				0.098±0.17	
714	1	0.107	0.980		
	2	0.150	0.994		
	3	0.124	0.976		0.005
	4	0.193	0.970		
	5	0.181	0.989		
	6	0.209	0.982		
				0.161±0.040	

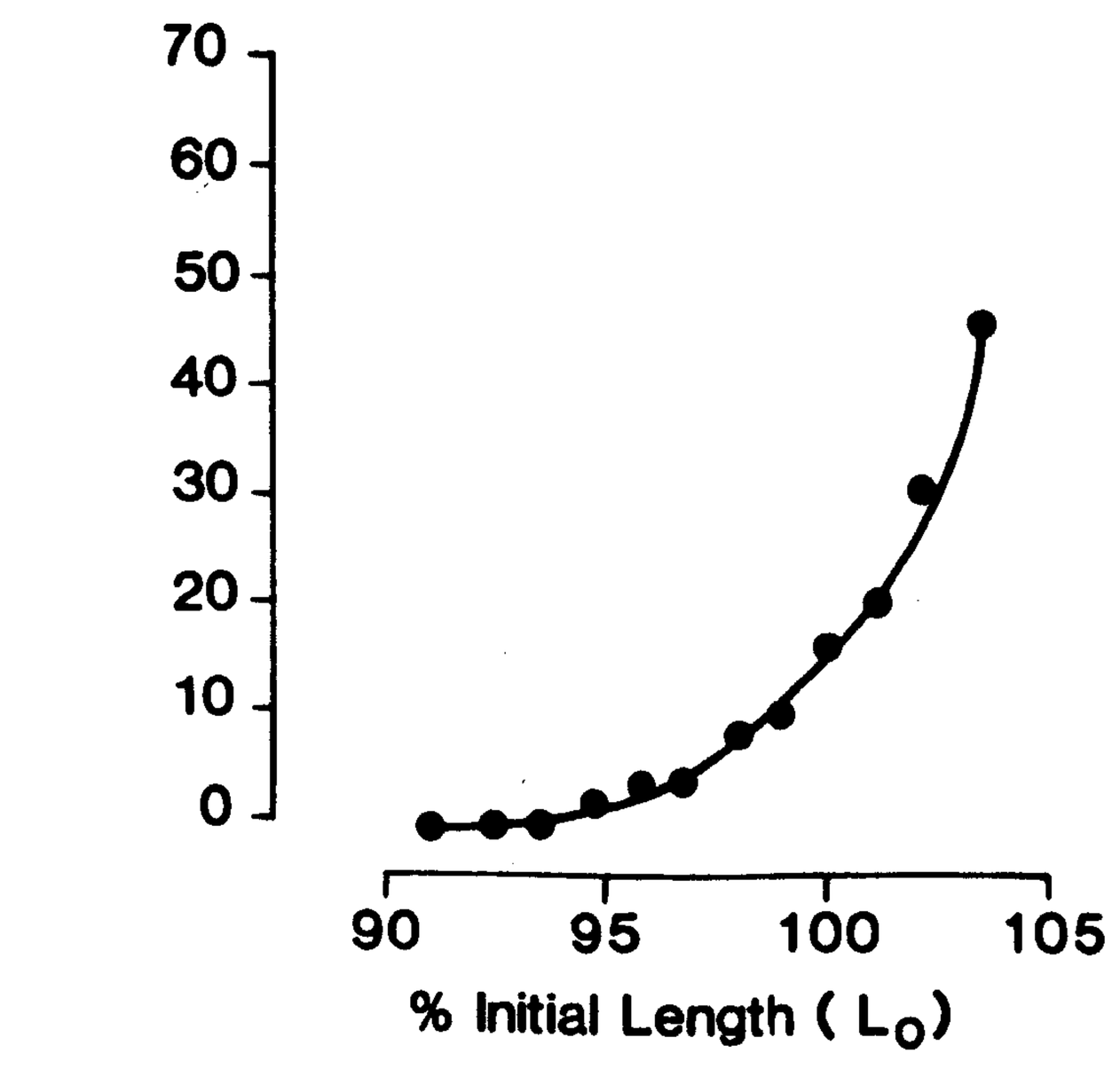
Fig. 5.5 An example of passive tension : length relationship in representative soleus muscles aged (a) 21 days (b) 387 days and (c) 714 days. Tension is expressed as a percentage of maximum twitch tension produced at optimum length.


70
60
50
40
30
20
10
90
95
100
105

(a)


% Passive Tension
70
60
50
40
30
20
10
0
90
95
100
105

(b)


70
60
50
40
30
20
10
0
90
95
100
105
% Initial Length (L_o)

(c)

Fig. 5.6 The log passive tension : length relationship in the soleus muscles aged (a) 21 days (b) 387 days and (c) 714 days allowed direct comparisons of the "slopes" of the relationship. Passive tension is expressed as log the percentage of maximum twitch tension produced at optimum length.

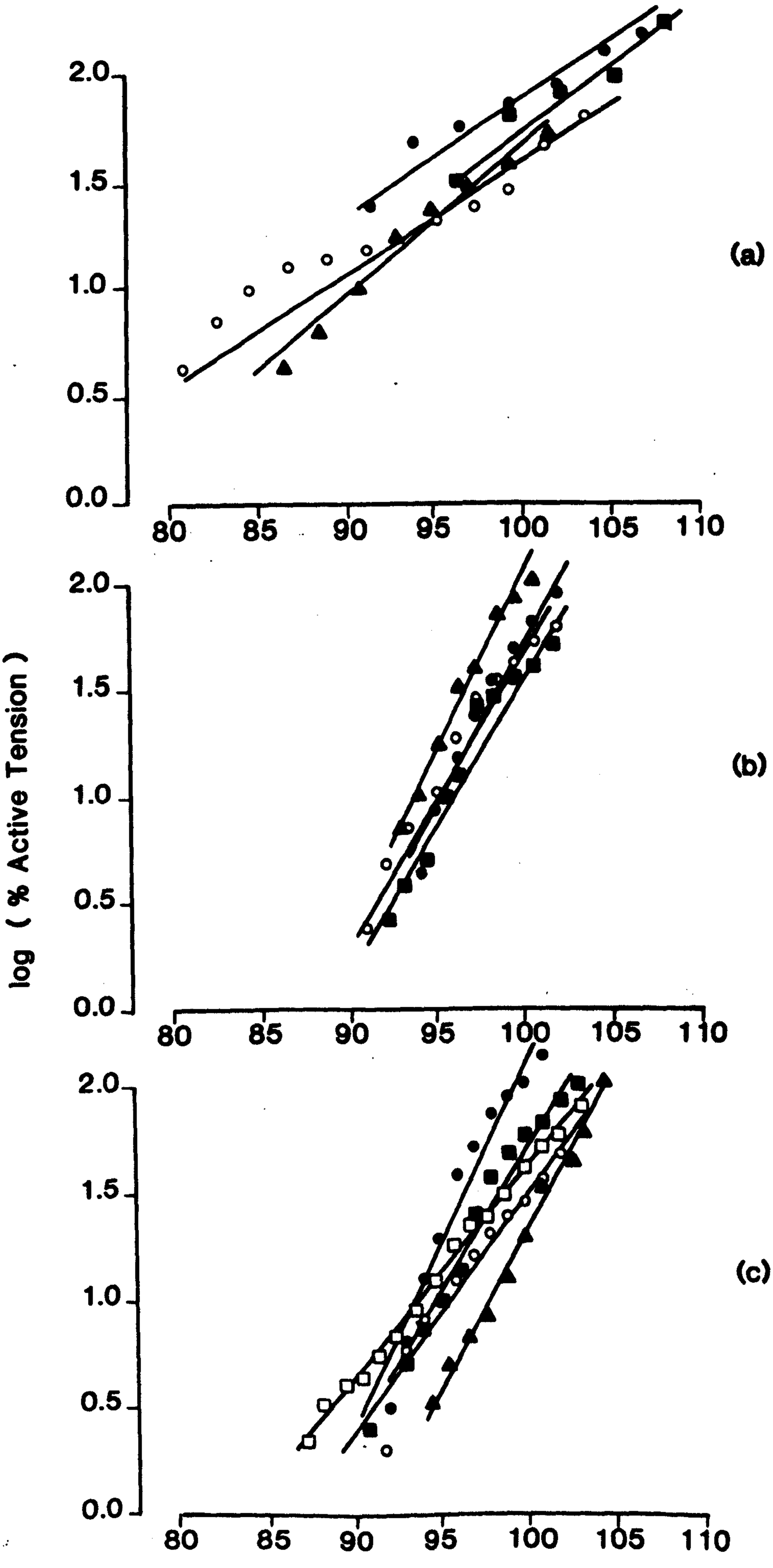

log (% Active Tension)
2.0
1.5
1.0
0.5
0.0
80
85
90
95
100
105
110
(a)
(b)
(c)
% Initial Length (L_o)

Table 5.2 Length:passive tension relation in the Soleus

Age (Days)	Animal No.	Regression Coefficient	Correlation Coefficient	Mean Regression Coefficient ± S.D.	Probability <
21					
	1	0.038	0.994		
	2	0.059	0.985		
	3	0.058	0.977		
	4	0.052	0.994		
				0.052 ± 0.010	
387					
	1	0.148	0.993		
	2	0.126	0.960		
	3	0.158	0.987		
	4	0.110	0.983		
				0.135 ± 0.022	0.001
714					
	1	0.128	0.971		
	2	0.095	0.998		
	3	0.117	0.978		
	4	0.089	0.994		
	5	0.145	0.997		
				0.115 ± 0.023	n.s.

Total collagen content in the whole muscle including both tendons increased continuously with age. Between the 21 day old weanling and the 84 day old animal there was a 13 fold increase in collagen in the muscle (Table 5.3). This increase continued at a much reduced rate in the 188 day old rat ($P < 0.001$) and then showed no significant increase until old age (716 days) where a further increase of 11% was observed ($P < 0.005$).

When collagen content was related to muscle weight there was still a significant increase in collagen per milligram muscle (73%) between the 21 day and 84 day old animals. After this, there was a trend towards a lower collagen content per milligram muscle, the reduction not significant until an age of 299 days was reached where a 14% reduction in collagen proportion was observed. Senile animals showed an increase in collagen concentration when compared with the adult animals but not when compared to the younger 188 day old animals. Results were similar whether dry or wet muscle weights were used.

Collagen to muscle ratios in the belly region of the extensor. digitorum longus where both tendons were removed changed with age; 84 day old animals showed a two fold increase in collagen over the younger 21 day old muscles. Collagen proportion continued to increase up to 188 days at a lower rate ($P < 0.001$) but was then reduced signficantly by 30% in adult animals. The senile extensor digitorum longus nevertheless had a higher concentration of collagen when compared to the 299 day old animals ($P < 0.001$).

(b) The soleus

Total collagen content in the soleus increased in a similar fashion to the extensor digitorum longus (Table 5.4). The 84 day old animal showed a 13 fold increase in absolute collagen content over the 21 day old animal. This increase continued in 188 day old rats.($P < 0.05$) then remained level up to 299 days. Senile soleus muscles showed a 29% increase

Table 5.3 Collagen content of the developing and ageing Extensor digitorum longus

Age (Days)	No. of Animals	Absolute Collagen Content mg	Pr. <	Collagen in whole muscle				Collagen in muscle (Belly)			
				µg/mg dry muscle	Pr. <	µg/mg wet muscle	Pr. <	µg/mg dry muscle	Pr. <	µg/mg wet muscle	Pr. <
21	5	0.3966 ± 0.0532	0.001	68.96 ± 8.45	0.001	17.50 ± 1.70	0.001	20.54 ± 2.37	0.001	4.89 ± 0.87	0.005
84	5	5.0170 ± 0.3914	0.001	107.92 ± 6.97	n.s.	30.28 ± 2.04	n.s.	44.82 ± 8.66	0.001	12.03 ± 2.54	n.s.
188	5	6.0618 ± 0.2946	n.s.	102.07 ± 7.21	0.005	28.29 ± 2.09	0.005	58.99 ± 1.72	0.001	15.73 ± 0.76	0.05
299	5	6.0474 0.4784	0.005	87.51 ± 5.22	0.005	24.40 ± 1.41	0.005	42.12 ± 6.81	0.001	11.04 ± 1.77	0.005
716	5	6.7436 0.1697		104.41 ± 7.75		28.45 ± 2.31		61.39 ± 5.23		18.33 ± 5.81	

All values are ± S.D.

Table 5.4 Collagen Content of the developing and ageing soleus

Age (Days)	No. of Animals	Abolsute Collagen Content mg	Pr. <	Collagen in whole muscle				Collagen in muscle (Belly)			
				µg/mg dry muscle	Pr. <	µg/mg wet muscle	Pr. <	µg/mg dry muscle	Pr. <	µg/mg wet muscle	Pr. <
21	5	0.3786 ± 0.1546	0.001	47.68 ± 8.29	0.001	18.18 ± 7.08	0.05	36.16 ± 10.83	n.s.	8.80 ± 2.33	n.s.
84	5	5.0308 ± 0.8993	0.05	106.96 ± 19.32	n.s.	29.33 ± 5.19	n.s.	33.17 ± 4.78	n.s.	8.29 ± 1.11	n.s.
188	5	7.4218 ± 1.6947	n.s.	125.27 ± 26.11	n.s.	34.13 ± 8.41	n.s.	44.97 ± 13.43	n.s.	10.99 ± 3.50	n.s.
299	4	7.5473 ± 2.2005	0.05	100.73 ± 23.22	0.05	28.61 ± 7.91	0.05	34.70 ± 5.22	0.05	8.77 ± 1.37	0.05
716	5	9.7524 ± 1.3380		132.86 22.34		39.74 ± 5.92		49.96 ± 11.25		12.50 ± 2.46	

All values are ± S.D.

in collagen at 716 days of age compared to 299 day muscles.

Collagen content related to muscle weight demonstrated a trend similar to the extensor digitorum longus. Collagen content per milligram of muscle continued to increase between the ages of 21 through 188 days. The changes were only statistically significant between the weanlings and the 84 day animals where a 61% increase was observed. The senile soleus demonstrated 39% higher concentrations of collagen compared to adult animals ($P < 0.05$).

The analysis of variance indicated that the variations in the belly region of the soleus were only significant when collagen was expressed in terms of wet muscle weight ($P < 0.045$) rather than dry muscle weight ($P < 0.055$). This was due to the slightly higher variability in samples expressed as μg collagen per mg dry muscle which was rather surprising. The statistical analysis showed no significant increase in collagen concentrations except at extreme age where a 43% increase was observed.

A comparison of the two skeletal muscles revealed higher concentrations in the belly of the senile extensor digitorum longus. When the tendons were taken into account senile soleus muscles showed higher concentrations of collagen. This suggested that most of the collagen in the soleus came from the tendons.

(c) The heart

Collagen in the heart of developing and ageing rats increased in absolute terms with age. All increases were statistically significant (Table 5.5) except when 188 and 299 day animals were compared. Senile hearts contained 49% more collagen when compared to adult hearts.

Expressed in relative terms (μg collagen per mg dry muscle) collagen concentrations increased by 44% early in life between 21 and 84 day old animals ($P < 0.01$) and remained relatively unchanged until old age where senile hearts contained 57% more collagen when compared to adult hearts.

The reductions in relative collagen content of the two skeletal

Table 5.5 Collagen content of the developing and ageing heart

Age (Days)	No. of Animals	Absolute Collagen Content µg	Pr. <	Collagen µg/mg dry muscle	Pr. <	Collagen µg/mg wet muscle	Pr. <
21	5	836.40 ± 141.57	0.001	15.11 ± 1.92	0.01	3.64 ± 0.86	n.s.
84	5	4891.60 ± 651.83	0.05	21.82 ± 3.34	n.s.	4.74 ± 0.88	n.s.
188	5	6787.00 ± 914.21	n.s.	22.00 ± 1.15	n.s.	5.15 ± 0.26	n.s.
299	5	7425.60 ± 1640.17	0.001	21.52 ± 3.78	0.001	4.98 ± 0.90	0.001
716	5	11076.90 ± 1637.21		33.89 ± 5.12		7.41 ± 1.10	

All values are ± S.D.

muscles around adulthood were not pronounced in the heart. The slight variations in the heart estimates at this age were well within the normal errors and variations expected in such a biochemical technique. This was important since it probably indicated a different response of the heart to connective tissue accumulation.

III Microchromometric estimation of connective tissue

(a) The extensor digitorum longus

Endomysial connective tissue expressed as counts per unit area showed a significant correlation to age as revealed by the analysis of variance. The amount of connective tissue per unit area was doubled by the time the animal was 84 days old (Table 5.6). Animals aged 508 days showed a 44% increase over the 299 day old adult animals. Senile animals exhibited a further increase of 24% when compared with the 508 day group.

When endomysial connective tissue was related to the number of fibres present in the field counted, similar trends appeared (Table 5.6). Young muscle fibres had the least amount of connective tissue associated with them. However by 84 days the amount of connective tissue per fibre increased by more than 8 fold, an indication of the rapidity at which connective tissue builds up around fibres. The readings were probably slightly over estimated by the reduced number of fibres per unit area due to normal increases in fibre dimensions with age. Ageing and senile animals accumulated connective tissue at a relatively high rate increasing by 54% between 299 and 508 days and later by another 19% between 508 and 754 days.

Perimysial connective tissue in the extensor digitorum longus estimated over a larger muscle area showed a trend towards reduced connective tissue counts per unit area in 84 day old animals as compared to 21 day old weanlings (Table 5.6). This trend was reversed in older animals; 508 day animals showed a 34% increase over 299 day adults and a further 69% was observed in senile animals as compared to 508 day old rats.

Table 5.6 Connective tissue content of developing and ageing Extensor digitorum longus

Age (Days)	No. of Animals	Endomysium				Perimysium	
		count/unit area*	Pr. <	count/fibre	Pr. <	count/unit area+	Pr. <
21	4	14.31 ± 2.71	0.0001	0.44 ± 0.10	0.0001	49.16 ± 4.22	n.s.
84	5	32.95 ± 5.83	n.s.	3.87 ± 0.69	n.s.	44.82 ± 7.91	n.s.
185	5	31.58 ± 4.74	n.s.	4.48 ± 0.79	n.s.	46.98 ± 4.51	n.s.
299	5	30.76 ± 3.50	0.001	4.70 ± 0.46	0.0001	44.28 ± 6.86	0.005
508	5	44.48 ± 4.32	0.005	7.22 ± 0.78	0.01	59.19 ± 4.59	0.0001
758	5	55.02 ± 6.66		8.62 ± 1.18		99.78 ± 11.67	

All values are ± S.D., * unit area = 0.0144mm^2, + unit area = 0.6006mm^2

(b) The soleus

The connective tissue of the endoysium in the soleus increased progressively with age (Table 5.7). The 84 day old soleus contained 61% more endomysial connective tissue per unit area as compared to 21 day old animals. Changes thereafter were not significant up to 299 days of age were endomysial connective tissue increased by 27% between the ages of 299 and 508 days. There was a further 34% increase between 508 and 754 days.

When expressed in terms of endomysial connective tissue per fibre, the soleus demonstrated a similar trend as per unit are (Table 5.7). A seven-fold increase in endomysial connective tissue per fibre was recorded when the two youngest groups were compared. No more statistically significant increases were evident until 508 days where a 36% increase over the estimate of 299 day animals was observed. Senile animals showed an additional increase of 29% over 508 day old animals. Although the general trend of both estimates of endomysuim were similar, the rate of increase in the estimate expressed in terms of fibre number was modified by the number of fibre counted per field which changed with age, especially in very young animals.

The group comparison revealed a significant decrease in perimysial. connective tissue early in life ($P<0.05$). The decrease exhibited between the ages of 21 and 84 days was followed by statistically insignificant variations in perimysial connective tissue up to 299 days (Table 5.7). Aged and senile animals (508 and 754 days respectively) showed increased perimysial counts with age, the increase being much lower than those shown by the extensor digitorum longus over the same period. Nevertheless perimysial connective tissue in both senile muscles was of similar levels. Endomysial connective tissue on the other hand was slightly higher in the senile soleus as compared to senile extensor digitorum longus. When the percentage change of the two skeletal muscles in weanling and senile animals was examined, the extensor digitorum longus showed a proportionally higher increase in both endo and perimysial connective tissue. The soleus

Table 5.7 Connective tissue content of developing and ageing soleus

Age (Days)	No. of Animals	Endomysium				Perimysium	
		count/unit area*	< Pr.	count/fibre	< Pr.	count/unit area+	< Pr.
21	5	21.68 ± 5.33	0.0001	0.97 ± 0.35	0.001	66.02 ± 6.51	0.05
84	5	35.01 ± 3.62	n.s.	7.05 ± 0.82	n.s.	56.56 ± 6.34	n.s.
185	5	37.23 ± 2.18	n.s.	7.74 ± 1.07	n.s.	62.98 ± 3.71	n.s.
299	5	37.88 ± 1.37	0.001	8.31 ± 0.80	0.01	67.43 ± 5.77	0.05
508	5	48.25 ± 4.47	0.0001	11.26 ± 2.35	0.005	78.58 ± 11.42	0.01
758	5	64.81 ± 5.15		14.49 ± 1.92		94.09 ± 1.53	

All values are ± S.D., * unit area = 0.0144mm^2, + unit area = 0.6006mm^2

showed only a 299% increase in endomysium as opposed to 384% in the extensor digitorum longus and a 142% increase in soleus perimysium as opposed to 203%. This indicated a higher rate of connective tissue accumulation in the extensor digitorum longus.

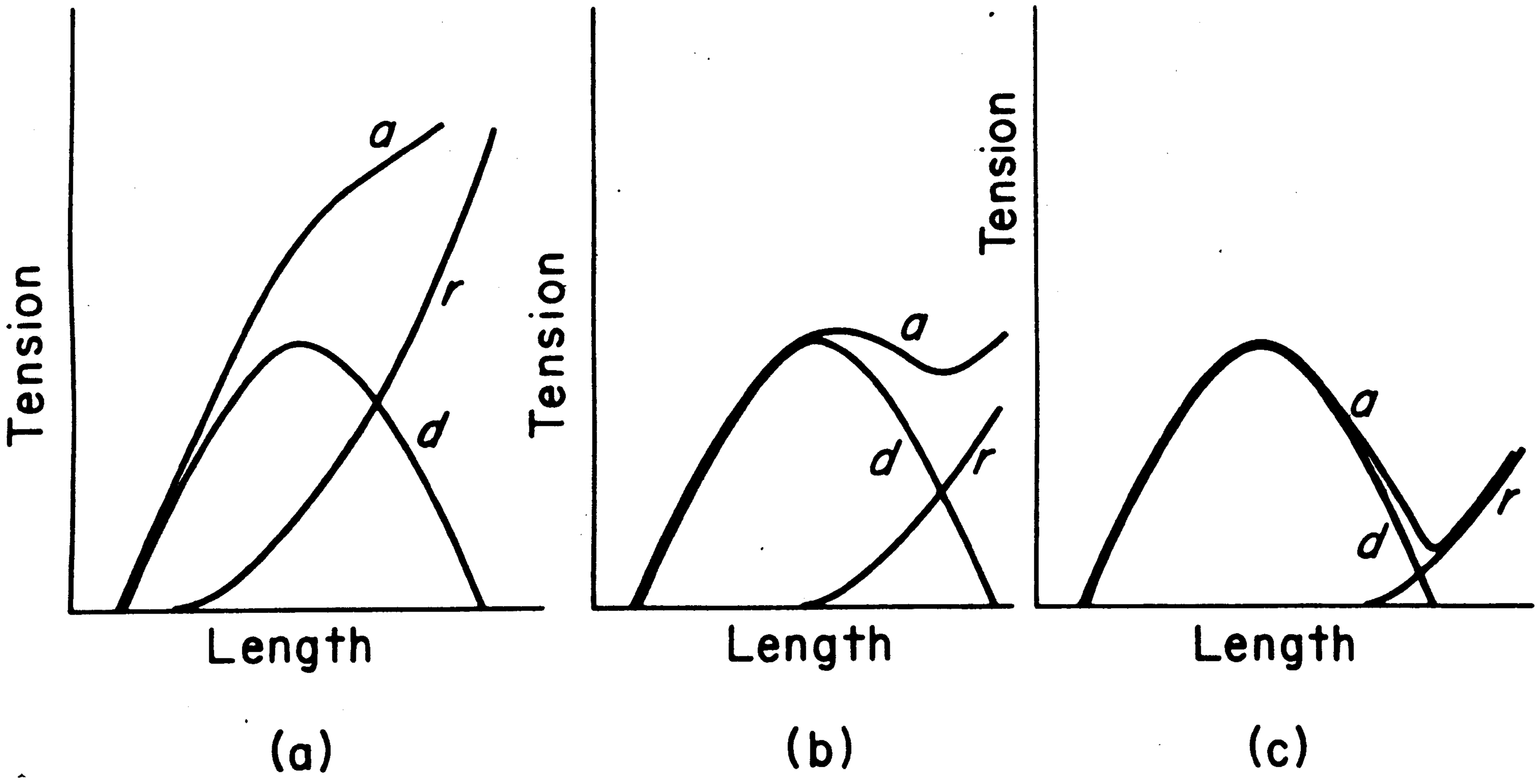
DISCUSSION

The passive mechanical characteristics of developing and ageing skeletal muscles changed appreciably in the two muscles investigated. The extensibility of the extensor digitorum longus decreased progressively with age. Higher tensions resulted in the muscle from the elastic component for small increases in length as the animal aged. The exponential relation as described earlier was steeper in senile animals when compared to either adult or young animals. The soleus exhibited a similar decrease in compliance early in life however it remained unchanged in senile muscles. Unfortunately, the behaviour of different mammalian muscles under passive tension conditions has not been investigated extensively. The little evidence extrapolated from frog muscles suggested that different muscles respond at different rates to length increases. The semitendinosus for example reached optimal length for maximal isometric tension well before any passive tension was detected (Fig. 5.7), while the gastrocnemius developed considerable passive tension at optimal length (Wilkie 1976). The development of passive tension is dependant on muscle size, anatomy and connective tissue quantity and distribution.

Collagen, the chief component in connective tissue is of extremely low compliance and compares favourably with copper. The breaking stain for collagen has been reported to be 6×10^{-7} Nm^{-2} compared to 4.5×10^{-9} Nm^{-2} for copper. This low compliance implies that small changes in the quantity of collagen in a muscle would decrease the compliance of that tissue considerably.

Biochemical estimates of absolute collagen content in the three muscles investigated showed progressive increases with age. Relative

Fig. 5.7 Tension : length curves from different types of muscle. In each case "r" is the passive tension : length curve, "a" is the active tension : length curve and "d" is the extra tension developed on stimulation (d=a-r). Note that although "d" is the same in the three muscles "r" starts developing well before optimum length is reached in the gastrocnemius of the frog(a) and at optimum length in the sartorius of the frog(b). In the semitendinosus(c) "r" appears well past the optimum length for active tension (After Wilkie 1976).


Tension
a
r
d
Length
(a)
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(c)

collagen content (μg/mg muscle) presented a more complex picture. In cardiac muscle, collagen stabilised around 80 days post partum but then increased by 50% in senile animals. This result in senile animals was in agreement with Mohan and Radha (1980) who showed an increase in cardiac collagen of similar levels. The same authors showed also a decrease in collagen solubility and hydroxyproline released at 65°C. Similar qualitative changes were reported by Saski et al (1976) for cardiac muscle but total collagen content displayed no significant changes. The latter study was carried out on human hearts as opposed to rat hearts. The discrepancy in the two studies may be due to qualitative changes in the aged hearts of different species.

Skeletal muscles presented more complex quantitative changes. Collagen concentrations increased during early development and then dropped around adulthood in the extensor digitorum longus. The decrease was only relative since absolute collagen content remained unchanged when compared to younger age groups. The decrease in concentration suggested a high rate of collagen accumulation around 188 days accompanied by a normal rate of growth in muscle tissue, thus giving an impression of high collagen proportion at that age. The ratio was later normalised when muscle tissue increases caught up with the collagen surge, alternatively collagen synthesis in the adult animal (299 days) lagged behind while muscle tissue continued to increase, this produces the same effect. An examination of both possibilities favoured the second, for both the extensor digitorum longus and the soleus increased in weight at a faster rate than collagen accumulation at adulthood. Schaub (1963) obtained similar results when examining collections of different muscles from various anatomical regions of the rat. Back musoles had a minimum collagen concentration between the ages of 5-12 months while abdominal and aductor hind leg muscles had the lowest collagen around

5-6 months. Extensor hind leg muscles on the other hand showed minimum values for collagen around 2-4 months post partum. These variations highlight the importance of function and location in dictating and influencing the ratio of collagen to muscle.

The inclusion of tendons in estimates of collagen in the soleus and extensor digitorum longus showed as expected a considerable increase over "pure" muscle estimates. However, the contribution of tendon to collagen concentration was different in the two muscles. The tendons from the extensor digitorum longus contributed similar levels of collagen throughout the animals life. Whole muscle collagen increased by 27% between the ages of 21 days and 299 days and by an extra 19% in senile animals. The soleus on the other hand increased by 11% and 32% over the same periods (estimates are dry muscle weight).

Since collagen determination in the muscle's belly reflect mainly interstitial connective tissue (endo, peri and epimysium) it probably represents the parallel elastic component. On the other hand collagen concentration of whole muscle (including tendons) produces an estimate of tendon contribution since tendon contains a high proportion of total muscle collagen. This estimate gives an idea of the changes in the series elastic component.

Histological examination of developing extensor digitorum longus and soleus muscles presented a comparable view of alterations accompanying the ageing process. Earlier studies examined overall changes in both endomysium and perimysium without any distinction. Furthermore Chiakulas and Pauly (1965) used an indirect method of estimating connective tissue, where any area not occupied by muscle was considered to be connective tissue. In broad terms, the extensor digitorum longus and the soleus behaved in similar fashions. The experiments described here confirm that estimates of endomysial connective tissue showed an increase early in life. The soleus continued to show an increase at a very reduced rate until

adulthood, beyond that endomysial connective tissue increased quickly reaching several times the weanling values. Senile extensor digitorum longus muscles had higher connective tissue compared to adult or young animals. Even when endomysial connective tissue was expressed in terms of fibre number, which was considerably larger per unit area in young animals, similar results were obtained.

Perimysial connective tissue in the soleus showed an initial decrease followed by slower increasessteepening again in senile animals. The extensor digitorum longus showed similar trends but the initial decrease was not statistically significant. The general trend of increased connective tissue was in agreement with a study conducted on human rectus abdominis muscles (Inokuchi et al 1975). In this latter study no distinction was made between endo and perimysial connective tissue, but since the trend in both types of connective tissue later in life was the same, it follows that the combined trend would be similar and comparable to the earlier study. The study reported by Chiakulas and Pauly where total connective tissue changes early in life were examined appears to show perimysial connective tissue changes only. On these bases reductions in connective tissue proportions between 21 day animals and 185 day animals (3 and 24 weeks in their study) are comparable. The trend observed early in life in the 3 rat muscles that they investigated was similar to the one observed here.

In this study the two approaches of estimating connective tissue using both biochemical and histological techniques produced comparable results. The histological technique was more sensitive to positional changes and revealed the relative abundance of endo and perimysial connective tissue. On the other hand, the biochemical approach supplied more accurate quantitative results and had a slightly better accuracy when concentrations and distributions were closely matched because of the larger sample size.

Both histological and biochemical results agreed on the increase in collagen with age in the two muscles under study. At the three ages investigated, the mechanical behaviour of the muscle was directly relatable to collagen concentrations. The extensor digitorum longus had an "average slope" for the passive tension:length relationship of 0.066 and a collagen content of 68.96µg/mg. The soleus with a lower collagen content (47.68µg/mg) showed a shallower slope (0.052). As collagen increased approaching adulthood to 87.51µg/mg in the extensor digitorum longus so did the slope of the log passive tension reaching 0.098. The soleus had a similar relationship (100.73µg/mg for a slope of 0.135) the higher collagen concentration in the soleus dictated a steeper slope. The trend continued in old age for the extensor digitorum longus, a slope of 0.161 was associated with the increased collagen concentration of 104.41µg/mg. The senile soleus deviated from this apparent relationship, showing a statistically unchanged "slope" for a statistically significant increase in collagen. The different response of the soleus may be due to the different accumulation rate of connective tissue in the belly region of the muscle. The extensor digitorum longus accumulated collagen in this region at a slightly higher rate than the soleus (66% for the extensor digitorum longus opposed to 42% in the soleus based on wet muscle weights). Such a difference would reduce the compliance of the extensor digitorum longus at a higher rate than the soleus.

The relationship between collagen proportion and extensibility was further modified by the reported changes in the ageing collagen molecules, namely cross-linking. Cross-linking has been suggested in senile collagen (Verzar 1963) and has been shown to alter the biochemical and mechanical properties of the protein. Cross-linking appears to increase the resistance of collagen to degrading enzymes (Mohan and Radha 1980) to urea degradation (Harrison and Archer 1978) and increase its tensile strength and decrease its elasticity (Verzar 1963). Such changes allow

modest increases in collagen to have considerable mechanical impact on passive tension. On the other hand cross-linking appears to reach a maximum value in some tendons around maturity (Davison 1978). Whether cross-linking continues in endomysial and perimysial collagen or whether it continues in the whole muscle undetected as suggested by some authors (Page106) is not clear at this time.

Senile muscles with lower physical activity will probably have a higher collagen concentration than active muscles as has been demonstrated in immobilisation experiments (Williams and Goldspink 1981). This implies that more active muscles may reduce collagen cross-linking by virtue of their activity. Also disuse atrophy may result in an increase in the ratio of connective tissue to muscle tissue.

As stated above connective tissue within a muscle contributes to either the series elastic component (the tendon and sarcomere cementing proteins)or the parallel elastic component (endo, peri and epimysium). An increase in either of the two components will produce the observed effect of reducing the compliance of the muscle. This model however does not reproduce to any degree the expected dampening induced by cross-linked, aged collagen. Under optimal length conditions a muscle with simple geometry will have both the series and parallel elastic component producing passive tension, when the contractile component shortens the contractile elements are working against the series elastic component and parallel to the parallel elastic component, but if the parallel elastic component is not under tension as is the case in the semitendinosus of the frog (Fig. 5.7), it will behave as a "compressible spring" and work against the contractile component resisting any further shortening. In the first case when the contractile component relaxes it regains the original configuration assisted by both the series and parallel elastic components, but when the parallel elastic component acts as a compressible element it will find it more difficult to regain its original precontraction

dimensions because it does not have any tension stored in the parallel elastic component. Furthermore, it will probably resist the passive relaxation of the contractile elements.

In ageing muscle the increase in parallel elastic component would therefore be expected to affect the contraction process, the effect being further magnified in aged collagen as a result of its increased rigidity. Cross-links between collagen strands and the extra packing density will act as a restraining jacket enveloping fibres as well as bundles and limiting their freedom.

The passive mechanical behaviour and properties of young as well as ageing muscles is far from clear. Further investigation of the effect of connective tissue and its distribution on the mechanical performance of muscle is required before more suitable models can be suggested.

CHAPTER VI

GENERAL DISCUSSION

Skeletal muscle in the present study has demonstrated considerable plasticity in responding to the changing functional demands. The young muscles investigated in this study increased rapidly in size to cope with the growing demands and loads on the skeletal and muscle systems. However, different muscles behaved in different ways. The young extensor digitorum longus lost muscle fibres but compensated for the loss by rapid increases in fibre diameter. The soleus on the other hand did not show any change in total fibre number but still showed fibre diameter increases. These observations indicated that at least early in life the greater dimensions achieved by the developing muscles are due to fibre diameter increases rather than fibre number increases. This is in agreement with earlier studies that indicated that the number of fibres is fixed at birth or just after birth and that the increase in muscle size is due almost entirely to an increase in the diameter of individual fibres (Goldspink 1972).

The fibre loss exhibited in the developing extensor digitorum longus was difficult to explain in functional terms. The loss was probably a continuation of the fusion process observed in very young muscles undergoing maturation. Nerve fibre numbers in the young nerve supplying the extensor digitorum longus did not show any reductions neither did the

muscle exhibit any denervation atrophy that could account for the muscle fibre loss.

The observed changes in fibre diameter did not always occur uniformly within the muscle, this was due to the heterogenity of the two muscles under investigation. The soleus having approximately a 1:1 ratio of slow oxidative to fast oxidative glycolytic fibres around weaning underwent a change towards a predominantly slow oxidative fibre population. Within the two populations in this muscle, fibre diameter changes were very closely matched. The extensor digitorum longus on the other hand had three fibre types; slow oxidative, fast oxidative glycolytic and fast glycolytic, the latter two of which were the dominant within the muscle. From the early period in life fast glycolytic fibres increased in diameter at a higher rate than either slow oxidative or fast oxidative fibres. The ratios of the three fibre types however remained unchanged throughout the animal's life. In spite of differences in the number of fast oxidative glycolytic and fast glycolytic fibres both types occupied a similar total cross-sectional area of the adult extensor digitorum longus. The discrepancy in the rate at which the two main fibre populations grew gave rise to a bimodal distribution within this muscle. This bimodality has been reported by other workers and was attributed to the existence of two phases of muscle fibres (Rowe and Goldspink 1969). The present study demonstrated that the two phases are identifiable with the two dominant fibre types within the muscle.

Changes in the aged and senile muscles were not as clear cut as the ones observed in young animals. In both muscles total fibre number changed little between the adult and senile stage. These findings are not in agreement with some reports of gross fibre loss in senile skeletal muscles (Gutmann and Hanzlikova 1966) but are in agreement with other studies that indicated an unchanged fibre number in some senile muscles (Rowe 1969). The discrepancy may be due to the increased occurrence of

splitting in ageing muscles. Such a change causes an apparent increase in the number of fibres in a section at the expense of fibre diameter. The word apparent is used because it is not known whether these splits are complete. Indeed it is likely that they are restricted to that particular region of the muscle. Because splitting does occur the data for muscle fibre number are difficult to interpret. However in this study total muscle fibre cross-sectional area for each fibre type has been used. This was derived from mean fibre diameters and fibre numbers and is in any case the most meaningful measure from a functional point of view. Both the extensor digitorum longus and the soleus were alike in showing a shift towards higher oxidative cross-sectional area during ageing. In the senile extensor digitorum longus fast oxidative glycolytic fibres contributed 58% of the total cross-sectional area while fast glycolytic fibres contributed 41%. This contrasted with the 43% fast oxidative glycolytic and 55% fast glycolytic cross-sectional area in the young muscle. The soleus suffered considerable loss of fast glycolytic "fibre cross-sectional area" leaving the slow oxidative fibres relatively intact.

This indicates either a selective degeneration process or a transformation of fibre types or both.

Frequency distribution of fibre diameters in ageing muscles displayed a characteristic spread. Unlike young muscles where there was a well-defined peak and more or less symmetrical tails, the senile distribution displayed a plateau often with assymmetrical tails. The reasons for this configuration are the underlying changes in fibres; senile muscles contain fibres which are undergoing both "splitting" and compensatory "hypertrophy". Both create tails that spread over a wide range of diameters.

The rate at which ageing progresses in the various systems varies between individuals. In muscles ageing is presumably modified by external factors such as exercise and diet, therefore unlike a controlled experiment, a group of animals with a given chronological age may have slightly

different biological ages. Also ageing is a multi-organ process therefore changes in other systems particularly the nervous system are likely to affect muscle tissue to a greater or lesser extent. Some changes may also mimic ageing as is the case in denervation atrophy. Muscles exhibiting mild denervation atrophy can produce changes in skeletal muscles similar to senile atrophy. The distinction between the two situations is not easy to make on the basis of morphometric measurements only.

One other major component in the muscle, besides the muscle fibres, is connective tissue, apparently contributing in some cases as much as 32% of the cross-sectional area of muscles (Inokuchi et al 1975). Connective tissue is important as it has a direct bearing on the mechanical performance of muscle. The techniques employed in this study included measurements of the passive mechanical performance of muscle under tension as well as biochemical estimates of whole and tendon free muscle collagen, in addition to qualitative histological estimates of connective tissue in cross-section. These three techniques gave some insight into changes in the developing and ageing muscle. Mechanically the compliance of the extensor digitorum longus and the soleus decreased with age up to adulthood. The compliance decreased even further in the senile extensor digitorum longus. The changes corresponded to an increased accumulation of connective tissue in both whole muscle and in cross-section. Bearing in mind that maximum active muscle performance is around early adulthood further accumulation of connective tissue beyond this point in time will affect the performance of muscle adversely. Rather surprisingly the soleus which remained highly active into senility did not show a lower compliance with extreme age although it showed a high rate of connective tissue accumulation. This implies that passive tension is related somehow to the activity and physiological function of the muscle and possibly the nature of the accumulated connective tissue. In the absence of activity

the accumulation and remodelling of connective tissue may result in a stiffer muscle with even lower compliance as indicated by the work of Williams and Goldspink (1978).

Connective tissue in adult muscles appears to change at a very slow rate, the rate only accelerating with advancing senility. This raises the question as to whether connective tissue accumulation is accelerated in old age by other factors such as decreased activity. Ageing muscles may respond positively to exercise up to a limit beyond which they fail to show any improvements in weight (McCafferty and Edington 1974) this situation may be explained in terms of a shifting balance of connective tissue to muscle fibre area. This is especially true when connective tissue becomes more difficult to degrade in vivo because of a lower enzyme activity and highly insoluble molecular arrangements. However, the exact relationship between connective tissue and active isotonic contraction is far from clear.

At the three representative ages used the mechanical performance of the developing and ageing soleus and the extensor digitorum longus differed. When the length:tension relationships were examined the extensor digitorum longus showed higher tensions per muscle length increment than the soleus. These results were easily explained in terms of sarcomere length changes and optimum overlap of contractile filaments. The relationship between length and tension was of interest, this steepened with age in the extensor digitorum longus whilst for some reason it remained unchanged in the soleus.

The twitch tension of the extensor digitorum longus appeared to remain unchanged whilst that of the soleus continued to increase between adulthood and senility (387 days and 714 days respectively). Maximum tetanic tension on the other hand decreased in the extensor digitorum longus and remained unchanged in the senile soleus. The different behaviour of the extensor digitorium longus and the soleus cannot be explained in terms

of reductions in "fibre cross-sectional area" contributing to tension alone. The rate at which tetanic tension developed was lower in senile animals in both muscles. This indicated the possibility that maximum tension developed is limited by other factors as well as muscle cross-sectional area. A point that should not escape attention is the effect of age on the packing of contractile material within the muscle fibres. Fibre hypertrophy can no doubt affect both packing density of contractile material as well as the organisation of that material.

Another feature of the developing and ageing skeletal muscle was the speeding up with growth and the subsequent slowing down, with senility, of the activation and contractile processes. The extensor digitorum longus demonstrated a speeding up of latent periods and times to peak tension in mature adult animals as compared with young ones, the same parameters slowed down in the senile muscle. A less marked effect was observed in the soleus but a trend was evident.

An unexpected finding for the senile extensor digitorum longus was the trend towards improved resistance to fatigue. When expressed as time for a 50% drop in maximum tetanic tension, the senile extensor digitorum longus demonstrated a higher fatigue resistance. However, in absolute terms the senile extensor digitorum longus was less able to maintain tension In other words it could not maintain a high force, e.g. 150 grams for the same length of time as an adult muscle.

As far as the senile soleus was concerned it produced a maximum tetanic tension comparable to that of an adult muscle and its half fatigue time was virtually unchanged.

The fibre type shift as well as the fibre type make up of the muscle appears to play an important role in preserving the stamina of muscle into old age. Oxidative fibres are usually associated with a better blood supply and therefore a better perfusion rate. Whether this plays a major role is difficult to asses. In addition, the efficiency of

oxidative pathways is known to be better than that of glycolytic ones for producing energy; if this situation holds true for ageing muscles, this could be interpreted as an adaptive transformation in favour of less energy expenditure. Nevertheless the selective atrophy of some fibre types namely the fast glycolytic fibres is probably just a reflection of changes in recruitment patterns with advancing age.

The work described in this study was an attempt to understand and relate some of the many alterations associated with developing and ageing skeletal muscles. Although some of the changes have been described and quantified, questions remain as to the underlying mechanisms particularly those that involve impaired mechanical performance. The process of ageing is in itself difficult to define although in general terms, we can describe it as a process of unfavourable progressive change which starts at different points of time in different systems. Whether the deterioration of muscle function is the sum effect of many minor changes in a multitude of associated systems is a matter for conjecture. The options discussed in this study serve to restrict the number of possible factors affecting the ageing process. Further investigation of some of the suggested explanations is required before conclusive evidence can be reached. The use of some of the procedures described in this study on dietry restricted animals, where the life span has been extended by more than 100% could provide invaluable insight into the nature of senile changes and hence it would probably be instructive to study the combined effects of exercise and ageing.

CHAPTER VII

SUMMARY

1. Age associated changes in developing and ageing rat skeletal muscles were studied in an effort to establish factors responsible for the decline of strength and activity of senile amimals.

2. Two representative skeletal muscles were used: The extensor digitorum longus, a fast contracting mixed muscle, and the soleus, a slow contracting muscle with predominantly slow oxidative fibres. In the course of the study other muscles were also used for indicators of trends but not detailed analyses.

3. Muscle weights decreased in the senile extensor digitorum longus and the soleus.

4. The soleus exhibited a shift in muscle fibre type from a mixed population of slow oxidative and fast oxidative glycolytic to a predominantly slow oxidative fibre. Total fibre number did not show any significant change with age. During development however the extensor digitorum longus demonstrated a considerable loss of fibres early in life but the ratio of the three fibre types did not change much with age.

5. Fibre diameters in both muscles increased with age up to adulthood with the fast glycolytic fibres exhibiting the highest rate of

increase. In the extensor digitorum longus the slow oxidative fibres grew slowly but continued to increase in diameter even in senile muscles. Fast oxidative glycolytic fibres in the soleus decreased in diameter dramatically in senile muscles.

6. A combination of fibre number and diameter changes revealed that the senile extensor digitorum longus had a higher cross-sectional area of fast oxidative glycolytic fibres compared to adult animals. Thus in both muscles there was a shift to a more oxidative type of fibre.

7. Frequency analysis of fibre diameter distribution were carried out. The study revealed the existence of a bimodal distribution in the extensor digitorum longus. This was shown to be the result of the different growth rates of fast oxidative glycolytic and fast glycolytic fibres. The distribution in senile muscles showed a larger number of both very small fibres and very large ones and no distinct peak inbetween. This is due to the presence of splitting and hypertrophied fibres.

8. Measurements of DNA content of young extensor digitorum longus and soleus muscles were made. They indicated a peak DNA content at 90 days. After 244 days total DNA content in the extensor digitorum longus decreased, but DNA to protein ratio remained unchanged. The soleus did not show a similar decrease.

9. Nerve fibre numbers of the specific nerves supplying both the extensor digitorum longus and soleus were counted. No significant changes were detected in either muscle with age.

10. The contractile properties of the soleus and the extensor digitorum longus were examined <u>in vivo</u> at three representative ages (21, 387 and 714 days). Measurements of single twitch parameters as well as tetanic contractions were made under isometric conditions.

11. The latent period of the extensor digitorum longus decreased as the

muscle matured and then increased in senile muscles. Time to peak in a single twitch was slower in both senile extensor digitorum longus and soleus muscles.

12. The relationship between muscle length and twitch tension in the extensor digitorum longus became steeper with age. Senile muscles produced more active tension per length increment. In the soleus this relationship was steeper in adult animals as compared to younger ones but did not change in senile animals.

13. Twitch tension in the extensor digitorum longus increased up to adulthood. No significant decrease was detected in senile muscles. On the other hand in the soleus twitch tension continued to increase from weaning to senility.

14. Maximum tetanic tension increased between weaning and adulthood but decreased again in the senile extensor digitorum longus muscles. The rate at which tetanus developed was highest in adult muscles and lowest in very young muscles. In the soleus both maximum tetanic tension and the rate of tension development increased early in life but did not change thereafter.

15. The ratio of twitch to maximum tetanic tension decreased with age up to adulthood then increased thereafter into senility in both the extensor digitorum longus and soleus.

16. Fatigue resistance measured as the time to 50% maximum tetanic tension was highest in the very young extensor digitorum longus and showed a trend towards better fatigue resistance in the senile muscle as compared to the adult muscle. But in absolute terms, senile muscles were less fatigue resistant, that is to say they were unable to maintain a high force for the same length of time as an adult muscle. The soleus on the other hand, although showing similar fatigue trends as the extensor digitorum longus for young and adult muscles, did not change appreciably in old age.

17. The passive mechanical properties of the soleus and the extensor digitorum longus were investigated and related to the connective tissue changes of developing and ageing muscles. Connective tissue estimates were obtained using biochemical as well as microchromometric techniques.

18. The length:passive tension relationship in the extensor digitorum longus showed a trend towards increasing steepness with age. The trend was later confirmed in senile muscles where for small increments in length, considerable passive tensions were produced. The observations indicated a lower compliance of muscles as ageing progressed.

19. In the case of the soleus the young muscles behaved not unlike the extensor digitorum longus showing significant increases in the steepness of the length:tension relationship. However, the tendency towards greater stiffness did not continue in senility.

20. Using biochemical techniques absolute collagen content was found to increase in all muscles with age. Both the extensor digitorum longus and the soleus were alike in showing increases in collagen to muscle ratios from weaning to senility. This observation held true whether tendon free estimates were considered or whether tendons were included with the muscle belly.

21. The microchromometric techniques revealed similar changes in the two muscles. Endomysial connective tissue did not reach maximum values until around 84 days and remained unchanged until adulthood where further increases were registered as the muscles aged. The extensor digitorum longus accumulated endomysial connective tissue at a higher rate than the soleus.

22. Perimysial connective tissue did not change significantly in the extensor digitorum longus up to adulthood where it started and continued to increase well into senility. In the case of the

young soleus it started at higher values as compared to the extensor digitorum longus but fell immediately afterwards and did not increase until late adulthood and senility.

23. In general the decreased extensibility of the muscle with age could be related to the increase in connective tissue.

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BIBLIOGRAPHY

ADELMAN, R.C., STEIN, G., ROTH, G.S. & ENGLANDER, D. (1972)

Age dependent regulation of mammalian DNA synthesis and cell proliferation in vivo.

Mechanisms of Ageing and Development, 1: 49-59.

ALDER, W.H. (1974)

An "Auto immune" theory of age. In: Theoratical Aspects of Aging, ed. Rockstein, M. New York: Academic Press.

ALLBROOK, D.B., HAN, M.F. & HELLMUTH, A.E. (1971)

Population of muscle satellite cells in relation to age and mitotic activity.

Pathology, 3: 233-243.

ALNAQEEB, M.A. & GOLDSPINK, G. (1980)

Interrelation of muscle fibre types , diameter and number in ageing white rats.

J. Physiol. 310: 56P.

ANDREW, W., SHOCK, N.W., BARROWS, C.H. & YIENGST, M.J. (1959)

Correlation of age change in histological and chemical characteristics in some tissues of the rat.

J. Geront, 14(4): 405-414.

ASHMORE, C.R. & DOERR, L. (1971)

Postnatal development of fibre types in normal and dystrophic skeletal muscle of the chick.

Exp. Neurol. 30: 431-446.

BAB, H. (1948)

The process of ageing.

Br. Med. J. 1: 1000-1001.

BAILY, D.A., BELL, R.D. & HOWARTH, R.E. (1973)

The effect of exercise on DNA and protein synthesis in skeletal muscle of growing rats.

Growth, 37: 323-331.

BANCROFT, W.D., FARNHAM, E.C. & RUTZLER, J.E. Jr. (1934a)

Preliminary tests with sodium rhodanate on rabbits and chickens.

Science, 80: 549.

BANCROFT, W.D., FARNHAM, E.C. & RUTZLER, J.E. Jr. (1935)

One aspect of the longevity problem.

Science, 81: 152-153.

BANCROFT, W.D. & RUTZLER, J.E. Jr. (1934b)

Reversible coagulation in living tissue.

Proc. natn. Acad. Sci. U.S.A. 20: 501-509.

BANU, G. (1922)

Recherches Physiologiques sur le Development Neuromusculair. Paris: Maretheux.

BARANY, M. (1967)

ATPase activity of myosin correlated with speed of muscle shortening.

J. gen. Physiol. 50: 197-216.

BARNARD, R.J., EDGERTON, V.R., FURUKAWA, T. & PETER, J.B. (1971)

Histochemical, biochemical and contractile properties of red, white and intermidiate fibres.

Am. J. Physiol. 220: 410-414.

BARNARD, R.J., EDGERTON, V.R. & PETER, J.B. (1970)

Effect of exercise on skeletal muscle: I Biochemical and histochemical properties.

J. appl. Physiol. 28(6): 762-766.

BARNARD, R.J., EDGERTON, V.R. & PETER, J.B. (1970)

Effect of exercise on skeletal muscle: II Contractile properties.

J. appl. Physiol. 28(6): 767-770.

BASS, A., GUTMANN, E. & HANZLIKOVA, V. (1975)

Biochemical and histochemical changes in energy-supply enzyme pattern of muscle of the rat during old age.

Gerontologia, 21: 31-45.

BENDALL, J.R. (1969)

Mechanics of contraction. In: Muscles, Molecules and Movement, P: 132, London: Heinemann Educational Books Ltd.

BERG, B.N., WOLF, A. & SIMMS, H. (1962)

Degenerative lesion of spinal roots and peripheral nerves in ageing rats.

Gerontologia, 6: 72-80.

BERGAUER, V. (1924)

Beitrage zum studium der protoplasmahysteresis.

Arch. mikrosk. Anat. EntwMech. 101: 489,508,512.

BERNSTEIN, J. (1898)

Zur theorie des wachstums und der befruchtung.

Arch. EntwMech. 7: 511-521.

BERTOLINI, A.M. (1962)

Modifications of cellular enzymatic systems during aging.

Gerontologia, 6: 175-187.

BICK, E.M. (1961)

Aging in the connective tissues of the human musculo-skeletal system.

Geriatrics, 16: 448-453.

BIDDER, G.P. (1925)

The mortality of plaice.

Nature, 115: 495-496.

BIRREN, J.E. (1965)

Behaviour, Aging and the Nervous System, Eds. Welford, A.T.and Birren, J.E. PP: 191-216. Illinois: Charles C. Thomas.

BJORKERUD, S. (1964)

Isolated lipoprotein granules: A survey of a new field. In: Advances in Gerontological Research, ed. Strehler, B.L. Vol 1, P: 257. New York: Academic Press.

BJORKSTEN, J., ANDREWS, F. & PRAHL, H.F. (1962)

Anhydrous hydrogen floride as a tool in studying cross-linkages in proteinaceous substances accumulating with age.

Finska Kemistsamfundets Meddelanden, 71(3): 69-76.

BLACKMAN, F.F. (1909)

The manifestations of the principles of chemical mechanics in the living plant. In: The Report of the 78th Meeting of the British Association for the Advancement of Science.

BRADFONBRENER, M., LANDOWNE, M. & SHOCK, N.W. (1955)

Changes in cardiac output with age.

Circulation, 12: 557-566.

BRODY, S. (1924)

The kinetics of senescence.

J. gen. Physiol. 6: 245-257.

BROOKE, M.H. & KAISER, K.K. (1970)

Muscle fibre types: how many and what kind.

Archs. Neurol. 23: 369-379.

BROOKE, M.H. & KAISER, K.K. (1970)

Three "myosin adenosine triphosphatase" systems: the nature of their pH liability and sulfhydryl dependence.

J. Histochem. Cytochem. 18: 670-672.

BROOKE, M.H. & KAISER, K.K. (1974)

The use and abuse of muscle histochemistry. In: The Trophic Functions of the Neuron.

Ann. N.Y. Acad. Sci. 228: 121-144.

BROOKE, M.H., WILLIAMSON, M.D. & KAISER, K.K. (1971)

The behaviour of four fibre types in developing and reinnervated muscle.

Archs. Neurol. 25: 360-366.

BROOKS, A.L., MEAD, D.K. & PETERS, R.F. (1973)

Effect of aging on the frequency of metaphase chromosome abberations in the liver of chinese hamster.

J. Geront. 28(4): 452-454.

BUHLER, A. (1904)

Alter und Tod.

Biol. Zbl. 24: 65-91, 113-120.

BULLER, A.J., ECCLES, J.C. & ECCLES, R.M. (1960a)

Differentiation of fast and slow muscles in the cat hind limb.

J. Physiol. 150: 399-416.

BULLER, A.J., ECCLES, J.C. & ECCLES, R.M. (1960b)

Interactions between motoneurons and muscles in respect of the characteristic speeds of their responses.

J. Physiol. 150: 417-439.

BULLER, A.J. & LEWIS, D.M. (1965)

Furthur observations on the differentiation of skeletal muscles in the kitten hind limb.

J. Physiol. 176: 355-370.

BURKE, R.E., LEVINE, D.N., TSAIRIS, P. & ZAJAC, F.E. (1973)

Physiological types and histochemical profiles in motor units of the cat gastrocnemius.

J. Physiol. 234: 723-784.

BURKE, R.E. & TSAIRIS, P. (1974)

The correlation of physiological properties with histochemical characteristics in single muscle units.

Ann. N.Y. Acad. Sci. 228: 145-159.

BURKE, W.E., TUTTLE, W.W., THOMPSON, C.W., JANNEY, C.D. & WEBBER, R.J. (1953)

The relation of grip strength and grip strength endurance to age.

J. appl. Physiol. 5: 628-630.

CACCIA, M.R., HARRIS, J.B. & JOHNSON, M.A. (1979)

Morphology and physiology of skeletal muscle in ageing rodents.

Muscle and Nerve, 2: 202-212.

CAMPBELL, M.J., McCOMAS, A.J. & PETITO, F. (1973)

Physiological changes in aging muscles.

J. Neurol. Neurosurg. Psychiat. 36: 174-182.

CARREL, A. & EMBELING, A.H. (1921)

Antagonistic growth principles of serum and their relation to old age.

J. exp. Med. 38: 419-425.

CATHCART, E.P., HUGHES, D.E.R. & CHALMERS, J.G. (1935)

The physique of man in industry. Industrial Health Research Board, Report No. 71, London: H.M. Stationary Office.

CHIAKULUAS, J.J. & PAULY, J.E. (1965)

A study of postnatal growth of skeletal muscle in rat.

Anat. Rec. 152: 55-62.

CHILD, C.M. (1911)

A study of senescence and rejuvenescence based on experiments with planarians.

Arch. EntwMech. Org. 31: 537-616.

CHILD, C.M. (1914)

Starvation, rejuvenescence and acclimation in Planaria dorotocephala.

Arch. EntwMech. Org. 38: 418-446.

CHISHOLM, G.M., TERRADO, E.M. & GAINER, J.L. (1971)

Physiological transport in relation to aging.

Nature, 230: 390-391.

CLOSE, R. (1964)

Dynamic properties of fast and slow skeletal muscles of the rat during development.

J. Physiol. 173: 74-95.

CLOSE, R. (1967)

Properties of motor units in fast and slow skeletal muscles of the rat.

J. Physiol. 193: 45-55.

CLOSE, R. (1972)

Dynamic properties of mammalian skeletal muscles.

Physiol. Rev. 52(1): 129-197.

COMFORT, A. (1979)

The Biology of Senescence. New York: Elsevier North Holland Inc.

COMMONER, B., HEISE, J.J., LIPPINCOT, B.B., NORBERG, R.E., PASSONEAU, J.V. & TOWNSEND, J. (1957)

Biological activity of free radicals.

Science, 126: 57-63.

CONKLIN, E.G. (1912)

Cell size and nuclear size.

J. exp. Zool. 12: 1-98.

COTTREL, L. (1940)

Histologic variations with age in apparently normal peripheral nerve trunks.

Archs. Neurol. Psychiat. 43: 1138-1150.

COWDRY, E.V. (1952)

Ageing of individual cells. In: Cowdry's Problems of Aging, ed. Lansing, A.I. Baltimore: Williams and Wilkins.

CRITCHLEY, M. (1956)

Neurological changes in the aged.

Res. Publs Ass. Res. nerv. ment. Dis. 35: 198-223.

DAM, H., PRANGE, I. & SONDERGAARD, E. (1952)

Muscular degeneration (white striation in muscles) in chicks reared on vitamin E deficient, low fat diet.

Acta path. microbiol. scand. 31: 172-184.

DANIELLI, J.F. (1956)

On the ageing of cells in tissues. In: Experimentelle Alterforschung, ed. Verzar, F. Experientia Suppl. IV: 55.

DAVISON, P.F. (1978)

Bovine tendons aging and collagen cross-linking.

J. Biol. Chem. 253(16): 5635-5641.

DELAGE, Y. (1903)

L'Heredite et les Grandes Problemes de la Biologie.
Paris: Scheleicher.

DEMANGE, E. (1886)

Etudes Clinique et Anatomopathologiques de la Viellesse.
Paris: Bailliere.

DENNY-BROWN, D.E. (1929)

The histological features of striped muscles in relation to it's functional activity.

Proc. R. Soc. B 104: 371-411.

DHAR, N.R. (1932)

Senescence, an inherent property of animal cells.

Q. Rev. Biol. 7: 68-76.

DREYFUS, J.C., SCHAPIRA, G. & BOURLIERE, F. (1954)

Modifications cliniques du muscle au cours de la senescence chez le rat.

C. r. Seanc. Soc. Biol. 148: 1065-1067.

DUBOWITZ, V. (1967)

Cross-innervated mammalian skeletal muscle: Histochemical, physiological and biochemical observations.

J. Physiol. 193: 481-496.

DUBOWITZ, V. & BROOKE, M.H. (1973)

Muscle Biopsy: a modern approach. Modern Problems in Neurology. New York: Saunders, W.B.

EISENBERG, B.R. & MOBLEY, B.A. (1975)

Size changes in single muscle fibres during fixation and embedding.

Tissue and Cell, 7(2): 383-387.

EISIG, H. (1887)

Monographie der Capitelliden, Fauna et Flora des Golfes von Neapel. Vol. 16. Berlin: R. Friedlander & Sohn.

EMERSON, A.E. (1960)

Evolution After Darwin, ed. Tax, S. P: 307-348. Chicago: University of Chicago Press.

ENESCO, M. & PUDDY, D. (1964)

Increase in the number of nuclei and weight in skeletal muscle of rats of various ages.

Am. J. Anat. 114: 235-244.

ENGEL, W.K. (1962)

The essentiality of histo- and cytochemical studies in the investigation of neuromuscular disease.

Neurology, 12: 778-784.

ENGEL, W.K. (1970)

Selective and non-selective succeptibility of muscle fibre types.

Archs Neurol. 22: 97-117.

ENGEL, W.K. (1974)

Fibre type nomenclature of human skeletal muscle for histochemical purposes.

Neurology, 24: 344-348.

ERMINI, M. (1976)

The energy metabolism of the ageing skeletal muscle.

Aktuelle Gerontologie, 6(4): 151-156.

ERMINI, M. (1976)

Aging changes in mammalian skeletal muscle.

Gerentology, 22(4): 301-316.

FAILLA, G. (1957)

Considerations bearing on possible accummulated radiation doses for occupational exposure. The aging process and cancerogenesis.

Radiology, 69: 23-27.

FAILLA, G. (1958)

The ageing process and cancerogensis. In: Genatic Concept for the Origion of Cancer.

Ann. N.Y. Acad. Sci. 71: 1124-1140.

FAULKNER, J.A., MAXWELL, L.C., BROOKE, D.A. & LIEBERMAN, D.A. (1971)

Adaptation of guinea pig plantaris muscle fibres to endurance training.

Am. J. Physiol. 221: 291-297.

FINDLEY, T. (1949)

Role of neurohypophysis in the pathogensis of hypertension and some allied disorders associated with ageing.

Am. J. Med. 7: 70-84.

FISHER, M.B. & BIRREN, J.E. (1947)

Age and strength.

J. appl. Psychol. 31: 490-497.

FROKLIS, V.V. (1969)

The role of self-regulation and adaption in the mechanism of aging. Proceedings of the 8th International Congress of Gerontology, Washington, Vol. I: 529-560.

FROKLIS, V.V. & BOGASTSKAYA, L.N. (1968)

The energy metabolism of myocardium and it's regulation in animals of various ages.

Exp. Gerontol. 3: 199-210.

FROKLIS, V.V., MARTYNENKO, O.A. & ZAMOSTYAN, V.P. (1976)

Aging of the neuromuscular apparatus.

Gerontology, 22(4): 244-279.

FUJII, K. & KUROSU, H. (1979)

Age related changes in the reducible cross-links of connectin from human skeletal muscle.

Biochem. biophys. Res. Commun. 89(3): 1026-1032.

FUJISAWA, K. (1974)

Some observations on the skeletal musculature of aged rats: I Histochemical aspects.

J. Neurological Sciences, 22: 353-366.

FUJISAWA, K. (1975)

Some observations on the skeletal musculature of aged rats: II Fine morphology of diseased muscle fibres.

J. Neurological Sciences, 24: 447-469.

FUJISAWA, K. (1976)

Some observations on the skeletal musculature of aged rats: III Abnormalities of terminal axons found in motor end-plates.

Exp. Gerontol. 11: 43-47.

GAUTHIER, G.F., LOWEY, S. & HOBBS, A.W. (1978)

Fast and slow myosin in developing muscle fibres.

Nature, 274: 25-29.

GELFANT, S. & GROVE, G.L. (1974)

Cycling and non-cycling cells as an explanation for the aging process. In: Theoratical Aspects of Aging, ed. Rockstein, M. New York: Academic Press.

GELFANT, S. & SMITH, J.G. Jr. (1972)

Aging: Non-cycling cells, an explanation.

Science, 178: 357-361.

GHIRINGHELLI, F., MIRA, E. & GERZELI, G. (1963)

Histochemical activities of glandular cells in old rats.

Giornale di Gerontologia, 11: 1097-1108.

GOLD, P.H., GEE, M., NORDGREEN, R. & STREHLER, B.L. (1966)

A reexamination of the efficiency of oxidative phosphorylation versus age in rat liver, kidney and heart. Proceedings of the 7th International Congress on Gerontology, Wein, Vol 8: 103-105.

GOLDBERG, P.B. (1978)

Cardiac function of Fischer 344 rats in relation to age. In: Aging in Muscle, ed. Kaldor, G. & DiBattista, W. P: 87-100. New York: Raven Press.

GOLDSPINK, G. (1961)

Fixation of muscle.

Nature, 192: 1305-1306.

GOLDSPINK, G. (1962)

Studies on postmitotic growth and development of skeletal muscle.

Proc. R. Ir. Acad. 62B: 135-150.

GOLDSPINK, G. (1970)

The proliferation of myofibrils during muscle fibre growth.

J. Cell Sci. 6: 593-603.

GOLDSPINK, G. (1972)

Studies of postembryonic growth and development. In: The Structure and Function of Muscle, ed. Bourne, G.H. Vol 1, New York: Academic Press.

GOLDSPINK, G., GELDER, S., CLAPISON, L. & OVERFIELD, P. (1973)

Pre and post-rigor fixation of muscle.

J. Anat. 114(1): 1-6.

GOLDSPINK, G. & WARD, P.S. (1979)

Changes in rodent muscle fibre types during post-natal growth, undernutrition and exercise.

J. Physiol. 296: 453-469.

GRANT, R.A. (1964)

Estimation of hydroxyproline by the auto-analyser.

J. clin. Path. 17: 685-686.

GRIFFITHS, T.R. (1973)

A new unifying theory for the initiation of aging mechanisms and processes.

Mechanisms of Ageing and Development, 2: 295-307.

GUTH, L. (1973)

Fact and artefact in the histochemical procedure for myofibrillar ATPase.

Expl Neurol. 41: 440-450.

GUTH, L. & SAMAHA, F.J. (1969)

Qualitative differences between actomyosin ATPase of slow and fast mammalian muscle.

Expl Neurol. 25: 138-152.

GUTH, L. & SAMAHA, F.J. (1970)

Procedure for the histochemical demonstration of actomyosin ATPase.

Expl Neurol. 28: 365-367.

GUTH, L. & YELLIN, H. (1971)

The dynamic nature of the so called "fibre types" of mammalian skeletal muscle.

Expl Neurol. 31: 277-299.

GUTHRIE, R.D. (1969)

Senescence as an adaptive trait.

Perspect. Biol. Med. 12: 313-324.

GUTMANN, E. & HANZLIKOVA, V. (1965)
Age changes of motor endplates.
Gerontologia, 11: 12-24.

GUTMANN, E. & HANZLIKOVA, V. (1966)
Motor unit in old age.
Nature, 209: 921-922.

GUTMANN, E. & HANZLIKOVA, V. (1976)
Fast and slow motor units in ageing.
Gerontology, 22: 280-300.

GUTMANN, E., HANZLIKOVA, V. & JAKOUBEK, B. (1968)
Changes in the neuromuscular system during old age.
Exp. Gerontol. 3: 141-146.

GUTMANN, E., HANZLIKOVA, V. & VYSKOCIL , F. (1971)
Age changes in cross-striated muscle of the rat.
J. Physiol. 219: 331-343.

GUTMANN, E. & MELICHNA, J. (1972)
Contractile properties of different skeletal muscles of the rat during development.
Physiologia bohemoslovaca, 21: 1-8.

GUTMANN, E., MELICHNA, J. & SYROVY, I. (1974)
Developmental changes in contraction time, myosin properties and fibre pattern of fast and slow skeletal muscles.
Physiologia bohemoslovaca, 23: 19-27.

GUTMANN, E. & SYROVY, I. (1967)
Metabolic differentiation of the anterior and posterior latissimus dorsi of the chicken during development.
Physiologia bohemoslovaca, 16: 232-243.

GUTMANN, E. & SYROVY, I. (1974)
Contraction properties and myosin ATPase activity of fast and slow muscles of the rat.
Gerontologia, 20: 239-244.

HAHN, H.P. von & VERZAR, F. (1963)
Age dependent thermal denaturation of DNA from bovine thymus.
Gerontologia, 7(2): 105-108.

HAKH, I.W.D. & WESTLING, E.H. (1934)
A possible cause of old age.
Science, 79: 231.

HALLGREN, H.M., BUCKLEY, G.E. III , GILBERSTEN, V.A. & YUNIS, E.J. (1973)

Lymphocyte phytohemagglutinin responsiveness immunoglobulins and autoantibodies in aging humans.

J. Immun. 111(4): 1101-1107.

HANSEMANN, D. von (1893)

Studien uber die Spezifitat, den Altruismus und die Anaplasie der Zellen, Berlin: A. Hirschwald.

HARMAN, D. & PIETTE, L.H. (1966)

Free radical theory of aging: Free radical reactions in serum.

J. Geront. 21: 560-570.

HARRIS, M. (1971)

Mutation rates in cells at different ploidy levels.

J. Cell Physiol. 78: 117-184.

HARRIS, R. (1975)

Cardiac changes with age. In: The Physiology and Pathology of Human Aging, eds. Goldman, R. & Rockstein, M. P: 109-122, New York: Academic Press.

HARRISON, D.E. & ARCHER, J.R. (1978)

Measurement of changes in mouse tail collagen with age: Temperature dependence and procedural detail.

Exp. Gerontol. 13: 75-82.

HAYASHI, M. & FREIMAN, D.G. (1966)

An improved method of fixation for formalin sensitive enzymes with special reference to myosin ATPase.

J. Histochem. Cytochem. 14: 577-581.

HAYFLICK, L. (1965)

The limited in vivo lifetime of human diploid cell strains.

Expl Cell Res. 37: 614-636.

HAYFLICK, L.(1974)

Cytogerontology. In: Theoratical Aspects of Aging, ed. Rockstein, M. New York: Academic Press.

HAYFLICK, L. & MOORHEAD, P.S. (1961)

The serial cultivation of human diploid cell strains.

Expl Cell Res. 25: 585-621.

HEGARTY, P.V.J. & NAUDE, R.T. (1973)

Effects of freezing, thawing and fixatives on the diameters of fibres from pre and post-rigor skeletal muscles.

Proc. R. Ir. Acad. 73B(6): 87-94.

HEGARTY, P.V.J. & HOOPER, A.C. (1971)

Sarcomere lenghth and fibre diameter in four different mouse skeletal muscles.

J. Anat. 110(2): 249-257.

HEILBRUNN, L.V. (1943)

An Outline of General Physiology. London: W.B. Saunders.

HENNEMAN, E. & OLSON, C.B. (1965)

Relations between structure and function in the design of skeletal muscle.

J. Neurophysiol. 28: 581-598.

HOH, J.F.Y. & YEOH, G.P.S. (1979)

Rabbit skeletal myosin isoenzymes from fetal fast-twitch and slow twitch muscles.

Nature, 280: 321-322.

HOLEHAN, A. (1981)

Effects of ageing and dietry restriction on reproduction in the female CFY rat. Ph.D. Thesis, University of Hull.

HOWARD-FLANDERS, P. & BOYCE, R.P. (1966)

DNA repair and genatic recombination: studies on mutants of E. coli defective in these processes.

Radiation Research, Supplement 6: 156-184

HOWELLS, K.F. & GOLDSPINK, G. (1974)

The effect of age and exercise on the succinic dehydrogenase content of individual muscle fibres from fast, slow and mixed hamster muscles.

Histochemistry, 38: 195-201.

HUXLEY, A.F. (1974)

Muscular contraction.

J. Physiol. 243: 1-43.

IHEMELANDU, E.C. (1980)

Decrease in fibre number of dog pectineus muscle with age.

J. Anat. 130(1): 69-73.

INGRAM, D.J.E. (1958)

Free Radicals as studied by Electron Spin Resonance. New York: Academic Press.

INOKUCHI, S., ISHIKAWA, H., IWAMOTO, S. & KIMURA, T. (1975)

Age-related changes in the histological composition of the Rectus Abdominis muscle of the adult human.

Human Biology, 47(2): 231-249.

JACKSON, D.S. & CLEAREY, E.G. (1967)
The determination of collagen and elastin.
Meth. biochem. Analysis, 15: 25-72.

JENNINGS, H.S. (1913)
The effect of conjugation in Paramecium.
J. exp. Zool. 14: 279-391.

KELLY, A.M. & RUBINSTEIN, N.A. (1980)
Why are fetal muscles slow.
Nature, 288: 265-269.

KELLY, S.S. (1978)
The effect of age on neuromuscular transmission.
J. Physiol. 274: 51-62.

KHAN, M.A., PAPADIMITRIOU, J.M., HOLT, P.G. & KAKULAS, B.A. (1972b)
A modified histochemical technique for the histochemical localisation of myosin ATPase.
Stain Technol. 47: 277-281.

KIRK, J.E. (1959)
Enzyme activities of human arterial tissue.
Ann. N.Y. Acad. Sci. 72: 1006-1015.

KMENT, S.A., LEIBETSEDER, J. & BURGER, H. (1966)
Gerontologische untrsuchungen an rattenherzmitochondrien.
Gerontologia, 12: 193-199.

KNORRING, J.V. (1970)
Effect of age on the collagen content of the normal rat myocardium.
Acta physiol. scand. 79: 216-225.

KORENCHIEVSKY, V. (1961)
Physiological and Pathological Ageing, ed. Bourne, G.H. P: 41, New York: Hafner.

KRAG, C.L. & KOUNTZ, W.B. (1950)
Stability of body function in the aged: I Effect of the exposure of the body to cold.
J. Geront. 5: 227-235.

KRAG, C.L. & KOUNTZ, W.B. (1952)
Stability of the body function in the aged: II Effect of the exposure of the body to heat.
J. Geront. 7: 61-70.

KUGELBERG, E. (1973)

Histochemical composition, contraction speed and fatiguability of rat soleus motor units.

J. Neurological Sciences, 20: 177-198.

KUGELGERB, E. (1976)

Adaptive transformation of rat soleus motor units during growth.

J. Neurological Sciences, 27: 269-289.

KUGELBERG, E. & EDSTROM, L. (1968)

Differential histochemical effects of muscle contraction on phosphorylase and glycogen in various types of fibres: relation to fatigue.

J. Neurol. Neurosurg. Psychiat. 31: 415-423

LACK, D. (1965)

Evolutionary ecology.

J. Ecol. 53(2): 237-245.

LAMB, M.J. & MAYNARD-SMITH, J. (1964)

Radiation and ageing in insects.

Exp. Gerontol. 1: 11-20.

LANSING, A.I. (1942)

Some effects of hydrogen ion concentration, total salt concentration, calcium and citrate on longevity and fecundity in the rotifer.

J. exp. Zool. 91: 195-211.

LANSING, A.I. (1948)

Evidence for ageing as a consequence of growth cessation.

Proc. natn. Acad. Sci. U.S.A. 34(6): 304-310.

LANSING, A.I. (1951)

Some physiological aspects of aging.

Physiol. Rev. 31: 274-284.

LARSSON, L. (1978)

Morphological and functional characteristics of the ageing skeletal muscle in man.

Acta physiol. scand. Supplement 457.

LARSSON, L. & KARLSSON, J. (1978)

Isometric and dynamic endurance as a function of age and skeletal muscle characteristics.

Acta physiol. scand. 104: 129-136.

LARSSON, L., SJODIN, B. & KARLSSON, J. (1978)

Histochemical and biochemical changes in human skeletal muscle with age in sedentary males aged 22-65 years.

Acta physiol. scand. 103: 31-39.

LAYMAN, D. K., HEGARTY, P.V.J. & SWAN, P.B. (1980)

Comparison of morphological and biochemical parameters of growth in rat skeletal muscles.

J. Anat. 130(1): 159-171.

LEPESCHKIN, W.W. (1930)

Death and it's causes.

Q. Rev. Biol. 6(2): 167-177.

LEWES, G.H. (1877)

The Physical Basis of Mind. London: Trubner and Co.

LIEBERMAN, D.A., MAXWELL, L.C. & FAULKNER, J.A. (1972)

Adaptation of guinea pig diaphragm muscle to aging and endurance training.

Am. J. Physiol. 222(3): 556-560.

LOEB, J. (1908)

Uber den temperaturkoeffizienten fur die lebensdaur kattblutiger tiere.

Pflugers Arch. 124: 411-426.

LORAND, A. (1904)

Quelques considerations sur les causes de senilite.

C. r. Seanc. Soc. Biol. 57: 500-502.

LORAND, A. (1929)

La role de la l'autointoxication intestinale dans la production de la vieillesse.

Clinique, 24: 205.

LOWRY, C.V., KIMMEY, J.S., FELDER, S., CHI, M.-Y., KAISER, K., POSSONNEAU, P.N., KIRK, K. & LOWRY, O.H. (1978)

Enzyme patterns in single human muscle fibres.

J. biol. Chem. 253(22): 8269-8277

LOWRY, O.H., HASTINS, A.B., HULL, T.Z. & BROWN, A.N. (1942)

Histochemical changes associated with aging: II Skeletal and cardiac muscle in the rat.

J. biol. Chem. 143: 271-280.

LUFF, A.R. (1975)

Dynamic properties of fast and slow skeletal muscles in the cat and rat following cross-reinnervation.

J. Physiol. 248: 83-96.

LUFT, J.H. (1961)

Improvements in epoxy resin embedding methods.

J. biophys. biochem. Cytol. 9: 409-414.

LUTZ, H., WEBER, H., BILLETER, R. & JENNY, E. (1979)

Fast and slow myosin within single skeletal fibres of adult rabbits.

Nature, 281: 142-144. .

MAGNUS-LEVY, A. (1970)

Metabolism in old age. In: Metabolism and Practical Medicine, Chicago: C. von Noorden, Anglo-American Issue.

MAKINODAN, T. & PETERSON, W.J. (1964)

Growth and senescence of the primary antibody-forming potential of the spleen.

J. Immun. 93(6): 886-896.

MANDEL, P. (1961)

Metabolism of bovine arterial wall and it's changes in young animals. Metab. Parietis Vasorum, Papers in the 5th International Congress on Angiology. Prague.

MANN, W.S. & SALAFSKY, B. (1970)

Enzymic and physiological studies on normal and disused developing fast and slow cat muscles.

J. Physiol. 208: 33-47.

MAXWELL, L.C., FAULKNER, J.A. & LEIBERMAN, D.A. (1973)

Histochemical manifestations of age and endurance training in skeletal muscle fibres.

J. Physiol. 224: 356-361.

McCAFFERTY, W.B. & EDINGTON, D.W. (1974)

Skeletal muscle and organ weights of aged and trained male rats.

Gerontologia, 20: 44-50.

McCAY, C.M. (1952)

Chemical aspects of ageing and the effect of diet upon ageing. In: Cowdry's Problems of Aging, ed. Lansing, A.I. Chapter IV, P: 139-220, Baltimore: Williams and Wilkins.

McCOMAS, A.J. (1978)

Ageing. In: Neuromuscular Function and Disorders, P: 101-108, London: Butterworth and Co.

MECHANIC, G.L., KUBOKI, Y., SHIMOKAWA, H., HAKAMOTO, K., SASKI, S. & KAWANISHI, Y. (1974)

Collagen crosslinks: Direct qualitative determination of stable structural crosslinks in bone and dentin collagens.

Biochem. biophys. Res. Commun. 60: 756-763.

MEDAWAR, P.B. (1957)

The Uniqueness of the Individual. London: Methuen and Co.

MEDVEDEV, ZH. A. (1961)

Advances in Contemporary Biology, 51: 299.
Quoted by Shock, N.W. (1974). Physiological theories of aging. In: Theoratical Aspects of Aging, ed. Rockstein, M. New York: Academic Press.

MEDVEDEV, ZH. A. (1964)

The nucleic acid in development and aging. In: Advances in Gerontological Research, ed. Strehler, B.L. Vol 1: 181-206, New York: Academic Press.

MEIJER, A.E.F.H. (1970)

Histochemical methods for the demonstration of myosin ATPase in muscle tissue.

Histochemie, 22: 51-58.

MERRY, B.J. & HOLEHAN, A.M. (1981)

Serum profiles of LH, testosterone and 5α-DHT from 21 to 1000 days of age in ad libitum fed and dietry restricted rats.

Exp. Gerontol. 16: (In Press).

METALNIKOV, S. (1937)

La Lutte Contre la Mont. Paris: Gallimard.

METCHNIKOFF, E. (1904)

The Nature of Man. New York: Heinemann.

METCHNIKOFF, E. (1907)

The Prolongation of Life - Optimistic Studies. London: Heinemann.

MEYER, J. SPIER, E. & NEUWELT, F. (1940)

Studies in old age: IV The clinical significance of salivary, gastric and pancreatic secreations in the aged.

Archs intern. Med. 65: 171-177.

MINOT, C.S. (1908)

The Problem of Age, Growth and Death: A study of cytomorphosis. Based on lectures at The Lowell Institute, London.

MOHAN, S. & RADHA, E. (1980)

Age related changes in rat muscle collagen.

Gerontology, 26: 61-67.

MOLISCH, H. (1938)

The Longevity of Plants. New York: Botanical Gardens.

MONTGOMERY, T.H. (1906)

On reproduction, animal life cycles and the biological unit. Trans. Tex. Acad. Sci. 9: 75-94.

MOORE, M.J., REBIEZ, J.J., HOLDEN, M. & ADAMS, R.D. (1971)

Biometric analysis of normal skeletal muscle atrophy. Acta Neuropathol. 19: 51-69.

MOSS, F.P. & LEBLOND, C.P. (1970)

Nature of dividing nuclei in skeletal muscle of growing rats. J. Cell Biol. 44(2): 459-462.

MOSS, F.P. & LEBLOND, C.P. (1971)

Satellite cells as the source of nuclei in muscles of growing rats. Anat. Rec. 170(4): 421-435.

MUHLMANN, M.S. (1900)

Uber die Ursache des Alters. Wiesbaden: Bergman.

MUHLMANN, M.S. (1927)

Wachstum, altern und tod. Uber die ursache des alterns und des todes. Ergebn. Anat. EntwGesch. 27: 1-245.

MUNRO, N.H. (1969)

Mammalian Protein Metabolism. Vol III , New York: Academic Press.

MURAVOV, I.V. (1969)

Motor activity in old age. In: Bases of Gerontology, eds. Chebotarev, Mankovski and Froklis, P: 546-568. Moscow: Medizina (Russian).

NACHLAS, M.M., TSOU, K.C., DESOUZA, E., CHANG, C.S. & SELIGMAN, A.M. (1957)

Cytochemical demonstration of succininic dehydrogenase by the use of a new p-nitrophenyl substituted ditetrazole. J. Histochem. Cytochem. 5: 420-436.

NARAYANAN, N. & EAPEN, J. (1975)

Age related changes in the incorporation of ^{14}C Leucine into myofibrillar and sarcoplasmic proteins of red and white muscles. Ajebak, 53(1): 59-63.

NEEDHAM, A.E.J. (1949)

Growth and regeneration in Asellus aquaticus (L.) in relation to age, sex and season. J. exp. Zool. 112: 49-78.

NEMETH, P., HOFER, H.-W. & PETTE, D. (1979)

Metabolic heterogenity of muscle fibres classified by myosin ATPase.

Histochemistry, 63: 191-201.

NORRIS, A.H. & SHOCK, N.W. (1957)

Age changes in ventilatory muscles and metabolic response to submaximal exercise. Proceedings of the 4th International Congress on Gerontology, Vol 2: 512-522.

NORRIS, A.H., SHOCK, N.W & YIENGST, M.J. (1953)

Age changes in heart rate and blood pressure responses to tilting and standardized exercise.

Circulation, 8: 521-526.

OGATA, T. & MORI, M. (1964)

Histochemical study of oxidative enzymes in vertebrate muscles.

J. Histochem. Cytochem. 12: 171-182.

OLSON, C.B. & SWETT, C.P. (1966)

A functional and Histochemical characterization of motor units in heterogeneous muscle (flexor digitorum longus) of the cat.

J. comp. Neurol. 128: 475-497.

ONTELL, M. (1974)

Muscle satellite cells: A validated technique for light microscope identification and a qualitative study of changes in their population following denervation.

Anat. Rec. 178(2): 211-228.

ONTELL, M. & DUNN, R.F. (1978)

Neonatal muscle growth: A qualitative study.

Am. J. Anat. 152(4): 539-556.

ORGEL, L.E. (1963)

The maintenance of the accuracy of protein synthesis and it's relevance to ageing.

Proc. natn. Acad. Sci. U.S.A. 49: 517-521.

ORLANDER, J., KEISSLING, K.,-H., LARSSON, L., KARLSSON, J. & ANIANSSON, A. (1978)

Skeletal muscle metabolism and ultrastructure in relation to age in sedentary men.

Acta physiol. scand. 104: 249-261.

ORTON, J.H. (1928)

On rhythmic periods of shell growth in Ostrea edulis with a note on flattening.

J. mar. biol. Ass. U.K. 15(2): 365-427.

PACKER, L., DEAMER, D.W. & HEATH, R.L. (1967)

Regulation and deterioration of structure in membranes. In: Advances in Gerontological Research, ed. Strehler, B.L. Vol 2: 77-120, New York: Academic Press.

PADYKULA, H.A. & HERMAN, E. (1955)

Factors affecting the activity of ATPase and other phosphatases as measured by histochemical techniques.

J. Histochem. Cytochem. 3: 161-169.

PADYKULA, H.A. & GAUTHIER, F. (1967)

Morphological and cytochemical characteristics of fibre types in normal mammalian skeletal muscle. In: Exploratory Concepts in Muscular Dystrophy and Related Disorders, ed. Milhorat, T. Amesterdam: Excerpta Medical Foundation.

PEARL, R. (1928)

The Rate of Living. New York: Knopf.

PETER, J.B., BARNARD, R.J., EDGERTON, V.R., GILLESPIE, C.A. & STEMPEL, K.E. (1972)

Metabolic profiles of three fibre types of skeletal muscle in guinea pigs and rabbits.

Biochemistry, 11(14): 2627-2633.

PETERSON, I. & KUGELBERG, E. (1949)

Duration and form of action potential in the normal human muscle.

J. Neurol. Neurosurg. Psychiat. 19: 148-154.

PRYOR, W.A. (1973)

Free radical reactions and their importance in biological systems.

Fedn Proc. 32(8): 1862-1869.

PULLEN, A.H. (1977)

The distribution and relative sizes of fibre types in the extensor digitorum longus and the soleus of the adult rat.

J. Anat. 123(2): 467-486.

QUETELT, L.A.J. (1836)

Sur L'Homme et le Development de ses Facultes. Bruxelles: L. Hauman and Cie.

ROBERTSON, T.B. (1908a)

On the normal rate of growth of an individual and it's biochemical significance.

Arch. EntwMech. Org. 25: 581-613.

ROBERTSON, T.B. (1913)

On the nature of the autocatalyst of growth.

Arch. EntwMech. Org. 37: 497-508.

ROBERTSON, T.B. (1923)

The Chemical Basis of Growth and Senescence.
Philadelphia: J.B. Lippincott Co.

ROCKSTEIN, M. & BRANDT, K. (1962)

Muscle enzyme activity and changes in weight in ageing white rats.

Nature, 196: 142-143.

ROMANUL, F.C.A. (1964)

Enzymes in muscle: I Histochemical studies of enzymes in individual muscle fibres.

Archs Neurol. 11: 355-369.

ROTHBAUM, D.A., SHAW, D.J., ANGELL, C.S. & SHOCK, N.W. (1974)

Age differences in the baroreceptor response of rats.

J. Geront. 29(5): 488-492.

ROUX, W. (1881)

Der Kampf der Theile im Organismus. Leipzig: Engelmann.

ROWE, R.W.D. (1969)

The effect of senility on the skeletal muscles in the mouse.

Exp. Gerontol. 4: 119-126.

ROWE, R.W.D. & GOLDSPINK, G. (1969)

Muscle fibre growth in five different muscles in both sexes of mice: I Normal mice.

J. Anat. 104(3): 519-530.

RUBINSTEIN, L.J. (1960)

Age changes in muscle. In: Structure and Function of Muscle, ed. Bourne, G.H. Vol 2: 209-226. New York: Academic Press.

RUBNER, M. (1909)

Kraft und Stoff im Haushalte der Natur.
Liepzig: Akademische verlagsgesellschaft m.b.h.

RUGER, H.A. & STOESSIGER, B. (1927)

Growth curves of certain charateristics in Man.

Ann. Eugen. 2: 76-111.

SALMONS, S. & SRETER, F.A. (1976)

Significance of impulse activity in the transformation of skeletal muscle type.

Nature, 263: 30-34.

SALMONS, S. & VRBOVA, G. (1969)

The influence of activity on some contractile characteristics of mammalian fast and slow muscles.

J. Physiol. 201: 535-549.

SANDS, J., DOBBING, J. & GRATIX, C.A. (1979)

Cell number and cell size: Organ growth and development and the control of catch up growth in rats.

The Lancet, 8141: 503-505.

SASKI, R., SHIZUKA, I., YAMAGIWA, H., ITO, A. & YAMAGATA, S. (1976)

Ageing and hydroxyproline content in human heart muscle.

Tohoku J. Experimental Medicine, 118: 11-16.

SAVILL, T.D. (1896)

Senile decay, it's pathology and treatment.

Transactions of the Medical Society, 25: 271-274.

SCHAUB, M.C. (1963)

The ageing of collagen in the skeletal muscle.

Gerontology, 8: 16-35.

SCHAUB, M.C. (1964b)

Degradation of young and old collagen by extracts of various organs.

Gerontologia, 9: 52-60.

SCHIAFFINO, S. & BORMIOLI, S.P. (1973)

Histochemical characterisation of adenosine triphosphatases in skeletal muscle fibres by selective extraction procedure.

J. Histochem. Cytochem. 21(2): 142-145.

SCHIAFFINO, S., BORMIOLI, P. & ALOISI, M. (1976)

The fate of newly formed satellite cells during compensatory muscle hypertrophy.

Virchows Arch. Abt. B 21: 113-118.

SCHMITT, H.P. (1976)

Measurement of voluntary muscle fibre cross sections: A comparative study of different possible methods.

Microscopica Acta, 77(5): 427-440.

SCHWARZ, F. & WICHAN, I. (1966)

Membrane potentials of aging muscle cells.

Acta biol. med. 17: 96-98.

SELYE, H. (1959)

Aging. Symposia of the American Association for the Advancement of Science, ed. Shock, N.W. ,Chicago.

SELYE, H. (1962)

Calciphylaxis. Chicago: University of Chicago.

SEMENOWA-TJAN-SCHANSKAJA, W. (1941)

Die morphologischem veranderungen der peripheren nerven bein menschen im greisenalter.

Z. ges. Neurol. Psychiat. 172: 587-600.

SHOCK, N.W. (1974)

Physiological theories of aging. In: Theoratical Aspects of aging, ed. Rockstein, M. New York: Academic Press.

SILLAU, A.H. & BANCHERO, N. (1978)

Skeletal muscle fibre size and capillaries.

Proc. Soc. exp. Biol. Med. 158(2): 288-291.

SINEX, F.M. (1957)

Aging and the lability of irreplaceable molecules.

J. Geront. 12(2): 190-198.

SINEX, F.M. (1960)

Aging and the lability of irreplaceable molecules: The amid groups of collagen.

J. Geront. 15: 15-18.

SINEX, F.M. (1974)

The mutation theory of aging . In: Theoratical Aspects of Aging, ed. Rockstein, M., New York: Academic Press.

SJØGAARD, G., HOUSTON, M.E., NYGAARD, E. & SALTIN, B. (1978)

Subgrouping of fast twitch fibres in skeletal muscles of man: A critical appraisal.

Histochemistry, 58: 79-87.

SMITH-MAYNARD, J. (1962)

The causes of ageing.

Proc. R. Soc. B 157: 115-127.

SNOW, M.H. (1979)

The effect of ageing on satellite cells in skeletal muscles of mice and rats.

Cell and Tissue Research, 185(3): 399-408.

SOKAL, R.R. & ROHLF, F.J. (1969)

Biometry. San Francisco: W.H. Freeman and Co.

SONG, S.K., SHIMADA, N. & ANDERSON, P.J. (1963)

Orthogonal diameters in the analysis of muscle fibres size and form.

Nature, 200: 1220-1221.

SPURWAY, N.C. (1978)

Objective typing of mouse ankle-extensor muscle fibres based on histochemical photometry.

J. Physiol. 277: 47-48P.

SPURWAY, N.C. (1978)

Cluster analysis and the typing of muscle fibres.

J. Physiol. 280: 39-40P.

SRIVASTAVA, U. (1969)

Polyribosome concentration of mouse skeletal muscle as a function of age.

Archs Biochem. Biophys. 130: 129-139.

STARLING, E.H. & EVANS, L. (1968)

Principles of Human Physiology. Ed. Darson, H. and Eggleton, M.G. Philadelphia: Lea and Febiger.

STEENIS, G.V. & KROES, R. (1971)

Changes in the nervous system and musculature of old rats.

Vet. Pathol. 8: 320-332.

STEIN, J.M. & PADYKULA, H.A. (1962)

Histochemical classification of individual skeletal muscle fibres of the rat.

Am. J. Anat. 110: 103-124.

STEINACH, E. (1920)

Verjungung Durch Experimetelle Neubelebung der Alternden Pubertatsdruse, Berlin: Springer.

STEGEMANN, H. (1958)

Mikrobestimmung von hydroxyproline mit chloramin-T und paradimethylaminobenzaldehde.

Hoppe-Seylers Z. physiol. chem. 311: 41-45.

STREHLER, B.L., MARK, D.D., MILDVAN, A.S. & GEE, M.V. (1959)

Rate and magnitude of age pigment accumulation in the human myocardium.

J. Geront. 14(4): 430-439.

SWEAT, F., PUCHTLER, H. & ROSENTHAL, S.I. (1964)

Sirius Red F3BA as a stain for connective tissue.

Archs Path. 78(1): 69-73.

SYROVY, I. & GUTMANN, E. (1970)

Changes in speed of contraction and ATPase activity in striated muscle during old age.

Expl Geront. 5: 31-35.

SYROVY, I. & GUTMANN, E. (1977)

Differentiation of myosin in soleus and extensor digitorum longus muscle in different animal species during development.

Pflugers Archiv European J. of Physiology, 369: 85-89.

SZABO, I. (1931a)

Absterben und entwicklung.

Biologia gen. 7: 163.

SZABO, I. (1931b)

The three types of mortality curves.

Q. Rev. Biol. 6: 462-463.

TAUCHI, H., YOSHIOKA, T. & KOBAYASHI, H. (1971)

Age change of skeletal muscles of rats.

Gerontologia, 17: 219-227.

TOMANEK, R.J. & LUND, D.D. (1973)

Degeneration of different types of skeletal muscle fibres 1) Denervation.

J. Anat. 116(3): 395-407.

TRIBE, M.A. (1966)

Some physiological studies in relation to age in the blow fly Calliphora Erythrocephala Meig.

J. Insect Physiol. 12: 1577-1593.

TRIBE, M.A. (1967)

Age related changes in the respiratory physiology of flight muscle from blowfly Calliphora Erythrocephala.

Exp. Gerontol. 2: 113-121.

TUNELL, G. & HART, M.N. (1977)

Simultaneous determination of skeletal muscle fibre types I, IIA and IIB by histochemistry.

Archs Neurol. 34(3): 171-173.

UFLAND, J.M. (1933)

Einfluss des lebensalters, geschlechts, der konstitution und des berufs auf die kraft verschiedener muskelgruppen.

Arbeitsphysiologie, 6: 653-663.

VERZAR, F. (1956)

Das altern des collagens.

Helv. physiol. pharmac. Acta, 14: 207-221.

VERZAR, F. (1963)

The ageing of collagen.

Scient. Am. 208(4): 104-114.

VOGT, C. & VOGT, O. (1946)

Ageing of nerve cells.

Nature, 158: 304.

VORONOFF, S. (1920)

Etude des Myens de Relever l'Energie vitale et de Prolonger la Vie. Paris: Grasset.

VORONOFF, S. (1929)

Testicular Grafting from Ape to Man. London: Bretano.

VYSKOCIL, F. & GUTMANN, E. (1972)

Spontaneous transmitter release from nerve endings and contractile properties in the soleus and the diaphragm muscle of senile rats.

Experientia, 28: 280-281.

WALFORD, R.L. (1962)

Auto-immunity and aging.

J. Geront. 17: 281-285.

WALFORD, R.L. (1969)

Immunologic Theory of Aging. Chapter IV , Copenhagen: Munksgaard.

WARD, P.S. (1975)

Histochemical studies of development and disease processes in rodent skeletal muscle. Ph. D. Thesis, University of Hull.

WARTHIN, A.S. (1929)

Old Age, the Major Involution; the Physiology and Pathology of the Ageing Process. New York: Hoeber.

WEINBACH, E.C. (1960)

Biochemical changes in mitochondria associated with age. In: The Biology of Aging, ed. Strehler, B.L. P: 328-331. Washington: American Institute of Biological Sciences.

WEINSTOCK, J.M., EPSTEIN, S. & MILHORAT, A.T. (1958)

Enzyme muscular dystrophy: III In the hereditary muscular dystrophy in mice.

Proc. Soc. exp. Biol. N.Y. 99: 272-276.

WEISMANN, A. (1882)

Uber die Dauer des Lebens. Jena: G. Fischer.

WEISMANN, A. (1884)

Uber Leben und Tod. Jena: G. Fischer.

WEISMANN, A. (1891)

Essays upon Heridity and Kindered Biological Problems. London: Oxford University Press.

WEISMANN, A. (1892)

Das Keimplasma. Jena: G. Fischer.

WELFORD, A.T. (1965)

Behaviour, Aging and the Nervous System. Eds. Welford A.T. and Birren, J.E., P: 3-20, Springfield, Illinois: Charles C. Thomas.

WHALEN, R.J., SCHWARTZ, K., KOUVERET, P., SELL, S.M. & GROS, F. (1979)

Contractile protein isoenzymes in muscle development; identification of an embryonic form of myosin heavy chain.

Proc. natn. Acad. Sci. U.S.A. 76(10): 5197-5201.

WILKIE, D.R. (1976)

The tension-length curve. In: Muscle. London: Edward Arnold (Publishers) Ltd.

WILLIAMS, G.C. (1957)

Pleiotrophy, natural selection and the evolution of senescence.

Evolution, 11: 398-411.

WILLIAMS, P.E. & GOLDSPINK, G. (1978)

Changes in sarcomere length and physiological properties in immobilized muscle.

J. Anat. 127(3): 459-468.

WILLIAMS, P.E. & GOLDSPINK, G. (1981)

Changes in the connective tissue component of muscle during periods of decreases or increased activity.

J. Anat. Proceedings, 133(1): 133.

WILSON, D.L. (1974)

The programmed theory of aging. In: Theoratical Aspects of Aging, ed. Rockstein, M. New York: Academic Press.

WOODHEAD-GALLOWAY, J. (1980)

Collagen: the Anatomy of a Protein. London: Edward Arnold (publishers) Ltd.

WYNNE-EDWARDS, V.C. (1962)

Animal Dispersion in Relation to Social Behaviour. New York: Hafner.

YIENGST, M.J., BARROWS, C.H. & SHOCK, N.W. (1959)

Age changes in the chemical composition of muscle and liver in the rat.

J. Geront. 14(4): 400-404.

APPENDIX 1

41 B Diet

INGREDIENTS

Barley
Wheat
Maize
Soya bean meal extract
Dried milk powder
Wheat by-products
Dried yeast products
Mineral supplement
Vitamin supplement
English white fish meal

CALCULATED ANALYSIS

Crude protein	21.10%	Tryptophan	0.28%
Crude oil	3.25%	Glycine	1.57%
Crude fibre	3.18%	Histidine	0.49%
Digestible crude protein	17.60%	Threonine	0.78%
Digestible crude oil	2.48%	Isoleucine	1.10%
Digestible crude fibre	2.10%	Leucine	1.67%
Digestible carbohydrate	46.80%	Phenylalanine	0.98%
Gross energy	3875.00kcal/kg	Valine	1.09%
Metabolisable energy	3487.00kcal/kg	Tyrosine	0.69%
Satursted fatty acids	0.73%	Aspartic acid	1.64%
Linoleic acid	1.03%	Glutamic acid	3.96%
Other unsaturated acids	1.54%	Proline	1.55%
Calcium	1.32%	Serine	1.00%
Phosphorus	1.03%	Vitamin A	11810.28IU/kg
Sodium chloride	0.80%	Carotene	0.79ppm
Magnesium	0.22%	Vitamin B1	5.91ppm
Potassium	0.91%	Vitamin B2	5.91ppm
Sulphur	0.23%	Vitamin B6	1.97ppm
Iron	98.42ppm	Vitamin B12	9.48ug/kg
Copper	9.84ppm	Vitamin E	49.21ppm
Manganese	49.21ppm	Vitamin K	2.95ppm
Zink	19.68ppm	Folic acid	0.74ppm
Cobalt	984.21ug/kg	Nicotinic acid	29.53ppm
Iodine	1968.42ug/kg	Pantothenic acid	9.84ppm
Arginie	1.14%	Choline chloride	492.10ppm
Lysine	1.29%	Biotin	0.12ppm
Methionine	0.74%	Vitamin D3	1968.38IU/kg
Cystine			

(After Holehan,A. 1981)

APPENDIX 2

Fibre types in mammalian skeletal muscles, their staining characteristics and the correlation of the nomenclature used by various investigators (After Close 1972 and Ward 1975).

I Classifications

Ogata and Mori(1964)	White	Medium	Red
Stein and Padykula(1962)	A	B	C
Engel(1962,1970)	II	I	II
Romanul(1964)	I	III	II
Padykula and Gauthier(1967)	White	Intermidiate	Red
Kugelberg and Edstrom(1968)	Fast-twitch White	Slow-twitch Intermidaite	Fast-twitch Red
Henneman and Olson(1965)			
Olson and Swett(1966)			
Barnard et al(1971)			
Brooke and Kaiser(1970)	IIB	I	IIA (and IIC)
Peter et al(1972)	FG	SO	FOG
Ashmore and Doerr(1971)	α W	β R	αα R
Burke et al(1973)	FF	S	FR

II Morphological Properties

Mitochondrial content	Small	Intermidiate	Large
Z line	Narrow	Intermidiate	Large
Neuromuscular junction	Large and complex	Intermidiate	Small and simple

III Histochemical Properties

Distribution of SDH	Even network	Even network	Predominantly subsarcolemmal
Oxidative enzyme activities	Low	High	Intermidiate
Mitochondrial ATPase	Low	Intermidiate	High
Glycolytic activities	High	Low	Intermidiate
Myofibrillar ATPase (pH 9.4)	High	Low	High
pH sensitivity of ATPase	Alkali stable	Alkali labile	Alkali stable
Formaldehyde sensitivity of myofibrillar ATPase	Sensitive		Stable

---

Relative amount of histochemical staining in human skeletal muscle fibres (After Engel 1974).

Reaction	Muscle fibre type and reactivity	
	Type I	Type II
NADH-TR	High	Low
SDH	High	Low
Cytochrome oxidase	High	Low
Benzidene peroxidase	High	Low
Phosphorylase	Low	High
Glycogen (PAS)	Low	High
Myofibrillar ATPase (pH 9.4)	Low	High
Myofibrillar ATPase acid preincubation	High	Low
Oil Red O (Sudan Black B)	High	Low

APPENDIX 3

STATISTICAL PROGRAMS

The programs used for statistical analysis were single precision Fortran 66 programs. They can be implemented with no major change on ICL 1904S-George 3 Computer System. The programs listed and described below are:

1- General Statistics

2- Fibre Diameter General Statistics

3- Model I Analysis of Variance

4- Model II Analysis of Variance

1- General Statistics

This program calculates basic statistical parameters for the sets of data supplied.

A- Input (each on a new line in free format)

1. Number of sets of data (maximum=35 sets, 5 in the example).
2. Name of the first set of data (maximum=4 letters, 1E3X in the example).
3. Number of data pieces in the first set (maximum=100 numbers, 6 in the example).
4. Data in the first set (each number on a new line, 45.3,80.5,52.6 etc. in the example).
5. Repeat 2,3 and 4 for all sets of data.

B- Output

1. Name = as in step 2 input.
2. N = number of data pieces in each set, as in step 3 input.
3. Mean
4. Sum of X squared $(\Sigma X)^2$.
5. Square of standard deviation.
6. Variance.
7. Standard deviation.
8. Standard error.

```
      MASTER NSTAT
      DIMENSION XNAME(35),X(35,101),SUMX(35),SUMSQX(35),
     /XBAR(35),SUMXSQ(35),SSD(35),VAR(35),SD(35),SE(35),N(35)
      READ(1,200)J
      DO 10 I=1,J
      READ(1,300)XNAME(I)
      READ(1,200)N(I)
      DO 1000 K=1,N(I)
      READ(1,100) X(I,K)
1000  CONTINUE
   10 CONTINUE
      DO 30 I=1,J
      CALL GPSTATS(X,N(I),SUMX(I),SUMSQX(I),XBAR(I),
     /SUMXSQ(I),SSD(I),VAR(I),SD(I),SE(I),I)
   30 CONTINUE
      WRITE(2,1040)
      DO 40 I=1,J
      WRITE(2,1050) XNAME(I),N(I),XBAR(I),SUMXSQ(I)
     /,SSD(I),VAR(I),SD(I),SE(I)
   40 CONTINUE
      STOP
  200 FORMAT(I0)
  300 FORMAT(A4)
  100 FORMAT(F0.0)
 1040 FORMAT(//'*NAME*N *       MEAN       *     SIGMA(XSQ)     *     ',
     /'SUM OF SDS     *      VARIANCE      *      STD DEVS      *     ',
     /'STD ERRS      *')
 1050 FORMAT('*',A4,'*',I2,1H*,F16.6,1H*,F18.4,1H*,
     /F18.10,1H*,F18.10,1H*,F18.10,1H*,F18.10,1H*)
      END
      SUBROUTINE GPSTATS(X,N,SUMX,SUMSQX,XBAR,SUMXSQ,SSD,VAR,SD,SE,J)
      DIMENSION X(35,101)
      SUMX=0.0
      SUMXSQ=0.0
      DO 100 I=1,N
      SUMX=SUMX+X(J,I)
      XSQ=X(J,I)*X(J,I)
  100 SUMXSQ=SUMXSQ+XSQ
      XBAR=SUMX/N
      SUMSQX=SUMX*SUMX
      SSD=SUMXSQ-(SUMSQX/N)
      VAR=SSD/(N-1)
      SD=SQRT(VAR)
      SN=FLOAT(N)
      SQN=SQRT(SN)
      SE=SD/SQN
      RETURN
  104 FORMAT('  SUM OF SQ. DEVIATIONS=',F19.10)
  200 FORMAT(1H0,I4)
  300 FORMAT(1H0,F12.8)
  105 FORMAT('  N=',I4)
  106 FORMAT('  MEAN=',F19.10)
  107 FORMAT('  VARIANCE=',F19.10)
  108 FORMAT('  STD DEV=',F19.10)
  109 FORMAT('  STD. ERROR=',F19.10,//)
  110 FORMAT('  SIGMA(XSQ)=',F19.10)
      END
      FINISH
****
```

A- The Input

```
5
1E3X
6
45.3
80.5
52.6
62.3
40.6
43.3
1E1X
6
50.2
36.3
32.8
40.5
72.9
62.5
1E5X
7
9.6
42.7
53.1
61.3
42.4
40.8
56.6
1E4X
8
29.7
40.8
64.3
47.8
45.5
46.6
68.5
53.1
3E2X
10
27.5
29.5
51.1
33.8
19.3
31.9
48.1
44.8
34.8
46.1

****
```

B- The Output

NAME	N	MEAN	SIGMA(XSQ)	SUM OF SDS	VARIANCE	STD DEVS	STD ERRS
1E3X	6	54.100000	18703.6400	1142.7799997325	228.5559999458	15.1180686579	6.1719256847
1E1X	6	49.200000	15774.4800	1250.6399999853	250.1279999967	15.8154354981	6.4566245051
1E5X	7	43.785714	15158.7100	1738.3885715005	289.7314285822	17.0214990110	6.4335219035
1E4X	8	49.537500	20719.7300	1088.0187499520	155.4312499929	12.4672069844	4.4078233006
3E2X	10	36.690000	14427.1500	965.5889997482	107.2876666383	10.3579759913	3.2754796082

2- Fibre Diameter General Statistics

This program is a modification of General Statistics to handle large sets of data in a special form. The data is assumed to be in pairs where the mean of the two readings in a pair produce a Mean Diameter. The Mean Diameter is multiplied by a conversion factor to allow for magnification factors. The diameter data pairs are split into two sets, each set is inputted independently. In the example to follow, the pairs 49-53,42-47,51-49 and 45-58 are inputted as 49,42,51 and 45 in one set and 53,47,49 and 58 in another. If only one diameter is available per fibre, the diameter should be duplicated and divided into two sets. Since this program considers fibre types , diameters should be inputted in the following order: SO,FOG and FG.

A- Input (each on a new line in free format).

1. Name assigned to the execution (maximum=8 letters, N6E6N* in the example).
2. Number of fibre types (either 2 or 3, 3 in the example).
3. Conversion factor (use 1 if no conversion is required or a reduction/magnification factor, 0.8275 in the example).
4. Fibre diameters (maximum=500, 49 42,51 etc in the example).
5. Each set is followed by 9999.9 which is a stop.
6. Repeat 4 and 5 for all sets.

B- Output

1. Name assigned to the execution run, as in step 1 input.
2. Internal checks. The numbers indicate the number of fibre diameters in each inputted set.
3. N = number of fibre diameters in a group.
4. Mean = Mean Fibre Diameter.
5. Sigma (XSQ) = Sum of X squared $(\Sigma X)^2$.
6. Sum of square deviation.
7. Variance, standard deviation and standard error.

```
      MASTER MAJPR
      DIMENSION XAA(501),XBA(501),XCA(501),XAB(501),
     /XBB(501),XCB(501),XCONA(501),XCONB(501),XCONC(501),
     /XSTDA(501),XSTDB(501),XSTDC(501)
      READ(1,11)NAME
      READ(1,20)K
      READ(1,30)T
      CALL INPUT(XAA,NAA)
      CALL INPUT(XAB,NAB)
      CALL INPUT(XBA,NBA)
      CALL INPUT(XBB,NBB)
      WRITE(2,15)NAME
      IF(K.EQ.2) GO TO 200
      CALL INPUT(XCA,NCA)
      CALL INPUT(XCB,NCB)
      WRITE(2,50)NAA,NAB,NBA,NBB,NCA,NCB
      GO TO 250
  200 WRITE(2,50)NAA,NAB,NBA,NBB
  250 IF(NAA.NE.NAB) GO TO 1000
      IF (NBA.NE.NBB)GO TO 2000
      IF(K.EQ.2) GO TO 300
      IF(NCA.NE.NCB) GO TO 3000
  300 NA=NAA
      NB=NBA
      IF(K.EQ.2)GO TO 350
      NC=NCA
  350 CALL CONVERT(XAA,XAB,NA,T,XCONA)
      CALL CONVERT(XBA,XBB,NB,T,XCONB)
      IF(K.EQ.2) GO TO 400
      CALL CONVERT(XCA,XCB,NC,T,XCONC)
  400 WRITE(2,5001)
      WRITE(2,5002)
  450 CALL GPSTATS(XCONA,NA,SUMXA,SUMSQXA,XBARA,SUMXSQA,SSDA,
     /VARA,SDA,SEA)
      WRITE(2,5003)
      CALL GPSTATS(XCONB,NB,SUMXB,SUMSQXB,XBARB,SUMXSQB,SSDB,
     /VARB,SDB,SEB)
      IF(K.EQ.2) GO TO  99
      WRITE(2,5004)
      CALL GPSTATS(XCONC,NC,SUMXC,SUMSQXC,XBARC,SUMXSQC,SSDC,
     /VARC,SDC,SEC)
      GO TO 99
 1000 WRITE(2,1001)
      GO TO 99
 2000 WRITE(2,2001)
      GO TO 99
 3000 WRITE(2,3001)
   99 STOP
   11 FORMAT(A8)
   15 FORMAT(///15X,'*GROUP ',A8//)
   20 FORMAT(I0)
   30 FORMAT(F0.0)
   50 FORMAT(1H ,I3)
   10 FORMAT(F18.9)
 1001 FORMAT('  THE NUMERS OF THE TWO SETS OF DIAMETERS IN ,'
     /'GROUP A ARE NOT THE SAME')
 2001 FORMAT('  THE NUMERS OF THE TWO SETS OF DIAMETERS IN ,'
     /'GROUP B ARE NOT THE SAME')
 3001 FORMAT('  THE NUMERS OF THE TWO SETS OF DIAMETERS IN ,'
     /'GROUP C ARE NOT THE SAME')
 5001 FORMAT(/' *CONVERTED RESULTS'/)
```

```
 5002 FORMAT(' *GROUP SO')
 5003 FORMAT(' *GROUP FOG')
 5004 FORMAT(' *GROUP FG')
      END
      SUBROUTINE CONVERT(A,B,N,T,XCON)
      DIMENSION A(501),B(501),XCON(501)
      DO 100 I=1,N
  100 XCON(I)=(A(I)+B(I))*T/2
      RETURN
      END
      SUBROUTINE INPUT(X,N)
C     THIS SUBROUTINE INPUTS DATA INTO ARRAY AND COUNTS
C     THEM.9999.9 IS STOP.
      DIMENSION X(501)
      N=1
   20 READ(1,100)X(N)
      IF (X(N).EQ.9999.9)GO TO 10
      N=N+1
      GO TO 20
   10 N=N-1
      RETURN
  100 FORMAT(F0.0)
      END
      SUBROUTINE GPSTATS(X,N,SUMX,SUMSQX,XBAR,SUMXSQ,SSD,VAR,SD,SE)
      DIMENSION X(501)
      SUMX=0.0
      SUMXSQ=0.0
      DO 100 I=1,N
      SUMX=SUMX+X(I)
      XSQ=X(I)*X(I)
  100 SUMXSQ=SUMXSQ+XSQ
      XBAR=SUMX/N
      SUMSQX=SUMX*SUMX
      SSD=SUMXSQ-(SUMSQX/N)
      VAR=SSD/(N-1)
      SD=SQRT(VAR)
      SN=FLOAT(N)
      SQN=SQRT(SN)
      SE=SD/SQN
      WRITE(2,105)N
      WRITE(2,106)XBAR
      WRITE(2,110)SUMXSQ
      WRITE(2,104)SSD
      WRITE(2,107)VAR
      WRITE(2,108)SD
      WRITE(2,109)SE

      RETURN
  104 FORMAT('  SUM OF SQ. DEVIATIONS=',F19.10)
  200 FORMAT(1H0,I4)
  300 FORMAT(1H0,F12.8)
  105 FORMAT('                        N=',I4)
  106 FORMAT('                     MEAN=',F19.10)
  107 FORMAT('                 VARIANCE=',F19.10)
  108 FORMAT('                 STD. DEV=',F19.10)
  109 FORMAT('               STD. ERROR=',F19.10,//)
  110 FORMAT('               SIGMA(XSQ)=',F19.10)
      END
      FINISH
****
```

A- The Input

```
N6F.6N*
3
0.8275
49
42
51
45
50
14
45
45
9999.9
53
47
49
58
58
59
52
62
9999.9
57
83
46
100
51
61
55
9999.9
70
91
71
98
92
44
74
41
9999.9
105
71
91
70
91
115
150
113
86
9999.9
137
108
148
128
157
134
85
96
114
9999.9
****
```

B- The Output

```
                *GROUP N6E6N*

 8
 A
 A
 8
 9
 9
```

```
*CONVERTED RESULTS

*GROUP SO
                          N=    8
                       MEAN=       40.2889062497
                 SIGMA(XSQ)=    13144.7521640055
 SUM OF SQ. DEVIATIONS=           159.1844296455
                   VARIANCE=       22.7406328064
                   STD. DEV=        4.7687139573
                 STD. ERROR=        1.6859949884


*GROUP FOG
                          N=    8
                       MEAN=       56.3217187501
                 SIGMA(XSQ)=    26813.1716701986
 SUM OF SQ. DEVIATIONS=          1436.0836465353
                   VARIANCE=      205.1548066474
                   STD. DEV=       14.3232261257
                 STD. ERROR=        5.0640251610

*GROUP FG
                          N=    9
                       MEAN=       91.8984722225
                 SIGMA(XSQ)=    76922.2645330387
 SUM OF SQ. DEVIATIONS=           914.3017616272
                   VARIANCE=      114.2877202033
                   STD. DEV=       10.6905434943
                 STD. ERROR=        3.5635144981
```

3- Model I Analysis of Variance

The Model I Anova in this program is based on Box 9.1 (Sokal and Rohlf 1969) and was followed by an a priori group comparison (Box 9.8). The program carries out general statistics and calculates F-test and probability levels. The latter requires the NAG subroutine GØ 1BBF.

A- Input (each on a new line in free format).

1. Name assigned to the execution (maximum=8 letters, COLL EDL in the example).
2. Number of groups (maximum=30 groups, 5 in the example).
3. First set of data (maximum=30 numbers, 0.326, 0.369, 0.390 etc in the example).
4. Each set of data is followed by 9999.9 which is a stop.
5. Repeat 3 and 4 for the 2nd ,3rd ...nth set of data.

B- Output

1. Group statistics as in general statistics program.
2. Model I Anova Table.
3. Group comparison, with differences between mean, variance ratio, degrees of freedom and probability levels displayed. The comparisons are numbered as in " Group Statistics Table". Although comprehensive comparison is carried out, only a maximum of n-1 is allowed.

```
      MASTER MODEL1
      DIMENSION X(30,30),NP(30),TEMPX(30)
      DIMENSION SUMX(30),XBARBAR(30),SD(30),SE(30)
      READ(1,200)SAM
      READ (1,100)N
      DO 10 I=1,N
      CALL INPUT(TEMPX,NTEMP)
      NP(I)=NTEMP
      DO 20 J=1,NP(I)
      X(I,J)=TEMPX(J)
   20 CONTINUE
   10 CONTINUE
      CALL MOD1(X,N,NP,SUMX,XBARBAR,XMSRES,NDFRES,SD,SE,SAM)
      CALL ASSEMBLE(N,SUMX,XBARBAR,NP,XMSRES,NDFRES,SD,SE)
      STOP
  100 FORMAT(I0)
  200 FORMAT(A8)
      END

      SUBROUTINE INPUT(X,N)
      DIMENSION X(30)
      N=1
   20 READ(1,100)X(N)
      IF (X(N).EQ.9999.9)GO TO 10
      N=N+1
      GO TO 20
   10 N=N-1
      RETURN
  100 FORMAT(F0.0)
      END
      SUBROUTINE MOD1(XBAR,NA,NPA,SUMX,XBARBAR,XMSRES,NDFRES,SD,SE,SAM)
      DIMENSION XBAR(30,30),TMPXBAR(30),NPA(30),SUMX(30),
     /SUMSQX(30),XBARBAR(30),SUMXSQ(30),SSD(30),VAR(30),SD(30),SE(30)
      WRITE(2,27)
      WRITE(2,29)
      WRITE(2,28)
      WRITE(2,29)
      DO 40 I=1,NA
      DO 50 J=1,NPA(I)
      TMPXBAR(J)=XBAR(I,J)
   50 CONTINUE
      CALL GPSTATS(TMPXBAR,NPA(I),SUMX(I),SUMXSQ(I),XBARBAR(I),
     /SUMSQX(I),SSD(I),VAR(I),SD(I),SE(I),I)
   40 CONTINUE
      NT=0
      RESSQ=0.0
      T=0.0
      DO 70 I=1,NA
      NT=NT+NPA(I)
      S=S+SUMSQX(I)
      RESSQ=RESSQ+SSD(I)
      T=T+SUMX(I)
   70 CONTINUE
      TSQ=T*T
      TOTSSQ=S-(TSQ/NT)
      BETSSQ=TOTSSQ-RESSQ
      NDFBET=NA-1
      NDFRES=NT-NA
      NDFTOT=NT-1
```

```
      XMSBET=BETSSQ/NDFBET
      XMSRES=RESSQ/NDFRES
      VARAT=XMSBET/XMSRES
      WRITE(2,29)
      CALL FTEST(NDFBET,NDFRES,VARAT,PROB)
      WRITE(2,30)SAM
      WRITE(2,32)
      WRITE(2,31)
      WRITE(2,32)
      WRITE(2,21)NDFBET,BETSSQ,XMSBET,VARAT,PROB
      WRITE(2,24)NDFRES,RESSQ,XMSRES
      N1=1
      WRITE(2,26)NDFTOT,TOTSSQ
      WRITE(2,32)
      RETURN
   21 FORMAT(' *BET. AGES    *',I5,1H*,F10.1,1H*,F12.2,1H*,F9.3,
     /1X,1H*,F13.10,1H*)
   24 FORMAT(' *BET. MUSCLES*',I5,1H*,F10.1,1H*,F12.2,1H*,
     /F9.3,1X,1H*,F13.10,1H*)
   26 FORMAT(' *TOTAL        *',I5,1H*,F10.1,1H*,T45,1H*,T56,
     /1H*,T70,1H*)
   27 FORMAT(///26X,'*GROUP STATS*',1H /)
   28 FORMAT(2H *,'GR. * NO. *    MEAN    *  S.D.    '
     /,'*  S.E.    *  VARIANCE *  SQ. S. D *')
   29 FORMAT(1H ,68(1H*))
   30 FORMAT(////1H ,14X,'*MODEL I ANOVA TABLE FOR ',A8/)
   31 FORMAT(' *SOURCE',T15,'* D.F *SUM OF S.D* MEAN SQUARE* VAR.RAT. *'
     /,' PROBABILITY',T70,1H*)
   32 FORMAT(1H ,69(1H*))
      END
      SUBROUTINE GPSTATS(X,N,SUMX,SUMSQX,XBAR,SUMXSQ,SSD,VAR,SD,SE,J)
      DIMENSION X(30)
      SUMX=0.0
      SUMXSQ=0.0
      DO 100 I=1,N
      SUMX=SUMX+X(I)
      XSQ=X(I)*X(I)
  100 SUMXSQ=SUMXSQ+XSQ
      XBAR=SUMX/N
      SUMSQX=SUMX*SUMX
      SSD=SUMXSQ-(SUMSQX/N)
      VAR=SSD/(N-1)
      SD=SQRT(VAR)
      SN=FLOAT(N)
      SQN=SQRT(SN)
      SE=SD/SQN
      WRITE(2,110)J,N,XBAR,SD,SE,VAR,SSD
```

```
      RETURN
  104 FORMAT('  SUM OF SQ. DEVIATIONS=',F19.10)
  200 FORMAT(1H0,I4)
  300 FORMAT(1H0,F12.8)
  105 FORMAT('  N=',I4)
  106 FORMAT('  MEAN=',F19.10)
  107 FORMAT('  VARIANCE=',F19.10)
  108 FORMAT('  STD DEV=',F19.10)
  109 FORMAT('  STD. ERROR=',F19.10,//)
  110 FORMAT(2H *,I3,2H *,I4,2H *,F11.4,1H*,F9.4,
     /1H*,F9.4,1H*,F11.4,1H*,F11.3,1H*)
      END

      SUBROUTINE ASSEMBLE(NA,SXA,XBARBAR,NPA,XMSPSUB,NDFP,SD,SE)
      DIMENSION SXA(30),SE(30),XBARBAR(30),NPA(30)
      WRITE(2,1800)
      WRITE(2,1805)
      WRITE(2,1804)
      WRITE(2,1805)
      DO 250 I=1,NA-1
      K=I+1
      DO 260 J=K,NA
      DBM=XBARBAR(I)-XBARBAR(J)
      ABM=ABS(DBM)
      CALL COMPARE(XMSPSUB,NPA(I),NPA(J),SXA(I),SXA(J),NDFP,I,J,ABM)
  260 CONTINUE
  250 CONTINUE
      WRITE(2,1805)
      RETURN
 1800 FORMAT(////24X,'*GROUP COMPARISON*'/)
 1801 FORMAT(/1H ,'*AGE GR*  NO  *     MEAN     *     SUM  .  *')
 1803 FORMAT(2H *,I5,2H *,I5,2H *,F12.7,1H*,F12.6,1H*)
 1804 FORMAT(1H ,'*A VS B* DIF. BET. MEAN * VAR. RAT. * D.F. *PR.',
     /' AGE GR. ARE THE SAME*')
 1805 FORMAT(1H ,69(1H*))
      END
      SUBROUTINE COMPARE(DEN,N1,N2,S1,S2,NDF,I,J,ABM)
      X=(((S1*S1)/N1)+((S2*S2)/N2))-((S1+S2)*(S1+S2))/(N1+N2)
      F=X/DEN
      CALL FTEST(1,NDF,F,PR)
      WRITE(2,10)I,J,ABM,F,NDF,PR
      RETURN
   10 FORMAT(2H *,I2,2H &,I2,1H*,F16.6,1H*,F11.4,1H*,'1 & ',I2,1H*,
     /9X,F15.12,1H*)
      END
      SUBROUTINE FTEST(N1,N2,VARAT,PROB)
      IFAIL=0
      PROB=G01BBF(N1,N2,VARAT,IFAIL)
      RETURN
      END
      FINISH
****
```

A- The Input

```
COLL EDL
5
.326
.369
.390
.445
.453
9999.9
5.692
4.941
4.950
4.699
4.803
9999.9
6.054
5.610
6.287
5.999
6.359
9999.9
5.431
6.376
6.397
5.627
6.406
9999.9
6.512
6.929
6.667
6.888
6.722
9999.9
****
```

B- The Output

GROUP STATS

GR.	NO.	MEAN	S.D.	S.E.	VARIANCE	SQ. S. D
1	5	0.3966	0.0532	0.0238	0.0028	0.011
2	5	5.0170	0.3914	0.1750	0.1532	0.613
3	5	6.0618	0.2946	0.1318	0.0868	0.347
4	5	6.0474	0.4784	0.2139	0.2289	0.915
5	5	6.7436	0.1697	0.0759	0.0288	0.115

*MODEL I ANOVA TABLE FOR COLL EDL

SOURCE	D.F	SUM OF S.D	MEAN SQUARE	VAR.RAT.	PROBABILITY
BET. AGES	4	131.7	32.94	329.033	0.0000000000
BET. MUSCLES	20	2.0	0.10		
TOTAL	24	133.7			

GROUP COMPARISON

A VS B	DIF. BET. MEAN	VAR. RAT.	D.F.	PR. AGE GR. ARE THE SAME
1 & 2	4.620400	533.1777	1 & 20	0.000000000073
1 & 3	5.665200	801.5734	1 & 20	0.000000000073
1 & 4	5.650800	797.5036	1 & 20	0.000000000044
1 & 5	6.347000	1006.1200	1 & 20	0.000000000000
2 & 3	1.044800	27.2633	1 & 20	0.000041400606
2 & 4	1.030400	26.5170	1 & 20	0.000048791888
2 & 5	1.726600	74.4554	1 & 20	0.000000035405
3 & 4	0.014400	0.0052	1 & 20	0.943344877596
3 & 5	0.681800	11.6099	1 & 20	0.002793766762
4 & 5	0.696200	12.1054	1 & 20	0.002365853914

4- Model II Analysis of Variance

The Model II Anova is based on Box 10.4 and Box 9.8 for group comparison (Sokal and Rohlf 1969). A Model I Anova is carried out simultaneously for comparison purposes. The program is a nested model where variations between subgroups as well as groups is considered. The data is supplied after an initial analysis with a general statistics program.

The program can handle problems where more than one sample (diameter) is obtained in each subgroup (eg. muscle) and there is more than one subgroup in each group (eg. age group) and more than one group as demonstrated in the example.

A- Input (each on a new line in free format)

1. Name assigned to the execution (maximum=8 letters, ENDO SOL in the example).
2. Number of groups (age groups for example, maximum=15, 6 in the example).
3. Number of subgroups (muscles) within the first group (maximum= 30, 5 in the example).
4. Number of data pieces (diameters) within the first subgroup (muscle), 10 in the example.
5. Mean "diameter" of the first subgroup (muscle), 30.2400 in the example.
6. Sum of X squared for diameters, $\Sigma(XSQ)$ i.e. $(\Sigma X)^2$, 11699.52 in the example.
7. Repeat 4-6 for 2nd subgroup (muscle).
8. Repeat 4-6 for nth subgroup.
9. Repeat 3-8 for each group (age).

B- Output

1. Model II Anova Table.
2. Model II Anova Table corrected for Satterthwaite's approximation.

3. Variance component expressed as a percentage for each contributor.
4. Internal checks.Calculation 1 should equal calculation 2.
5. Group statistics as in general statistics.
6. Model I Analysis of Variance Table for the same data.
7. Group comparison using Model I variance ratio.
8. Group comparison using Model II variance ratio.

```
      MASTER MODEL2
      DIMENSION NOSQ(15,30),NOSQSUB(15)
      DIMENSION XNTA(15), SUMX(15,30),SUMXSQ(15,30),NO(15,30)
      DIMENSION XBAR(15,30),NPA(15)
      DIMENSION SUBGRDS(15,30),SUMXA(15),NTA(15),GRDS(15)
      DIMENSION SUMNX(15),XNBARBAR(15),SD(15),SE(15)
      READ(1,1040)SAM
      READ(1,1000)NA
      DO 100 I=1,NA
      READ(1,1000)NPA(I)
      DO 110 J=1,NPA(I)
      READ(1,1000)NO(I,J)
      READ(1,1010)XBAR(I,J)
      READ(1,1010)SUMXSQ(I,J)
      SUMX(I,J)=XBAR(I,J)*NO(I,J)
  110 CONTINUE
  100 CONTINUE
      GT=0.0
      SUMXSQOBS=0.0
      SUMSQSUB=0.0
      DO 120 I=1,NA
      DO 130 J=1,NPA(I)
      GT=GT+SUMX(I,J)
      SUMXSQOBS=SUMXSQOBS+SUMXSQ(I,J)
      SUBGRDS(I,J)=SUMX(I,J)*SUMX(I,J)/NO(I,J)
      SUMSQSUB=SUMSQSUB+SUBGRDS(I,J)
  130 CONTINUE
  120 CONTINUE
      SUMSQA=0.0
      DO140 I=1,NA
      SUMXA(I)=0.0
      NTA(I)=0.0
      DO 150 J=1,NPA(I)
      SUMXA(I)=SUMXA(I)+SUMX(I,J)
      NTA(I)=NTA(I)+NO(I,J)
  150 CONTINUE
      GRDS(I)=SUMXA(I)*SUMXA(I)/NTA(I)
      SUMSQA=SUMSQA+GRDS(I)
  140 CONTINUE
      MTN=0.0
      NT=0.0
      DO 160 I=1,NA
      NT=NT+NTA(I)
      MTN=MTN+NPA(I)
  160 CONTINUE
      CT=GT*GT/NT
      SSTOT=SUMXSQOBS-CT
      SSGR=SUMSQA-CT
      SSSUBGR=SUMSQSUB-SUMSQA
      SSWIN=SUMXSQOBS-SUMSQSUB
      NDFA=NA-1
      NDFSUB=MTN-NA
      NDFTOT=NT-1
      NDFWIN=NDFTOT-(NDFA+NDFSUB)
      XMS GR=SSGR/NDFA
      XMS SUB=SSSUBGR/NDFSUB
      XMS WIN=SSWIN/NDFWIN
      VARAT AGE=XMS GR/XMS SUB
      VARAT MUS=XMS SUB/XMS WIN
```

```
      CALL FTEST(NDFA,NDFSUB,VARAT AGE,PROB AGE)
      CALL FTEST(NDFSUB,NDFWIN,VARAT MUS,PROB MUS)
      WRITE(2,1321)SAM
      WRITE(2,1630)
      WRITE(2,1620)
      WRITE(2,1630)
      WRITE(2,1621)NDFA,SSGR,XMS GR,VARAT AGE,PROB AGE
      WRITE(2,1624)NDFSUB,SSSUBGR,XMS SUB,VARAT MUS,PROB MUS
      WRITE(2,1625)NDFWIN,SSWIN,XMS WIN
      WRITE(2,1630)
      WRITE(2,1626)NDFTOT,SSTOT
      WRITE(2,1630)
      N2=0
      N3=0
      Q4=0.0
      DO 170 I=1,NA
      NOSQSUB(I)=0
      DO 180 J=1,NPA(I)
      NOSQ(I,J)=NO(I,J)*NO(I,J)
      NOSQSUB(I)=NOSQSUB(I)+NOSQ(I,J)
  180 CONTINUE
      N2=N2+NOSQSUB(I)
      N3=N3+NTA(I)*NTA(I)
      XNTA(I)=FLOAT(NTA(I))
      Q=NOSQSUB(I)/XNTA(I)
      Q4=Q4+Q
  170 CONTINUE
      Q1=FLOAT(NT)
      Q2=FLOAT(N2)
      Q3=FLOAT(N3)
      XNPO=(Q4-(Q2/Q1))/NDFA
      XNO=(Q1-Q4)/NDFSUB
      XNBO=(Q1-(Q3/Q1))/NDFA
      VARCOMPM=(XMS SUB-XMS WIN)/XNO
      VARCOMPA=((XMS GR-XMS WIN)-(XNPO*VARCOMPM))/XNBO
      XMSCGR=XMS WIN+(XNPO*VARCOMPM)+(XNBO*VARCOMPA)
      XMSPSUB=XMS WIN+(XNPO*VARCOMPM)
      CVARAT AGE=XMSCGR/XMSPSUB
      VARCOEF=XNPO/XNO
      VARCOEFN=1.0-VARCOEF
      SUB1=VARCOEFN*XMS WIN
      SUB2=VARCOEF*XMS SUB
      XMSPCHSUB=SUB1+SUB2
      DFPNUM=XMSPCHSUB*XMSPCHSUB
      DFPDEN=(SUB1*SUB1/NDFWIN)+(SUB2*SUB2/NDFSUB)
      DFP=DFPNUM/DFPDEN
      NDFP=IFIX(DFP)
      CALL FTEST(NDFA,NDFP,CVARAT AGE,CPROB AGE)
      TOTVARC=XMS WIN+VARCOMPM+VARCOMPA
      PCVARCOMPA=100*VARCOMPA/TOTVARC
      PCVARCOMPM=100*VARCOMPM/TOTVARC
      PCVARCOMPF=100*XMS WIN/TOTVARC
      WRITE(2,1637)
      WRITE(2,1630)
      WRITE(2,1620)
      WRITE(2,1630)
      WRITE(2,1621)NDFA,SSGR,XMS GR,CVARAT AGE,CPROB AGE
      WRITE(2,1624)NDFP,SSSUBGR,XMSPSUB,VARAT MUS,PROB MUS
      WRITE(2,1625)NDFWIN,SSWIN,XMS WIN
```

```
      WRITE(2,1630)
      WRITE(2,1631)
      WRITE(2,1633)
      WRITE(2,1632)
      WRITE(2,1633)
      WRITE(2,1634)VARCOMPA,PCVARCOMPA
      WRITE(2,1635)VARCOMPM,PCVARCOMPM
      WRITE(2,1636)XMS WIN,PCVARCOMPF
      WRITE(2,1633)
      WRITE(2,1638)
      WRITE(2,1639)XMS GR,XMSCGR
      WRITE(2,1640)XMSPSUB,XMSPCHSUB
      CALL MOD1(XBAR,NA,NPA,SUMNX,XNBARBAR,XMSRES,NDFRES,SD,SE,SAM,
     /XMSBET)
      CALL ASSEMBLE(NA,SUMNX,XNBARBAR,NPA,XMSRES,NDFRES,SD,SE)
      WRITE(2,1806)
      XMSPWIN=XMSBET/CVARAT AGE
      CALL ASSEMBLE(NA,SUMNX,XNBARBAR,NPA,XMSPWIN,NDFP,SD,SE)
      STOP
1000  FORMAT(I0)
1010  FORMAT(F0.0)
1020  FORMAT(1H ,I5)
1030  FORMAT(1H ,F22.7)
1040  FORMAT(A8)
1321  FORMAT(////1H ,14X,'*MODEL II ANOVA TABLE FOR ',A8/)
1620  FORMAT(' *SOURCE',T15,'* D.F *SUM OF S.D* MEAN SQUARE* VAR.RAT. *'
     /,' PROBABILITY',T70,1H*)
1621  FORMAT(' *BET.  AGES  *',I5,1H*, F10.2,1H*,F11.3,1X,1H*,F9.3,1X,1H
     /*,F13.11,1H*)
1622  FORMAT(' BET MUSCLES *',T18,1H*,T29,1H*,T42,1H*,T52,1H*,T66,1H* )
1623  FORMAT(' (WITH. AGES)*',I3,1H*, F10.3,1H*,F11.7,1X,1H*,F3.3,1X,1H*
     /,F13.10,1H*)
1624  FORMAT(' *BET. MUSCLES*',I5,1H*,F10.2,1H*,F11.3,1X,1H*,F9.3,1X,1H*
     /,F13.11,1H*)
1625  FORMAT(' *BET. FIBRES *',I5,1H*,F10.2,1H*,F11.3,1X,1H*,T56,1H*,T70
     /,1H*)
1626  FORMAT(' *TOTAL       *',I5,1H*,F10.2,1H*,T45,1H*,T56,1H*,T70,1H*)
1627  FORMAT(' RESIDUAL    *',T18,1H*,T29,1H*,T42,1H*,T52,1H*,T66,1H*)
1628  FORMAT(' (REG.+UNK.) *',I3,1H*,T29,1H*,F11.7,1X,T52,1H*,T66,1H*)
1630  FORMAT(1H ,69(1H*))
1631  FORMAT(///1H ,9X,'*VARIANCE  COMPONENT*',/)
1632  FORMAT(1H ,'*SOURCE      *  VAR. COMP.  * PERCENT *')
1633  FORMAT(1H ,39(1H*))
1634  FORMAT(1H ,'*BET. AGES   *',F14.9,1H*,F9.6,1H*)
1635  FORMAT(1H ,'*BET. MUSCLES*',F14.9,1H*,F9.6,1H*)
1636  FORMAT(1H ,'*BET. FIBRES *',F14.9,1H*,F9.6,1H*)
1637  FORMAT(///1H ,2X,'*CORRECTED ANOVA II TABLE USING',
     /' SATTERTHWAITE''S APPROXIMATION*',1H /)
1638  FORMAT(///1H ,'INTERNAL  CHECKS* CALCULATION 1 * CALCULATION 2 *')
1639  FORMAT(1H ,'MEAN SQ. AGES   *',F15.9,1H*,F15.9,1H*)
1640  FORMAT(1H ,'COR. MS. MUSCLES*',F15.9,1H*,F15.9,1H*)
1700  FORMAT(///1H ,10X,'FOR AGE GROUP ',I2)
1701  FORMAT(1H ,15X,'MEAN=',F12.8)
1702  FORMAT(1H ,15X,'NO. =',I5)
1703  FORMAT(1H ,15X,'SUM =',F12.8)
1704  FORMAT(////1H ,10X,'COMPARING AGE GROUP ',I2,' WITH GROUP ',
     /I2)
1705  FORMAT(1H ,10X,'DIFFERENCE BETWEEN MEAN=',F16.12)
1805  FORMAT(1H ,69(1H*))
1806  FORMAT(1H ,////15X,'*COMPARISON USING MODEL II VARIANCE RATIO*')
      END
```

```
      SUBROUTINE MOD1(XBAR,NA,NPA,SUMX,XBARBAR,XMSRES,NDFRES,SD,SE,SAM,
     /XMSBET)
      DIMENSION XBAR(15,30),TMPXBAR(30),NPA(15),SUMX(15),
     /SUMSQX(15),XBARBAR(15),SUMXSQ(15),SSD(15),VAR(15),SD(15),SE(15)
      WRITE(2,27)
      WRITE(2,29)
      WRITE(2,28)
      WRITE(2,29)
      DO 40 I=1,NA
      DO 50 J=1,NPA(I)
      TMPXBAR(J)=XBAR(I,J)
   50 CONTINUE
      CALL GPSTATS(TMPXBAR,NPA(I),SUMX(I),SUMXSQ(I),XBARBAR(I),
     /SUMSQX(I),SSD(I),VAR(I),SD(I),SE(I),I)
   40 CONTINUE
      NT=0
      RESSQ=0.0
      T=0.0
      DO 70 I=1,NA
      NT=NT+NPA(I)
      S=S+SUMSQX(I)
      RESSQ=RESSQ+SSD(I)
      T=T+SUMX(I)
   70 CONTINUE
      TSQ=T*T
      TOTSSQ=S-(TSQ/NT)
      BETSSQ=TOTSSQ-RESSQ
      NDFBET=NA-1
      NDFRES=NT-NA
      NDFTOT=NT-1
      XMSBET=BETSSQ/NDFBET
      XMSRES=RESSQ/NDFRES
      VARAT=XMSBET/XMSRES
      WRITE(2,29)
      CALL FTEST(NDFBET,NDFRES,VARAT,PROB)
      WRITE(2,30)SAM
      WRITE(2,32)
      WRITE(2,31)
      WRITE(2,32)
      WRITE(2,21)NDFBET,BETSSQ,XMSBET,VARAT,PROB
      WRITE(2,24)NDFRES,RESSQ,XMSRES
      N1=1
      WRITE(2,26)NDFTOT,TOTSSQ
      WRITE(2,32)
      RETURN
   21 FORMAT(' *BET. AGES    *',I5,1H*,F10.2,1H*,F12.5,1H*,F9.3,
     /1X,1H*,F13.10,1H*)
   24 FORMAT(' *BET. MUSCLES*',I5,1H*,F10.2,1H*,F12.5,1H*,
     /F9.3,1X,1H*,F13.10,1H*)
   26 FORMAT(' *TOTAL        *',I5,1H*,F10.2,1H*,T45,1H*,T56,
     /1H*,T70,1H*)
   27 FORMAT(///26X,'*GROUP STATS*',1H /)
   28 FORMAT(2H *,'GR. * NO. *     MEAN    *  S.D.    '
     /,'*  S.E.    *  VARIANCE *  SQ. S. D *')
   29 FORMAT(1H ,68(1H*))
   30 FORMAT(////1H ,14X,'*MODEL I ANOVA TABLE FOR ',A8/)
   31 FORMAT(' *SOURCE',T15,'* D.F *SUM OF S.D* MEAN SQUARE* VAR.RAT. *'
     /,' PROBABILITY',T70,1H*)
   32 FORMAT(1H ,69(1H*))
      END
```

```
      SUBROUTINE GPSTATS(X,N,SUMX,SUMSQX,XBAR,SUMXSQ,SSD,VAR,SD,SE,J)
      DIMENSION X(30)
      SUMX=0.0
      SUMXSQ=0.0
      DO 100 I=1,N
      SUMX=SUMX+X(I)
      XSQ=X(I)*X(I)
  100 SUMXSQ=SUMXSQ+XSQ
      XBAR=SUMX/N
      SUMSQX=SUMX*SUMX
      SSD=SUMXSQ-(SUMSQX/N)
      VAR=SSD/(N-1)
      SD=SQRT(VAR)
      SN=FLOAT(N)
      SQN=SQRT(SN)
      SE=SD/SQN
      WRITE(2,110)J,N,XBAR,SD,SE,VAR,SSD
      RETURN
  104 FORMAT('  SUM OF SQ. DEVIATIONS=',F19.10)
  200 FORMAT(1H0,I4)
  300 FORMAT(1H0,F12.8)
  105 FORMAT('  N=',I4)
  106 FORMAT('  MEAN=',F19.10)
  107 FORMAT('  VARIANCE=',F19.10)
  108 FORMAT('  STD DEV=',F19.10)
  109 FORMAT('  STD. ERROR=',F19.10,//)
  110 FORMAT(2H *,I3,2H *,I4,2H *,F11.4,1H*,F9.4,
     /1H*,F9.4,1H*,F11.4,1H*,F11.3,1H*)
```

```
      SUBROUTINE ASSEMBLE(NA,SXA,XBARBAR,NPA,XMSPSUB,NDFP,SD,SE)
      DIMENSION SXA(15),SE(15),XBARBAR(15),NPA(15)
      WRITE(2,1800)
      WRITE(2,1805)
      WRITE(2,1804)
      WRITE(2,1805)
      DO 250 I=1,NA-1
      K=I+1
      DO 260 J=K,NA
      DBM=XBARBAR(I)-XBARBAR(J)
      ABM=ABS(DBM)
      CALL COMPARE(XMSPSUB,NPA(I),NPA(J),SXA(I),SXA(J),NDFP,I,J,ABM)
  260 CONTINUE
  250 CONTINUE
      WRITE(2,1805)
      RETURN
```

```
1800 FORMAT(////24X,'*GROUP COMPARISON*'/)
1801 FORMAT(/1H ,'*AGE GR*  NO  *     MEAN     *     SUM      *')
1803 FORMAT(2H *,I5,2H *,I5,2H *,F12.7,1H*,F12.6,1H*)
1804 FORMAT(1H ,'*A VS B* DIF. BET. MEAN * VAR. RAT. * D.F. *PR.',
     /' AGE GR. ARE THE SAME*')
1805 FORMAT(1H ,69(1H*))
     END
     SUBROUTINE COMPARE(DEN,N1,N2,S1,S2,NDF,I,J,ABM)
     X=(((S1*S1)/N1)+((S2*S2)/N2))-((S1+S2)*(S1+S2))/(N1+N2)
     F=X/DEN
     CALL FTEST(1,NDF,F,PR)
     WRITE(2,10)I,J,ABM,F,NDF,PR
     RETURN
  10 FORMAT(2H *,I2,2H &,I2,1H*,F16.6,1H*,F11.4,1H*,'1 & ',I2,1H*,
     /9X,F15.12,1H*)
      END
      SUBROUTINE FTEST(N1,N2,VARAT,PROB)
      IFAIL=0
      PROB=G01BBF(N1,N2,VARAT,IFAIL)
      RETURN
      END
      FINISH
****
```

A- The Input

```
ENDO SOL
6
5
10
30.2400
11699.52
10
17.9200
3399.04
10
17.6400
4052.64
10
23.4600
6479.14
10
19.1200
4729.28
5
20
32.5050
22347.05
15
36.9800
20971.47
13
40.0615
22415.88
20
34.5650
24948.19
20
30.9350
19877.23
5
10
35.6300
13103.43
12
37.2833
17148.62
20
39.4450
31698.21
20
34.5350
24488.47
20
39.2800
31238.20
```

```
5
20
36.2050
26552.05
10
36.8700
13820.53
20
37.8550
29065.03
15
39.0267
23164.26
20
39.4300
31662.00
5
20
42.9000
37882.94
10
53.2200
28792.60
20
44.9250
41458.65
20
52.1900
55613.86
20
48.0200
47298.28
5
20
61.8650
79074.39
20
67.0850
91945.69
20
63.5600
82542.22
20
59.1150
71475.07
20
72.4350
107502.95
*****
```

B- The Output

*MODEL I ANOVA TABLE FOR ENDO SOL

SOURCE	D.F	SUM OF S.D	MEAN SQUARE	VAR.RAT.	PROBABILITY
BET. AGES	5	5263.03	1052.60611	66.770	0.0000000001
BET. MUSCLES	24	378.35	15.76460		
TOTAL	29	5641.38			

GROUP COMPARISON

A VS B	DIF. BET. MEAN	VAR. RAT.	D.F.	PR. AGE GR. ARE THE SAME
1 & 2	13.333300	28.1924	1 & 24	0.000019011335
1 & 3	15.558660	38.3885	1 & 24	0.000002109227
1 & 4	16.201340	41.6254	1 & 24	0.000001134074
1 & 5	26.575000	111.9963	1 & 24	0.000000000087
1 & 6	43.136000	295.0779	1 & 24	0.000000000000
2 & 3	2.225360	0.7853	1 & 24	0.384306954627
2 & 4	2.868040	1.3044	1 & 24	0.264669087876
2 & 5	13.241700	27.8064	1 & 24	0.000020840875
2 & 6	29.802700	140.8537	1 & 24	0.000000000029
3 & 4	0.642680	0.0655	1 & 24	0.800184812717
3 & 5	11.016340	19.2456	1 & 24	0.000197440415
3 & 6	27.577340	120.6040	1 & 24	0.000000000044
4 & 5	10.373660	17.0656	1 & 24	0.000378175071
4 & 6	26.934660	115.0482	1 & 24	0.000000000160
5 & 6	16.561000	43.4941	1 & 24	0.000000804110

COMPARISON USING MODEL II VARIANCE RATIO

GROUP COMPARISON

A VS B	DIF. BET. MEAN	VAR. RAT.	D.F.	PR. AGE GR. ARE THE SAME
1 & 2	13.333300	27.9107	1 & 23	0.000023178083
1 & 3	15.558660	38.0049	1 & 23	0.000002737217
1 & 4	16.201340	41.2095	1 & 23	0.000001499324
1 & 5	26.575000	110.8771	1 & 23	0.000000000285
1 & 6	43.136000	292.1292	1 & 23	0.000000000002
2 & 3	2.225360	0.7775	1 & 23	0.387031712391
2 & 4	2.868040	1.2914	1 & 23	0.267489827904
2 & 5	13.241700	27.5285	1 & 23	0.000025345136
2 & 6	29.802700	139.4461	1 & 23	0.000000000030
3 & 4	0.642680	0.0648	1 & 23	0.801257075298
3 & 5	11.016340	19.0533	1 & 23	0.000226678399
3 & 6	27.577340	119.3988	1 & 23	0.000000000137
4 & 5	10.373660	16.8950	1 & 23	0.000427570472
4 & 6	26.934660	113.8986	1 & 23	0.000000000220
5 & 6	16.561000	43.0594	1 & 23	0.000001074100

*MODEL II ANOVA TABLE FOR ENDO SOL

*SOURCE	* D.F	*SUM OF S.D*	MEAN SQUARE*	VAR.RAT.	* PROBABILITY *
*BET. AGES	* 5*	83378.76*	16675.753 *	67.937	*0.00000000012*.
BET. MUSCLES	24*	5891.06*	245.461 *	3.756	*0.00000001239*
*BET. FIBRES	* 465*	30390.62*	65.356 *		*
*TOTAL	* 494*	119660.44*	*	*	*

CORRECTED ANOVA II TABLE USING SATTERTHWAITE'S APPROXIMATION

*SOURCE	* D.F	*SUM OF S.D*	MEAN SQUARE*	VAR.RAT.	* PROBABILITY *
*BET. AGES	* 5*	83378.76*	16675.753 *	66.103	*0.00000000000*
BET. MUSCLES	23*	5891.06*	252.269 *	3.756	*0.00000001239*
*BET. FIBRES	* 465*	30390.62*	65.356 *		*

VARIANCE COMPONENT

*SOURCE	* VAR. COMP.	* PERCENT *
*BET. AGES	* 200.501755197	*72.416717*
BET. MUSCLES	11.014269831 *	3.978106*
*BET. FIBRES	* 65.356171212	*23.605177*

INTERNAL CHECKS*	CALCULATION 1 *	CALCULATION 2 *
MEAN SQ. AGES	*16675.752841233*	16675.752841472*
COR. MS. MUSCLES*	252.269272000*	252.269272000*

GROUP STATS

*GR. *	NO. *	MEAN *	S.D. *	S.E. *	VARIANCE *	SQ. S. D *
* 1 *	5 *	21.6760*	5.3258*	2.3818*	28.3637*	113.455*
* 2 *	5 *	35.0093*	3.6221*	1.6198*	13.1193*	52.477*
* 3 *	5 *	37.2347*	2.1757*	0.9730*	4.7336*	18.934*
* 4 *	5 *	37.8773*	1.3733*	0.6142*	1.8859*	7.544*
* 5 *	5 *	48.2510*	4.4709*	1.9994*	19.9889*	79.956*
* 6 *	5 *	64.8120*	5.1474*	2.3020*	26.4962*	105.985*