The effects of ischaemia and denervation on mammalian muscle spindles

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THE EFFECTS OF ISCHAEMIA AND DENERVATION ON MAMMALIAN MUSCLE SPINDLES

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by

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To:

my daughter

Sally

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TABLE OF CONTENTS

ABSTRACT

The effects of ischaemia and denervation on rat muscle spindles have been investigated in a series of experiments in which the soleus muscle was either devascularized, or denervated, or devascularized and denervated at the same time. The structure of the experimental spindles was observed with the electron microscope at various post-operative periods.

Soleus muscle is revascularized 5 days after sectioning its supplying blood vessels. The temporary ischaemia thus produced results in the rapid concomitant degeneration of both extrafusal and intrafusal muscle fibres as well as their nerve terminals and supplying axons. The basal lamina of the intrafusal fibres remains intact and necrotic debris is removed by phagocytic cells. Satellite cells survive the temporary ischaemia, giving rise to numerous myoblasts within the basal-lamina tubes. The myoblasts fuse to form myotubes which, by the 14th day after devascularization, are maturing into thin muscle fibres, in the absence of any innervation. Reinnervation of the regenerating spindle begins 21 days after devascularization and is completed some 7 days later, during which time further differentiation of reinnervated intrafusal fibres occurs. Regenerated spindles vary considerably with respect to the extent of their sensory and motor innervation and equatorial nucleation and the reasons for this variation are discussed. Most regenerated ischaemic spindles and denervated spindles contain short, thin additional muscle fibres, the sources of which are also discussed.

The degeneration and regeneration of ischaemic muscle spindles are not affected by sectioning the muscle nerve at the time of devascularization. Differences are only seen at the later post-

operative stages when further differentiation of the intrafusal fibres does not occur, presumably because of the absence of reinnervating axon terminals. Instead, signs of denervation atrophy are seen in non-innervated regenerated ischaemic spindles.

In contrast to the rapid effects of devascularization, denervation results in a slow atrophy of intrafusal fibres. Equatorial nuclei are replaced by myofibrils. As long as one year after denervation only focal atrophy of intrafusal fibres is evident, contrasting markedly with the widespread extensive atrophy of extrafusal fibres.

The significance of these results is discussed in relation to other experimental studies of the regeneration or reinnervation of muscle spindles. Their possible clinical application in nerve and muscle repair is also highlighted.

PART I INTRODUCTION

CHAPTER 1: Rat muscle spindles

1.1 General structure:

The classical descriptions of muscle spindles arose from observations in cat muscles because of their suitability for both histological and physiological examination. Muscle spindles are mechanoreceptors that lie in parallel to the ordinary extrafusal muscle fibres of skeletal muscles of tetrapod vertebrates. They consist of a bundle of intrafusal muscle fibres, which are shorter and thinner than the extrafusal ones. This axial bundle of intrafusal fibres is enclosed by a cellular multi-lamellated capsule, which expands in the equatorial region to enclose the fluid-filled periaxial space. At the equatorial region, the intrafusal fibres are innervated by sensory axons, whereas the motor axons make their contacts at the polar regions. Intrafusal fibres differ in their number, size, histochemical properties, ultrastructure, and innervation between muscles of the same species and between different species (for more information see Barker, 1974).

1.2 Intrafusal muscle fibres:

As in other mammals, rat muscle spindles contain three types of intrafusal fibre, namely, bag_1, bag_2, and nuclear-chain fibres. They are classified on the basis of their morphology and histochemical properties (James, 1971a; Milburn, 1973; Soukup, 1976; Barker, Banks, Harker, Milburn & Stacey, 1976; Banks, Harker & Stacey, 1977; Kucera,

Dorovini-Zis & Engel, 1978). Each muscle spindle contains four intrafusal muscle fibres, with a combination of one bag_1, one bag_2, and two nuclear-chain fibres (Milburn, 1973; Soukup, 1976; Banks et al., 1977; Kucera et al., 1978).

The bag_2 fibre is the longest and thickest, whereas the bag_1 fibre is slightly shorter and significantly thinner in rat. The nuclear-chain fibres are the shortest and thinnest, and usually end within the limits of the capsule. Both of the nuclear-bag fibres extend far beyond it (Soukup, 1976).

In the polar regions, both bag_1 and bag_2 fibres are characterized by ill-defined myofibrils, which appear tightly packed with little interfibrillar sarcoplasm, containing few glycogen granules. In contrast, the myofibrils in nuclear-chain fibres are discrete with abundant interfibrillar sarcoplasm, containing scattered glycogen granules (Merrillees, 1960; Landon, 1966a; Ovalle, 1971, 1972a; Milburn, 1973; Soukup, 1976). In chain fibres, the mitochondria are larger and more numerous than in bag fibres (Landon, 1966a; Ovalle, 1971, 1972a; Milburn, 1973).

Merrillees (1960) describes elongated nuclei centrally placed and widely spaced along the polar region of all fibre types. However, according to Landon (1966a) these nuclei are peripherally placed. Landon also observed a few satellite cells lying in troughs on the surface of intrafusal fibres, and included within the basal lamina. However Maynard & Cooper (1973) maintain that abundant satellite cells accompany both nuclear-bag and nuclear-chain fibres in the myotube and polar regions.

The myotube region is marked by a gradual increase in the number of nuclei, and in the volume of the sarcoplasm surrounding them, together with a progressive loss of myofilaments towards the mid-equator.

In chain fibres, a single row of elongated nuclei (a nuclear chain) is maintained throughout the equatorial region. In bag fibres, an aggregation of vesicular nuclei (a nuclear bag) is present in the equatorial region (Merrillees, 1960; Landon, 1966a; Ovalle, 1972a; Milburn, 1973; Soukup, 1976). The nuclear bag of the bag_2 fibre consists of more nuclei (3-4 abreast on average) than that of the bag_1 fibre (2-3 abreast on average), (Soukup, 1976).

Intrafusal fibres also differ with respect to the condition of the M-line in their sarcomeres. The chain fibres possess a prominent M-line throughout their length. A distinct M-line is present in the polar regions of the bag_2 fibre and absent in the equatorial region (Barker et al., 1976; Banks et al., 1977; Kucera et al., 1978). The bag_1 fibre possesses a distinct M-line in the extreme extracapsular regions only, and lacks one in other regions (Kucera et al., 1978).

The three types of intrafusal muscle fibre differ when incubated for the demonstration of adenosine triphosphatase (ATPase). Regional variations in ATPase staining are observed along both types of bag fibres, but not along chain fibres (Kucera et al., 1978). The bag_1 fibre stains lightly with alkali ATPase along the inner two-thirds of its intracapsular region and becomes moderately dark when approaching the termination of the capsule and remains so while passing beyond it. Along the rest of its extracapsular region, the bag_1 fibre regains its light alkali ATPase staining. However, the bag_1 fibre stains lightly with acid ATPase in the encapsulated region, but invariably becomes dark outside the capsule (Kucera et al., 1978). On the other hand, the bag_2 fibre shows dark staining with alkali ATPase in its encapsulated region and becomes light outside the capsule. The bag_2 fibre stains darkly with acid ATPase in all regions (Kucera et al., 1978). Such regional variations are absent in the chain fibres. Throughout their

length, they stain darkly with alkali ATPase and lightly with acid ATPase (James, 1971a; Soukup, 1976; Barker et al., 1976; Banks et al., 1977; Kucera et al., 1978).

1.3 Capsule, periaxial space and axial sheath:

For most of their length the intrafusal fibres, together with the periaxial space, are surrounded by a fusiform capsule. An axial sheath encloses the axial bundle in the equatorial region (Landon, 1966a). The capsule is similar to the perineurium of the peripheral nerve (Shantha, Golarz & Bourne, 1968) and is composed of 6-9 layers of flattened epithelial cells (Landon, 1966a). These cellular layers are composed of very thin sheets of cytoplasm, which possesses a basal lamina on their surfaces, and contain small mitochondria, occasional ribosomes and numerous pinocytotic vesicles (Merrillees, 1960; Landon, 1966a; Milburn, 1973). The cells of each layer contain flattened nuclei and have closely-apposed edges to form terminal-bar tight junctions (Landon, 1966a), described by Merrillees (1960) as desmosomes. They serve to bring the sheets into intimate contact.

The capsule layers are separated by narrow spaces in which scattered collagen fibrils are present (Merrillees, 1960). Occasional elastic fibrils are also found (Milburn, 1973). The collagen fibrils on the external surface of the capsule lie in both circular and longitudinal planes, whereas those between the capsule layers are arranged in a circular plane only (Landon, 1966a). The number of layers in the capsule gradually decreases towards the polar regions, which become closely invested by a single layer as the capsule ends (Merrillees, 1960; Landon, 1966a). In the equatorial region, some of the inner layers cross the periaxial space obliquely, forming one or more subcompartments within it (Landon, 1966a). Fibrocytes are found

in the outer layers of the spindle capsule as well as in the outer layers of the perineurium of the spindle nerve trunk (Milburn, 1973).

The axial bundle is separated from the periaxial space by the axial sheath, which consists of endomysial cells. These cells resemble those of the capsule, but have thinner sheets of cytoplasm, fewer vesicles, and no basal lamina (Merrillees, 1960; Landon, 1966a; Milburn, 1973; Barker, 1974). They have terminal-bar tight junctions at their adjoining edges and form more or less complete envelopes around each intrafusal fibre at the equator. The envelope becomes less complete and surrounds two or more intrafusal fibres at the limits of the periaxial space (Landon, 1966a). Inside the axial sheath, and between the intrafusal fibres, are found myelinated and non-myelinated axons, collagen and elastic fibrils, and loose strands of basal lamina (Landon, 1966a;Milburn, 1973; Barker, 1974). Elastic fibrils are seen to run in parallel with the bag fibres, but few, if any, are seen around chain fibres (Landon, 1966a).

Small arterioles and capillaries are usually found between the outer layers of the capsule (Merrillees, 1960; Landon, 1966a; James & Meek, 1971; Milburn, 1973). Myelinated and non-myelinated axons of different diameters are usually seen penetrating the capsule, passing through the periaxial space to innervate the intrafusal fibres. A group of these axons forming the spindle nerve trunk is usually enclosed in a sheath similar to the capsule. Several other axons may enter the spindle individually (Merrillees, 1960).

According to James (1971b), the periaxial space in rat muscle spindles contains hyaluronic acid.

1.4 Sensory innervation:

In general, sensory nerve terminals are supplied by the large

axons that innervate the spindle. The largest of these axons, the Ia afferent, forms the primary ending which is located at the mid-equator of the intrafusal fibres. Smaller afferent axons form the secondary endings, which may be located on either sides of the primary ending (Barker, 1974).

All rat muscle spindles contain one primary ending; in addition some spindles contain one or two secondary endings (Porayko & Smith, 1968; Gladden, 1969). The primary ending is usually seen as tightly-wound spirals on both bag and chain fibres. The secondary endings appear as fine sprays of nerve terminals mainly at the juxta-equatorial regions of chain fibres (Porayko & Smith, 1968). Those secondaries next to the primary ending may take the form of rings and spirals (Gladden, 1969).

Sensory terminals are devoid of Schwann cells, and enclosed by the basal lamina of the intrafusal fibres. Basal lamina is absent from the junctional region between the sensory terminal and intrafusal fibre (Merrillees, 1960; Landon, 1966b).

1.5 Motor innervation:

According to Porayko & Smith (1968), each muscle spindle in rat lumbrical muscles, receives two motor axons; one innervating the bag fibres, the other innervating the chain fibres. They obtained no evidence for overlapping of the innervation between chain and bag fibres. Ovalle (1972b), on the other hand, has shown that two fusimotor axons innervate the opposite poles of the spindle, each of which is supplied by an additional one or two fusimotor axons.

It is generally agreed that in muscle spindles of cat and some other mammals, there are three types of fusimotor ending, namely: (1) small P_1 plates derived from collaterals of β axons which also

innervate extrafusal fibres; (2) large P_2 plates of γ axons; and (3) multiple γ trail endings (Barker, 1974). In rat lumbrical muscles, two types of fusimotor ending have been reported by Porayko & Smith (1968); one resembles the plate ending, the other is a fine single filament. However, the three types of fusimotor ending were described in rat tail muscles by Gladden (1969). There are conflicting reports about the distribution of these fusimotor endings on different intrafusal fibre types in rat spindles (for review, see Smith & Ovalle, 1972; Barker, 1974). In lumbrical muscles, smaller plates (presumably P_1) are according to Mayr (1969) localized on chain fibres, whereas the larger plates (P_2) terminate on bag fibres. The reverse is stated by Ovalle (1972b). Other workers (Karlsen, 1965; Porayko & Smith, 1968) suggest that the distribution is non-selective, but predominantly to bag fibres as in cat and rabbit spindles. The distribution of trail endings appears to be non-selective as well, although they seem to be predominantly on bag fibres (Mayr, 1969).

The P_1 plates are confined to the polar regions of intrafusal fibres and have nucleated sole-plates and a discrete subneural apparatus. The P_2 plates are without a nucleated sole-plate and found in the juxta-equatorial region, where the trail endings are also located (Gladden, 1969).

The fusimotor terminals are unlike the sensory terminals in that a basal lamina always interposes between the terminal and the muscle cell, and they are covered by Schwann cells.

CHAPTER 2: The effects of experimental lesions on muscle spindles

The examination of muscle spindles subjected to different experimental lesions is essential for a full understanding of their structure and function in the normal animal. Apart from pathological abnormalities (for review see Adams, Denny-Brown & Pearson, 1962; Cazzato & Walton, 1968; Patel, Lalitha & Dastur, 1968), several workers have studied muscle spindles subjected to different experimental lesions. Total and differential denervation gained special interest amongst these techniques as they gave some insight into the degree of dependency of muscle fibres on their nerve supply.

2.1 Nerve injury:

2.1.1 Total denervation:

Some light-microscopic investigations of the effects of peripheral nerve sectioning on muscles suggested that intrafusal fibres are unaffected (Sherrington, 1894; Batten, 1897; Sunderland & Ray, 1950; de Reuck, van der Eecken & Roels, 1973). In contrast to this, other studies suggested that parallel effects are seen in both intrafusal and extrafusal fibres (Onanoff, 1890; Tower, 1932; Chor, Dolkart & Davenport, 1937; Gutmann & Zelená, 1962). The general view however, is that degenerative changes take a longer time to appear in intrafusal fibres than in extrafusal fibres, presumably due to their small diameter (Adams, et al., 1962).

During long-term denervation, intrafusal fibres undergo slow and gradual atrophy. This includes a decrease in diameter, a loss of cross-striations, and shrinking of the myonuclei. These changes are

accompanied by thickening of the capsule and a reduction in the volume of the periaxial space (Tower, 1932).

2.1.2 Differential denervation:

In order to demonstrate how the components of a peripheral nerve share the responsibility for maintaining the trophic condition of the muscle spindle, several studies have been made in which either the motor or the sensory nerve supply were eliminated.

Following ventral-root section, degenerative changes are confined to the polar region of the muscle spindle (Tower, 1932; Boyd, 1962; Banker & Girvin, 1972). Nuclear-chain fibres were found to be more affected than nuclear bag fibres (Boyd, 1962; Maynard & Tipton, 1971). The motor axon terminals retract and become surrounded by Schwann cells which replace the endings (Banker & Girvin, 1972).

On the other hand, removal of the dorsal-root ganglia seems to affect the equatorial region only. The characteristic equatorial nucleation is lost and the fibres fill with myofibrils presenting morphological and histochemical properties similar to those of the polar region (Boyd, 1962; Kucera, 1980). Again, the nuclear-chain fibres are the first to be converted, followed by the nuclear-bag fibres (Boyd, 1962). Unlike normal bag fibres, the de-afferented bag fibres usually do not demonstrate regional staining variability with alkali ATPase along the fibre length (Kucera, 1980).

Muscle spindles appear normal after sympathetic denervation (Tower, 1932).

2.1.3 Regeneration of muscle spindles in reinnervated muscles:

The effect of temporary nerve injury on adult muscle spindles has recently been examined (de Reuck, et al., 1973; Ip & Vrbová, 1973;

Schröder, 1974a, b; Brown & Butler, 1976; Ip, Vrbová & Westbury, 1977; Schröder, Kemme & Scholz, 1979; Barker & Boddy, 1980) to determine the degree of restoration of intrafusal muscle fibres and their neural connections.

Reinnervation follows denervation caused by temporary nerve injury. Following sciatic nerve cut or crush in rat, atrophy of intrafusal muscle fibres is rarely encountered and is insignificant compared with that of the extrafusal fibres in the denervation phase, although both types show the same histochemical changes (de Reuck et al., 1973; Schröder, 1974a). Atrophic intrafusal fibres are filled with flecked vesicles or small mitochondria (Schröder, 1974a). There is a striking increase in the number of intrafusal fibres in some reinnervated muscle spindles. This increase is caused by denervation rather than reinnervation since it is also observed after permanent denervation (Gutmann & Zelená, 1962; Schröder, 1974a; Arendt & Asmussen, 1976; Schröder et al., 1979).

Reinnervation and recovery of function is fairly complete in cat muscle spindles after nerve crush (Ip & Vrbová, 1973; Brown & Butler, 1976; Ip et al., 1977). The sensory innervation of cat muscle spindles is restored less successfully and more slowly than the motor. Sensory and motor nerve endings in rat muscle spindles are well reconstituted following nerve crush (Schröder et al., 1979). Considerable hyper-innervation, especially in the early stages of reinnervation was noted in rat and cat muscle spindles (Brown & Butler, 1976; Schröder et al., 1979; Barker & Boddy, 1980).

2.2 Muscle injury:

The techniques used to initiate the degeneration of skeletal muscle are many and varied. The crudest method, that of muscle mincing

and implantation (e.g. Carlson, 1972), has been refined to produce techniques which achieve the degeneration of almost all of the muscle fibres in the muscle.

2.2.1 Muscle grafting:

This technique is carried out by sectioning the muscle tendons and neurovascular supply and then reconnecting the muscle at either the same site (orthotopic graft) or at the site of another muscle (heterotopic graft). In autografted soleus muscles of rat, Schmalbruch (1977) found muscle spindles in almost all regenerates. The spindle's capsule survives the damage but intrafusal fibres undergo necrosis and phagocytosis like extrafusal fibres. Regeneration of intrafusal fibres may be initiated by satellite cells. These muscle spindles however, lack sensory and motor innervation and distinction between nuclear-bag and nuclear-chain fibres is not possible (Schmalbruch, 1977). Zelená & Sobotková (1971), attributed the failure of muscle spindles to regenerate in minced muscle grafts to the destruction of their capsules and to an asynchrony in the time of the arrival of the reinnervating Ia afferents at the regenerating myotubes. This has been recently challenged in another light-microscopic study (Mackenson-Dean, Hikida & Frangowlakis, 1981) where muscle spindles are reported to form de novo in pigeon muscles lacking muscle spindles grafted into sites of muscles that normally contain spindles. The authors conclude that the generation of spindles in the grafted muscles suggests strongly that their development here does not require the presence of a capsule to act as a template.

The regeneration of muscle spindles in grafted muscles has been observed even where reinnervation of the graft was prevented by denervation (e.g. Rogers & Carlson, 1981). They found a considerable

reduction in the number of muscle spindles which they associated with the satellite-cell death in the centre of the graft following prolonged ischaemia. In the reinnervated grafts, Rogers & Carlson noted an increase in the number of nerve axons associated with the muscle spindles, but their conclusions were limited by the absence of any observations of the fine structure of the regenerated muscle.

2.2.2 Injection of local anaesthetics and toxins:

Following the injection of snake venom toxins in rat soleus muscles, most of the extrafusal muscle fibres degenerate (Harris & Johnson, 1978), but even in those muscles totally destroyed by the toxin, the intrafusal muscle fibres, when viewed with the light microscope, appear to survive. Their structural integrity is maintained and this resistant property is shared by immature extrafusal muscle fibres, blood vessels and satellite cells (see Harris, Johnson & MacDonnel, 1980). No observations have been made of the effects of the toxin on the sensory or motor nerve terminals of the spindle.

This resistance of the intrafusal muscle fibres contrasts markedly with their susceptibility to damage following the administration of other myotoxic agents. Milburn (1975, 1976) found that intrafusal muscle fibres degenerate and subsequently regenerate in the same manner as the surrounding extrafusal fibres following the intramuscular injection of bupivacaine (Marcain) into peroneus longus of rat. The sensory nerve terminals of the primary afferent axon are also destroyed by bupivacaine so that the intrafusal muscle fibres at least begin to regenerate in the absence of afferent innervation. Sensory innervation of the regenerating axial bundle is incomplete. The motor nerve terminals of the muscle spindle, like those of the extrafusal muscle fibres (Jirmanová, 1975) remain ultrastructurally intact, and

reinnervate the axial bundle by the end of the third week, when extrafusal neuromuscular junctions are also restored (Jirmanová & Thesleff, 1972; Jirmanová, 1975). Most regenerated muscle spindles differ from normal in two main aspects. Firstly, equatorial bags and chains of nuclei do not regenerate so that one of the classical distinguishing features of nuclear-bag and nuclear-chain fibres is lost. Milburn (1976) considered this to be related to the second main difference, that being the abnormalities in the sensory innervation of the regenerated spindles. Duchen & Queiroz (1981) have noted that the potent black widow spider venom acts on sensory nerve terminals causing degenerative changes similar to those at extrafusal motor end-plates, though delayed for several hours. Regeneration of sensory terminals rapidly follows and one week later well-formed annulospiral endings are seen.

2.2.3 Ischaemia:

The effects of ischaemia on skeletal muscles have been extensively investigated using different experimental techniques. Some of these techniques include the ligation of the abdominal aorta alone or in combination with the ligation of the supplying blood vessels to the experimental muscle (Brooks, 1922; Karpati, Carpenter, Melmed & Eisen, 1974; Janda, Linhart & Kasalicky, 1974; Hanzliková & Schiaffino, 1977; Hanzliková & Gutmann, 1979; Bass, Gutmann, Hanzliková & Teisinger, 1979). Ligation of the local blood supply to the experimental muscle alone has also been used (Allbrook & Aitken, 1951; Scully, Shannon & Dickersin, 1961; Reznik & Hansen, 1969; Kaspar, Wiesmann & Mumenthaler, 1969; Reznik & Engel, 1970; Letts & Sobrie, 1970; Kayaalp, Kucera & Smith, 1972; Rosenthal, 1972; Urbanová, Janda, Mrhová & Linhart, 1974; Hall-Craggs, 1978; Jaya, 1980; Ludatscher, Hashmonai, Monies-Chass & Schramek, 1981).

Other techniques used include the application of tourniquets or clamps around the thigh of the animal (Macfarlane & Spooner, 1946; Harman, 1947; Harman & Gwinn, 1949; Moore, Ruska & Copenhaver, 1956; Dahlbäck & Rais, 1966; Mäkitie & Teräväinen, 1977a). Damage to the nerve supply was difficult to avoid using some of these techniques.

Rapid degeneration followed by regeneration is observed after temporary ischaemia and is comparable to that in muscle grafts. The severity of degenerative changes is determined by the duration of the ischaemia. Mitochondrial alterations are the first sign of degeneration and begin as early as one hour after devascularization (Hanzliková & Schiaffino, 1977). Irreversible changes such as disappearance of the Z-line and disruption of cell membrane follow. Autolysis and phagocytosis of the remnants of the affected fibres are seen on the fourth day. At this time, regeneration, which follows revascularization, begins, indicated by the presence of activated satellite cells and young myotubes (Karpati et al., 1974; Mäkitie & Teräväinen, 1977b). Most of the muscle fibres regenerate within eighteen days, though morphological alterations such as variation in fibre size, split fibres and central nuclei are still observed 226 days after ischaemia (Mäkitie & Teräväinen, 1977a).

All of these observations were confined to extrafusal muscle fibres and no histological study of the effects of temporary ischaemia on intrafusal muscle fibres was found in the literature.

2.2.4 Disuse:

Immobilization has been studied by placing the legs of experimental animals within plaster casts, or by tenotomizing the muscles. Generally, these techniques cause atrophy of the muscle which is comparable to that seen after denervation (Herbison, Jaweed & Ditunno, 1978, 1979).

Tenotomy however, does not significantly affect the functional and morphological features of muscle spindles (Sunderland & Lavarack, 1959; Hnik, Beránek, Vyklicky & Zelená, 1963). Within one week after leg immobilization in a cast, the primary endings of rabbit muscle spindles show signs of degeneration, the capsules become swollen and cross-striations in intrafusal muscle fibres grow indistinctly (Esaki, 1966).

CHAPTER 3: The present study

3.1 Justification

The study of experimentally-induced regeneration of skeletal muscles, is of obvious importance for the understanding of various neuromuscular diseases and for reconstructive surgery. Recently, this field of research has gained increasing interest, and different techniques have been tried in the search for an effective experimental model for the degeneration-regeneration process in skeletal muscles (see chapter 2, 2.2). The complexity of the effects of the different techniques used to initiate muscle degeneration has been the subject, in part, of a major symposium (Muscle regeneration; Ed. A. Mauro, 1979). Initially, little attention was paid to the muscle spindles in regenerating muscle, but in recent years this void has been recognized and interest in this area renewed (Zelená & Sobotková, 1971; Milburn, 1976; Rogers & Carlson, 1981; Mackenson-Dean et al., 1981).

As noted before (chapter 2; 2.2.1), muscle spindles do not regenerate in minced muscle grafts (Zelená & Sobotková, 1971; Carlson, 1973). For those wishing to investigate the processes involved in muscle-fibre degeneration and regeneration, muscle grafting has the obvious disadvantage of disturbing the neuromuscular connections (see review by Carlson, Hanson-Smith & Magon, 1979). Thus by definition, the free muscle graft is denervated at the time of transplantation, and random reinnervation is inevitable, thus adding an uncontrollable factor to the model. On the other hand, the disadvantage of the myotoxic model lies in the fact that not all extrafusal muscle fibres and spindles are affected, and the effect is not uniform throughout the length of the individual intrafusal muscle fibres (Milburn, 1976).

Temporary ischaemia has been shown to cause the degeneration and

regeneration of extrafusal fibres (e.g. Karpati et al., 1974). The cause of the initial degeneration on which the transplant model (Hall-Craggs, 1978), and possibly the myotoxic model (Hall-Craggs, 1974), depends is ischaemic in origin, and these models are mimicked by devascularization of the muscle (Hall-Craggs, 1979). It was therefore considered that the use of a technique that produced temporary ischaemia might well achieve the same phenomenon in muscle spindles. The advantage of the ischaemic model is that the innervation is left intact, although some temporary peripheral damage is inevitable. Reinnervation of the muscle spindle is therefore much more easily controlled, and the model affords the opportunity either to denervate the regenerating spindle completely or to remove its motor or sensory supply by differential denervation.

3.2 The technique used:

Two important factors were borne in mind during the preparation of this study; firstly, that temporary ischaemia should be achieved with the least possible disturbance to the neuromuscular connections; and secondly, that all of the muscle spindles (and if possible extrafusal fibres) should be affected. With these considerations, several experimental techniques, which have been described before (chapter 2; 2.2.3), were excluded for different reasons.

The most common approach, i.e. ligature of the abdominal aorta (e.g. Karpati et al., 1974) also affects the arteries that supply the lower segments of the spinal cord. Thus, this technique could also result in secondary neurogenic damage to the muscle under study (Mäkitie & Teräväinen, 1977a). Ligature of the femoral or the common iliac artery does not produce total ischaemia of the muscle (Blunt, 1960) and may cause unnecessary suffering in the experimental animal. Models of

ischaemia that also affect nerves or tendons (Moore et al., 1956; Stenger, Spiro, Scully & Shannon, 1962; Mäkitie & Teräväinen, 1977a), introduce additional lesions which produce multiple effects on the muscle fibres (Hanzlíková & Gutmann, 1979). A controlled and reproducible experimental model was therefore necessary to study the effects of temporary ischaemia on muscle spindles.

Hall-Craggs's (1978) procedure of muscle devascularization, without sectioning the supplying nerves, was chosen so that the optimum conditions for revascularization and reinnervation could be preserved and be comparable between individual experiments. Hall-Craggs additionally ligated the tendons and soaked the muscle with bupivacaine to eliminate the surviving fibres at the muscle periphery. Serial transverse sections of soleus muscle (Fig. 6.1), showed that spindles are distributed throughout the muscle, and are not confined to the axial fasicles as in gastrocnemius, plantaris and tibialis anterior (Yellin, 1969). Several extrafusal muscle fibres overlie the most superficial spindles. The supplementary lesions of Hall-Craggs were therefore not considered essential to this study.

3.3 The animal used:

Rat was chosen as the experimental animal for the following reasons; firstly, the animal is readily available and easily bred; secondly, more information on muscle degeneration was already available for rat muscles than for any other experimental animal; and thirdly, the effects of ischaemia had been studied in rat extrafusal fibres, but not in muscle spindles.

Therefore, it was considered appropriate to fill this void, so that the effects of ischaemia on spindles could be compared with those

caused by other techniques.

The soleus muscle was chosen because its size is suitable for electron-microscopic techniques and because it had already been used by several investigators (Karpati et al., 1974; Hanzliková & Schiaffino, 1977; Hanzliková & Gutmann, 1979). In addition, the damage caused by ischaemia is much more severe in soleus muscle than in others (e.g. gastrocnemius); this may be related to the metabolic differences between tonic and phasic muscles (Karpati et al., 1974).

The one disadvantage of using rat muscles however, is the considerable confusion surrounding the extent, distribution, and morphology of the spindle's innervation (see chapter 1; 1.4 & 1.5). However, the advantages by far outweighed this single disadvantage.

3.4 Plan of study:

In all, five series of animals were used (Fig. 3.1). The following abbreviations are used for each series; Isch (Ischaemia), Dn (Denervation), Isch/Dn (Ischaemia + Denervation), Isch/Rn/Dn (Ischaemia followed by denervation after 28 days), and N (Normal).

3.4.1 Series Isch:

The main purpose of this series was to investigate the effects of temporary ischaemia on the muscle spindle. Spindles were observed in rat soleus muscles at intervals varying from 30 minutes to 182 days following devascularization of the muscle and from these observations the process of spindle degeneration and subsequent regeneration is described.

3.4.2. Series Dn:

In a second series, soleus muscles were permanently denervated by

sectioning the sciatic nerve using a technique that prevented reinnervation. Muscle spindles were observed at frequent intervals thereafter. This series therefore, separated out from series Isch any features of the degenerating and regenerating axial bundle that may have resulted from the peripheral damage to the spindle's innervation, caused by devascularization of the muscle.

3.4.3 Series Isch/Dn:

A third series of experimental animals was devised to elucidate the role of the spindle's innervation in the regeneration of the intrafusal fibres that follows temporary ischaemia. Soleus muscle was thus devascularized and frankly denervated at the same time, using a technique that allowed revascularization of the muscle, but prevented reinnervation. Muscle spindles were observed at frequent intervals following the combined lesions.

3.4.4 Series Isch/Rn/Dn:

This series of experimental animals was devised as a consequence of the results obtained from series Isch and Isch/Dn, and was aimed at comparing the properties of regenerating and developing intrafusal muscle fibres.

Intrafusal muscle fibres are generally totally dependant on their innervation for their formation and survival for a short period of time during muscle-spindle morphogenesis. Denervation of the muscle at this time results in the degeneration of the intrafusal muscle fibres, which then fail to regenerate (Zelená, 1957; Zelená & Hnik, 1960). In this experimental series, the regenerating intrafusal muscle fibres were denervated at a time when reinnervation of the regenerating muscle spindles had just begun (c. 28 days following ischaemia). Muscle

spindles were then observed at frequent intervals following their denervation.

3.4.5. Series N:

This series consisted of the observation of muscle spindles of soleus muscle of normal unoperated animals, and from soleus muscle of the contralateral limb of operated animals.

P A R T I I M E T H O D S

CHAPTER 4: Materials and methods

4.1 Number of animals used:

Adult male albino rats of the Wistar strain (initial weight 170-280g) were used in the study. Initially, ten rats were used in the development of the technique to achieve temporary ischaemia. A total of seventy eight animals was then used in four series of apparently successful experiments. They were divided into groups of 49, 8, 15 and 6, for series Isch, Dn, Isch/Dn and Isch/Rn/Dn respectively. At least two animals were used for each time period of the four series (see Table 4.1 for details). In addition, ten unoperated rats were used in the study of the normal muscle spindle (series N).

4.2 The muscle used:

Soleus is a flat muscle lying beneath the plantaris (Fig. 4.1). It originates by fleshy fibres from the lateral surface of the head of the fibula. It inserts by means of a slender tendon which unites with that of the gastrocnemius muscle to form the calcaneal tendon. It is innervated by a branch of the tibial nerve. Its blood supply is derived from the femoral artery through the external sural and fibular branch of the peroneal artery (Greene, 1959). The pedicle, which contains the nerve and blood supply, is connected to the middle third of the muscle.

4.3 Operating procedures:

Rats were anaesthetized with sodium pentobarbital (Nembutal),

Table 4.1

Number of rats and muscle spindles used in the experimental studies

Series / Time	Isch		Dn		Isch/Dn		Isch/Rn/Dn	
	R	Sp	R	Sp	R	Sp	R	Sp
30 min	2	3						
1 h	2	1						
2 h	2	2						
3 h	2	2						
6 h	2	3						
12 h	2	1						
1 d	4	2						
2 d	2	2			2	2		
3 d	2	3						
4 d	2	4						
5 d	4	3			2	2		
6 d	2	2						
8 d	2	4			2	3		
14 d	3	4			2	1	2	2
21 d	2	3	2	1	2	2		
28 d	5	9			2	2	2	1
42 d	2	6						
56 d	3	6	2	2	3	3	2	1
119 d	2	1						
156 d			2	1				
182 d	2	3						
364 d			2	2				
Total	49	64	8	6	15	15	6	4

Total no. of rats: 78

Total no. of spindles: 89

R : number of rats

Sp : number of spindles

min : minute

h : hour

d : day

Isch : Ischaemia

Dn : Denervation

Isch/Dn : Ischaemia + Denervation

Isch/Rn/Dn : Ischaemia + Denervation 28 days later

5 mg/100 g being injected intraperitoneally. Four types of operation were carried out using the Zeiss operating microscope and under aseptic conditions. All the operations were performed on the right hindlimb of the animals.

4.3.1 Series Isch:

An incision 2 cm long was made on the medial surface of the leg. Soleus muscle was exposed by lateral deflection of gastrocnemius and plantaris. It was then freed from its surroundings, except for its tendons and neurovascular pedicle, which enters at the junction of the middle and distal thirds of the muscle. The nerves and blood vessels in the pedicle were carefully dissected from each other for 1 cm proximal to the muscle, and all blood vessels encountered were cut. Great care was taken not to disturb the nerve supply. Furthermore, both tendons of the muscle were freed from the surrounding tissues up to their attachments, and all blood vessels passing through them were interrupted. By then, the light red colour of the muscle had changed rapidly to become deep red. The slight bleeding around the muscle was then cleared with cotton wool soaked in Ringer solution. After making sure that the bleeding had stopped, the wound was sutured in two layers, sprayed with a surgical plastic dressing (Nobecutane, BDH Ltd.), and dusted with sulphamethoxypridazine (Lederkyn, Cyanamid of Great Britain Ltd.).

4.3.2. Series Dn:

In this series of animals, the right sciatic nerve was approached by an incision 1 cm long made on the lateral surface of the thigh parallel to the femur. The surrounding muscles were deflected by a surgical clamp, and the nerve was freed over a length of 1 cm. Then

the nerve was sectioned with fine scissors. In order to prevent reinnervation, the proximal stump of the nerve was sutured under the skin. No bleeding was caused by this operation. The wound was then sutured and treated as in the first series.

4.3.3 Series Isch/Dn:

Animals of this series were operated as those in series Isch, but the whole neurovascular pedicle was cut. Additional denervation of the muscle was carried out at the same time, by sectioning the right sciatic nerve as in series Dn. The wounds of the two operations were then sutured.

4.3.4 Series Isch/Rn/Dn:

Similar operations to those of series Isch were carried out in the animals of this series. They were left for a period of 28 days, when reinnervation of muscle spindles was presumed to have taken place. The sciatic nerve of the previously operated limb was then sectioned and reinnervation prevented.

Only a few rats were lost during the operation mainly because they did not survive the anaesthesia. They were replaced by others. Infection was not recorded. The operated animals received the normal laboratory diet. Animals of series Isch and series Isch/Rn/Dn (before the second operation), moved without difficulty after the operation. Those of series Dn, Isch/Dn and Isch/Rn/Dn (after the second operation), did not use the operated limb in their movements.

4.4 Post-operative periods:

Rats were killed at different time intervals after the operation (Table 4.1). Those of series Isch were killed at 30 min. and 1, 2, 3, 6

and 12 hrs., and at 1, 2, 3, 4, 5, 6, 8, 14, 21, 28, 42, 56, 119 and 182 days after the operation. Animals of series Dn were killed on the 21st, 56th, 156th and 364th post-operative day. Those of series Isch/Dn were killed after 2, 5, 8, 14, 21, 28 and 56 days. Animals of series Isch/Rn/Dn were killed at 14, 28 and 56 days after the second operation. Normal muscles (series N) were taken from the contralateral side of all operated animals, and from both sides of the ten unoperated animals.

4.5 Methods for electron microscopy:

Soleus muscles of both sides were removed from the operated and normal animals under anaesthesia. The animals were killed later by overdose injection of the anaesthetic. All the specimens were treated in a similar manner as follows:

- Fixation:

The muscle was stretched slightly on a piece of cardboard and immersed immediately in Karnovsky (1965) fixative (as modified by M. Saito, personal communication) at 4°C. The fixative was prepared as follows:

2 g Paraformaldehyde
40 ml Distilled water ... Solution A

The paraformaldehyde was heated and dissolved with 2-6 drops of 1N.NaOH with continuous shaking.

10 ml 25% Glutaraldehyde
50 ml 0.2M Cacodylate buffer pH 7.3 ... Solution B

The two solutions were freshly prepared and cooled in a refrigerator, and mixed just before use. After 30 min., the muscle was cut into thin slices, parallel to the long axis of the muscle fibres, and placed in fresh fixative for another 90 min. in the refrigerator.

- Post-fixation:

The specimens were postfixed for 2 hrs. in the following solution in the refrigerator:

25 ml 4% Osmium tetroxide

25 ml Distilled water

50 ml 0.2M Cacodylate

- Dehydration:

The material was dehydrated at room temperature through a series of 50%, 70%, 95% and absolute alcohol, with three changes at each stage, and for a total of 20 min. at each concentration.

- Embedding:

The material was then embedded in Araldite using the following procedure:

50 ml Absolute alcohol
50 ml Propylene oxide — 30 min.

Propylene oxide 30 min. with three changes

50 ml Propylene oxide
50 ml Araldite — 30 min. at 45°C

The material was then placed in Araldite, which was changed after 30 min., and left overnight in a 45°C oven and finally transferred to a 60°C oven for two days.

4.6 Sectioning and staining techniques:

All material was sectioned on a Reichert OMU3 ultra-microtome, using glass knives. Thick (approx. 1 µm) transverse sections, stained with toluidine blue in 1% borax were scanned with a light microscope for muscle spindles. Once located, the spindle was serially cut in

alternate thin (usually ten sections of silver-to-gold interference colour) and thick sections (covering a length of 20 or 50 μm from the equatorial or polar regions of the spindle, respectively). By this technique of alternate electron- and light-microscopic examination of serial transverse sections, a complete picture of the spindle could be gained.

For longitudinal sectioning, the spindle was first located in transverse thick sections, the block was then re-orientated through 90° and thick sections were cut and examined until the spindle capsule was visible. The intrafusal muscle fibres were then serially-sectioned for electron-microscopic study.

Thin sections were doubly stained with uranyl acetate followed by lead citrate (Reynolds, 1963) and subsequently examined and photographed with an AEI 801 electron microscope. Thick sections stained with toluidine blue were photographed using a Microflex UFX camera mounted on Nikon Biological microscope OPTIPHOT.

PART III RESULTS

CHAPTER 5: Normal muscle spindles

Much of the description that follows of the ultrastructure of the spindle components confirms the observations made by Merrillees (1960), Landon (1966 a, b), Ovalle (1971, 1972 a, b) and Milburn (1973) in rat spindles, and those in other species (see Barker, 1974). For the sake of brevity, detailed acknowledgement has been ommitted.

5.1 Intrafusal muscle fibres:

In spindles from rat soleus muscle, the usual number of intrafusal fibres is four with the combination of one bag_1, one bag_2, and two chain fibres. Only a few spindles contain five intrafusal fibres. At the equator, the bag_2 fibre has the largest diameter, the bag_1 fibre is of a medium diameter, and the chain fibres are the thinnest. These diameter differences are not always maintained, particularly at the poles where chain fibres may become as big as bag fibres (Fig. 5.1). The bag_2 fibre is the longest, the bag_1 fibre is slightly shorter, and chain fibres are the shortest. Both bag_2 and bag_1 fibres extend far beyond the ends of the capsule, but chain fibres usually end within its limits. Elastic fibrils are more abundant around the bag_2 fibre than the bag_1 and chain fibres.

Subsarcolemmal nuclei and occasional central myonuclei are present in the polar regions of all intrafusal fibres. As in cat spindles (Banks, 1981), satellite cells can also be seen in all regions, and these are more associated with bag fibres than chain

fibres. A chain of myonuclei is present in the equatorial region of the chain fibres, whereas bag fibres have a bag of myonuclei which contains 3-4 and 2-3 nuclei abreast in bag_2 and bag_1 fibres, respectively. The nuclei of the chain and bag are surrounded by sarcoplasm rich in organelles bounded by an outer rim of myofilaments (Fig. 5.2).

Mitochondria are large and numerous in chain fibres and in longitudinal section some may extend over three sarcomeres. In the bag_2 fibre, they are smaller and fewer than those in chain fibres. In the bag_1 fibre, they are usually the same as in the bag_2 fibre, but in some regions they appear as big as those in chain fibres.

In the polar region of chain fibres, the myofibrils are discrete with abundant interfibrillar sarcoplasm, containing numerous glycogen granules. In the bag_2 fibre, they are tightly packed with little interfibrillar sarcoplasm, containing few glycogen granules. In the bag_1 fibre, the myofibrils are usually as in the bag_2 fibre, but in some regions they are discrete as in chain fibres.

The sarcoplasmic reticulum is more developed in chain fibres than in both types of bag fibre, although it is more abundant in the bag_1 compared with the bag_2. Junctional couplings of the sarcoplasmic reticulum and transverse tubule system, usually in the form of triads, are frequently encountered in chain fibres and very sparse in bag fibres. Triads are, however, more numerous in the bag_1 than bag_2 fibre. In all types of intrafusal fibre, dilated cisternae of sarcoplasmic reticulum have been observed, apposed to both the sarcolemma and mitochondria. They are more numerous in chain fibres than in bag fibres. Micropinocytotic vesicles are commonly seen beneath the sarcolemma in all intrafusal fibres. Microladders can occasionally

be seen at the periphery of chain fibres especially near the sensory terminals.

The chain fibres possess a distinct M-line in all regions, whereas the bag_2 fibre has a distinct M-line in the polar region but lacks a distinct M-line in the equatorial region. The bag_1 fibre lacks a distinct M-line for most of its length. The existence of a distinct M-line in the extreme polar region of the bag_1 fibre, as reported by Kucera et al. (1978), was not confirmed in this study as no clear evidence was obtained. The Z-line is more prominent in the bag_1 fibre than in the bag_2 and chain fibres. Many of the features described above are illustrated in Fig. 5.3.

The association between bag_2 and chain fibres that is reported in cat spindles (Barker et al., 1976) was not seen in rat spindles, but a close association between the two chain fibres in the equatorial region, and a more intimate and common association between the bag_2 and bag_1 fibre was observed (Figs. 5.4, 5.5 & 5.10B). The two bag fibres become closely associated in the myotube regions where they share a common basal lamina. A satellite cell is usually present between the fibres, and it is occasionally accompanied by a subsarcolemmal nucleus in one of the muscle fibres. The association lasts for a relatively short distance, and is usually found on both sides of the nuclear bag. The association between the two chain fibres is less intimate, although they sometimes share a common basal lamina. It is also usually found on both sides of the nuclear chain. Satellite cells and subsarcolemmal nuclei are usually absent from the association zone of chain fibres. In regions where no association is present, the intrafusal fibres are well separated and each occupies a separate compartment enclosed within the axial sheath. The association of bag fibres and the presence of satellite cells between them is also

described by Maynard & Cooper (1973).

5.2 Capsule, periaxial space and axial sheath:

The capsule consists of 4-7 layers of flattened cells in the mid-equatorial region. The number of layers decreases towards the poles. The capsule layers are composed of very thin cellular sheets covered by basal lamina. Areas of close contact can occasionally be seen to bind the edges of two adjacent cellular sheets. In some regions, a pair of capsule layers lack a basal lamina on their inner surfaces, forming a channel. The capsule cells contain flattened nuclei, small mitochondria, endoplasmic reticulum, ribosomes, caveolae intracellulares, and micropinocytotic vesicles, some of which open to the surface. The narrow spaces between the capsule layers contain numerous collagen fibrils of longitudinal and circular orientation. Elastic fibrils are also present, but are fewer in number than collagen fibrils. Occasionally, fibrocytes can be seen between the capsule layers. The external surface of the capsule is surrounded by numerous collagen fibrils and, to a less extent, by elastic fibrils of varied orientation (Fig. 5.6).

The spindle nerve trunk penetrates the capsule, usually at the equator, and is enclosed by a perineurium that is composed of cells similar to those of the capsule and is continuous with them. Different types of individual axons can also be found between the capsule layers. In the equatorial region of some spindles, the innermost layer of the capsule diverges from the others and crosses the periaxial space obliquely to form one or more subcompartments within it. The periaxial space is devoid of any structures except crossing axons and occasional fibrocytes.

The axial sheath is composed of endomysial cells which are similar to the capsule cells, but they lack a basal lamina and their nuclei have an irregular oval shape. The cytoplasmic sheets are thinner and contain fewer clear vesicles and caveolae. Vesicles filled with fine dense granules are more numerous than in the capsule cells. The cytoplasm around the nucleus is rich with organelles such as ribosomes, endoplasmic reticulum, Golgi apparatus, and occasional centrosomes. As in the capsule cells, the edges of the endomysial cells are joined together by areas of close contact, but form a less elaborate envelope than the capsule layers. In the mid-equator, each intrafusal fibre has a separate compartment. At the end of the myotube region where this envelope approaches its termination, it becomes even looser and surrounds more than one intrafusal fibre. Areas around the intrafusal fibres and among the endomysial cells contain loose strands of basal lamina, myelinated and unmyelinated axons, preterminal axons, elastic and collagen fibrils (Fig. 5.7).

Small arterioles and capillaries are usually few in number and are found between the outer layers of the capsule or close to it, but were not seen in the periaxial space or within the axial sheath.

5.3 Sensory terminals:

As noted earlier (chapter 1; 1.4), all rat spindles possess one primary ending, and in addition some spindles contain one or two secondary endings. In cross sections of the equatorial region, most terminals of the primary afferent have the shape of half or incomplete rings. They are distributed to all intrafusal fibres. Other terminals are lenticular or triangular in outline and are bounded by lips of

sarcoplasm. Round terminals can also be seen, either partly or completely enclosed by the lips depending on the extent to which they indent the muscle fibre. Terminals situated at the juxta-equatorial region of the bag_2 and chain fibres are of shapes other than incomplete rings. They may represent the terminals of secondary afferents or the irregular tips of the primary ending. The bag_1 fibre is usually devoid of any sensory terminals in this region.

In longitudinal sections, the sensory endings appear as a series of convex studs rising at intervals from the surface of the muscle fibre. They lie in gutters of varying depth and are bounded by lips of sarcoplasm. Occasionally, some are completely enclosed by these lips. In some sections, the terminals appear as bands crossing the width of the muscle fibre.

Sensory terminals are not covered by Schwann cells, but the basal lamina of the muscle fibre is continuous over their free surfaces. The adjacent cell membranes of the terminal and muscle fibre are separated by a gap of at least 10 nm that is devoid of basal lamina. The axolemma of the terminal has a corrugated appearance which is not seen in motor terminals. As described by Landon (1966b), the axoplasm of sensory terminals has an amorphous or finely fluffy appearance, sometimes with a central clear zone containing a skein of neurofilaments. The terminals however, have fewer neuro-filaments than the parent axons. The mitochondria are dense, numerous and unevenly distributed. Few small vesicles of 25-250 nm diameter some of them with dense cores are present. Fine tubules similar to those reported by Merrillees and Landon can also be seen. In addition, sensory terminals occasionally contain one or more myelin figures. In the region where bag_2 and bag_1 fibres are associated or move close to one another, a sensory terminal on one muscle fibre is commonly seen to

cross by a narrow, short band of axoplasm to another terminal on the other muscle fibre. Such cross terminals were not seen between two associated chain fibres; instead only a common sensory terminal is present. Many of the above observations are illustrated in Figs. 5.2, 5.8-5.10.

5.4 Motor terminals:

Twenty-two motor terminals from four spindles were examined with the electron microscope. Every intrafusal fibre receives at least one motor terminal in one pole or the other. The terminals are classified on the basis of the type of intrafusal fibre they innervate.

Since chain fibres usually terminate within the capsule limits, their motor terminals are situated in either the juxta-equatorial or sleeve region. The plasma membranes of the motor terminal and the muscle fibre are separated by a synaptic cleft of 40-150 nm wide. All motor terminals on chain fibres have junctional folds and sole-plates, which is not always the case in those on bag fibres. Sole-plates are well developed, containing one or more nuclei, accumulations of mitochondria, ribosomes, rough and smooth endoplasmic reticulum, and different types of sarcoplasmic vesicles. Some of the small round vesicles, of 40-110 nm diameter, have dense cores, but most have clear contents. Larger clear vesicles of about 100 nm diameter have an oval or flat shape. Round large vesicles of 200-500 nm diameter with granular dense cores are also present. Sole-plate vesicles however, are much less numerous than those in the motor terminals. One or two myelin figures can occasionally be seen. The sole-plates of some junctions contain several myofilaments running at right angles to the

sarcomeres. The junctional folds are shallow and unbranched and often difficult to recognize in low-power electron micrographs. Motor terminals lie in shallow troughs or sit on the surface of the muscle fibre. They appear to have a greater surface area of contact with the chain fibre than those innervating the bag fibres (Fig. 5.11).

Two kinds of motor end-plates on bag_1 fibres were identified (Fig. 5.12). Firstly, those situated in the juxta-equatorial region with synaptic clefts of 60-140 nm wide lack junctional folds. Three out of five of the junctions had post-junctional specializations of the sarcoplasm. Secondly, one end-plate was found in the extra-capsular region, with a synaptic cleft 40-75 nm wide. It had relatively deep unbranched junctional folds and a well-developed sole-plate with a nucleus. It lay in a trough on the surface of the muscle fibre.

Motor end-plates on the bag_2 fibres are situated in the juxta-equatorial and sleeve region. No terminals were seen in the extra-capsular polar region. The synaptic clefts range between 20-140 nm in width. They generally lack junctional folds or have few hardly recognizable ones. Some have a poorly developed sole-plate (sometimes with a nucleus), others lack any subneural specialization (Fig. 5.13).

The ultrastructure of the spindle's motor nerve terminals seems to be the same regardless of the type of intrafusal fibre innervated. Terminals on chain fibres however, have more numerous mitochondria than those innervating bag fibres. Motor terminals in general have fewer mitochondria than sensory terminals, but more vesicles. These vesicles are of different types, the most abundant being the small

clear vesicles of 20-80 nm diameter. A few small vesicles have moderately dense cores. Some larger vesicles (120-190 nm in diameter) with clear or dense cores can also be seen. An occasional multi-lamellated structure containing very small vesicles (20 nm in diameter) and larger ones (40-100 nm in diameter) is present. The external surface of all motor terminals is covered by a Schwann cell or Schwann-cell processes and the basal lamina of the muscle fibre passes through the synaptic cleft.

CHAPTER 6: General effects of temporary ischaemia on extrafusal muscle fibres

Following devascularization, the colour, size and texture of soleus muscle alters. Its normal light red colour changes to deep red, pink and cream before returning to the normal colour on revascularization. The time course for these changes varies from animal to animal depending upon the efficiency of any ingrowing collateral circulation and the restoration of blood supply. The development of a pink colouration occurs within 30-60 minutes if the slight extramuscular haemorrhage is swabbed during the operation; if this is not done, it takes up to 2-3 days for its development. The subsequent cream colouration fades when the muscle becomes fully revascularized after 5-8 days.

The colour changes are linked with the texture changes of the muscle. The appearance of the pink colouration is accompanied by a noticeable rigidity in the muscle. Later, the muscle becomes more fragile and attaches to neighbouring tissues up to day 21 when the muscle fibres become fully regenerated. Oedema develops within the first five days, after which the muscle undergoes a gradual atrophy up to the 14th day when it is no more than half its original width. By 21-28 days, the original width is restored.

Three distinct zones of muscle fibres could be observed in transverse sections of the whole muscle examined with a light microscope four days after devascularization (Fig. 6.1), these being; (1) a peripheral zone consisting of a few (0-5) layers of apparently normal extrafusal fibres, (2) a large intermediate zone of regenerating muscle cells occupying most of the cross-sectional area and (3) a

smaller central zone consisting of degenerating muscle fibres.

The following ultrastructural changes are generally similar to those reported in literature (see e.g. Karpati et al., 1974).

6.1 0-3 days:

Normal soleus muscle consists of an homogeneous population of pleomorphic fibres characterized by distinct wide Z-lines, faint M-lines and numerous mitochondria (Tomanek & Lund, 1973).

The effects of devascularization are seen as early as 30 minutes after the operation. Aggregations of enlarged mitochondria are observed at the periphery of muscle fibres, particularly at those edges that face capillaries. Many satellite cells appear separated by a cleft from the muscle sarcoplasm. Occasional lipid droplets are also present. Apart from this the muscle fibres appear normal (Fig. 6.2). At 1-2 hours, the above signs become evident in an increased number of muscle fibres.

By 3 hours, changes in sarcoplasm and contractile material have already taken place (Figs. 6.3-6.5). The enlarged mitochondria contain dense osmiophilic bodies (Reznik & Hansen, 1969). Some of these mitochondria have cristae with an irregular orientation and with tubular or vesicular configuration (Hanzliková & Schiaffino, 1977). The cristae of other mitochondria contain dense material previously described as rod-like structures (Stenger, Spiro, Scully & Shannon, 1962; Reznik & Hansen, 1969), intracristal plates (Karpati et al., 1974) or paracrystalline inclusions (Hanzliková & Schiaffino, 1977).

Advanced degeneration was seen as early as 3 hours when myofibrils are absent from the periphery or the entire area of some fibres. Persisting myofibrils are disorganized and with no Z-lines. The

sarcotubular system is changed to vesicles, vacuoles and occasional tubules. The nuclei are pyknotic and may occupy a central position. Their chromatin is condensed and sometimes located at the periphery but separate from the nuclear membrane. The inner membrane of some nuclei shows invaginations that run deep inside the condensed chromatin. The sarcolemma is either absent or reduced to an occasional strand.

At 6 hours, the majority of muscle fibres are affected. The degree of damage varies considerably; it is slight in some fibres and severe in others, with a wide range in between. Enlarged mitochondria are numerous and contain dense osmiophilic bodies and rod-like structures (Fig. 6.6), that can also occasionally be seen in sarcoplasmic tubules close to these mitochondria (Fig. 6.7). Dense osmiophilic bodies and, to a less extent, rod-like structures may also be found in normal-sized mitochondria. Whorls of densely packed membranes and tubules are occasionally observed near a myonucleus or under the basal lamina (Fig. 6.8). Similar structures are reported by Reznik (1973) in cold injured mouse gastrocnemius muscle. Occasionally, disintegration and streaming of the Z-line can be seen in some fibres. Other fibres undergo hyaline degeneration (Fig. 6.9), and are filled with a mass of disintegrated myofilaments in which scattered mitochondria, lipid bodies and sarcoplasmic vacuoles of varying size are present.

Some fibres have completely degenerated and only remnants may be present inside the preserved basal lamina (Fig. 6.10). The basal lamina usually survives but in some places it degenerates and necrotic sarcoplasm spills into the interstitial space (Fig. 6.11, 6.12).

By 1-2 days, the degeneration process is well advanced. As described by Jirmanová & Thesleff (1972), the contractile material

undergoes a variety of changes (Figs. 6.12, 6.13); in some regions, the myofibrils retain their I- and A-filament banding but the Z-lines completely disappear. In other regions, the fibrils are broken and filaments disintegrate and are eventually removed. In some areas, short bundles of filaments are bound by preserved Z-lines. Severely-affected fibres are now more numerous than at 6 hours, and they are also demarcated by preserved basal lamina.

By 6 hours, the interstitial space is invaded by erythrocytes and various types of inflammatory cell. Bundles of dense fibrin threads are occasionally observed. Invasion of the necrotic fibres by inflammatory cells was first seen at this stage (Fig. 6.9B). By day 2, inflammation and haemorrhage have increased considerably. Numerous erythrocytes, monocytes, polymorphonucleated cells, bundles of fibrin threads and occasional platelets can be seen both outside and inside the degenerating fibres (Figs. 6.14-6.17). Many fibrin bundles are attached to the outer surface of the basal lamina tubes. The inflammatory cells and erythrocytes apparently enter through ruptures in the basal lamina.

Degenerating motor end-plates were seen as early as 6 hours (Fig. 6.18). The axon terminals retract and degenerate and become enclosed by Schwann cells which contain necrotic material. The subneural apparatus also degenerates and the preserved basal lamina, of the former synaptic folds, appears as protrusions projected towards the retracted axon terminal.

Autolysis and phagocytosis result in the complete degeneration of extrafusal fibres leaving folded tubes of basal lamina and occasional satellite cells. The extent of damage is not uniform in all muscle fibres at any one time, and various regions of an individual fibre

may behave in different ways. A few muscle fibres exhibit minimal degenerative changes even at day 3.

6.2 3-5 days:

This stage is characterized by two major events. Firstly, revascularization of the muscle begins and is generally completed by the 5th day. Secondly, regeneration of muscle fibres begins at day 3 when late degeneration is still in progress. The latter mainly concerns the removal of necrotic debris. By day 4, most of the debris has been removed. Presumptive myoblasts were first seen at day 2 (Fig. 6.15), but they are more numerous at day 3.

At day 3, the preserved tubes of basal lamina contain macrophages, fibrocytes, leucocytes and occasional erythrocytes (Fig. 6.19,6.20). Occasional nascent myotubes can also be seen in some basal-lamina tubes. In addition, presumptive myoblasts are also found at the periphery of these tubes. They contain large oval nuclei, numerous ribosomes, a few mitochondria and a poor endoplasmic reticulum (Fig. 6.21). It is not unusual at this stage to observe a mass of cytoplasm containing two or four nuclei (Fig. 6.22). Such configurations may indicate satellite-cell fusion as suggested by James (1973).

At day 4, the regenerating muscle fibres are irregular in shape, consisting of a bundle of myoblasts and nascent myotubes enclosed in the original basal lamina (Fig. 6.23). The newly-formed myotubes contain large, irregular-shaped central nuclei each usually containing one nucleolus. Myofibrils are assembled gradually so that, in nascent myotubes, the paucity of the myofibrils leads to a "patchy" appearance. Peripheral nuclei can be seen in some myotubes and may indicate that myoblasts fuse with myotubes.

Satellite cells, myoblasts and nascent myotubes are also present beneath the basal lamina of partially damaged muscle fibres (Fig. 6.24A, B). The foci of necrosis in such fibres are repaired by the activation of the neighbouring satellite cells, as noted by Reznik (1969). In Fig. 6.24A, the sarcoplasm appears to extend under the basal lamina of the damaged area.

6.3 5-21 days:

There is a striking increase in the number of capillaries at this stage (Mäkitie, 1977). By day 5, the number of erythrocytes in the interstitial space has reduced and the fibrin threads have completely disappeared but macrophages are still numerous.

Regeneration is advanced by the 5th day (Figs. 6.25, 6.26). Regenerating muscle fibres are now composed of well-formed myotubes with central myonuclei contained within the original basal lamina which have folds in many places. Occasional myoblasts and satellite cells are also present at the periphery of the myotubes. Two separate myotubes are occasionally observed to lie parallel within the basal lamina tube. Round mononuclear cells lie independently in the interstitial space. They are enclosed by a basal lamina and resemble myoblasts.

By day 14, myotubes are less numerous and are replaced by muscle fibres. However, these are smaller in diameter and have an irregular shape compared with normal fibres, and are often characterized by the retention of central nuclei (Fig. 6.27). Occasional satellite cells lie at the periphery of these fibres.

Regenerating motor end plates devoid of axon terminals were seen at day 5 (Fig. 6.28). Small nerve trunks containing Schwann-cell

processes, enclosed in a tube of basal lamina, were occasionally seen at a distance from the end-plates. Post-junctional folds are well-formed and lined with a basal lamina. The newly-formed sole-plates consist of numerous ribosomes, mitochondria, sarcoplasmic tubules, vesicles, myelin figures and one or two nuclei. Intramuscular nerve trunks were also seen at this stage (Fig. 6.29). They contain collagen fibrils and basal lamina tubes of varying sizes. Each tube contains a Schwann cell, with a nucleus, or Schwann-cell processes. The cytoplasm of these Schwann cells contains lipid droplets, myelin figures, vacuoles and a few mitochondria.

6.4 21-28 days:

By day 21, some muscle fibres are fully restored to normal diameter, but many small fibres are also present. Central nuclei are still present in some fibres. The interstitial space is reduced, but still bigger than normal.

Reinnervation was first seen at day 21 (Fig. 6.30). Intramuscular nerve trunks are present and contain myelinated and unmyelinated axons. Relatively small motor terminals probably become reestablished exclusively at the sites of original myoneural junctions. Two observations suggest this. Firstly, extensive well-formed post-junctional folds were observed in a regenerated muscle fibre in the absence of any axon terminal. Secondly, the reinnervating terminals do not always make a direct contact with the regenerating muscle fibre. In some regions, there is a relatively wide gap separating the terminal and muscle fibre, each of which retains its own basal lamina. The axon terminals are covered with Schwann cells and contain numerous small vesicles, a few larger ones,

several mitochondria, multilamellated structures containing vesicles and dense myelin figures. The subneural sarcoplasm is moderately developed. It contains numerous mitochondria, ribosomes, poor endoplasmic reticulum, small vesicles and a single nucleus.

6.5 28-182 days:

Many muscle fibres attain normal diameters by this stage, although some remain small (Fig. 6.31). Most myonuclei are at the periphery of the fibres but some retain the central position. Peripheral myoblasts and satellite cells are occasionally observed. A few macrophages can be seen in the interstitial space. Motor terminals are normal and well established at this stage (Fig. 6.32).

Two types of small unusual muscle fibre are found among the extrafusal fascicles. The first type consists of muscle fibres which have a fragmented appearance (Fig. 6.33). The well-developed myofibrils at the periphery of these fibres are surrounded by invaginated basal lamina and by processes of cells that invade the reticular system of the fibre. This dual invasion by basal lamina and cell processes effectively dismantles the fibre by stripping off each myofibril.

The second type consists of muscle fibres that show signs of atrophy similar to those described in series Dn (chapter 9: 9.1). At day 56, some fibres of this type have already lost peripheral myofibrils with a wide space among the persisting ones (Fig. 6.34). Their nuclei are pyknotic and irregularly-shaped with nuclear projections. By day 182, these fibres are very atrophied and their basal lamina is folded (Fig. 6.35). Labyrinths and helical complexes are also found in some fibres (Fig. 6.36). The contractile material of other fibres (Fig. 6.37) is disorganized and has a granular

appearance which is probably caused by disintegration of the Z-line. In addition, these fibres show sarcolemmal invagination and cellular invasion. Post-junctional folds devoid of axon terminals are observed in some fibres (Fig. 6.34B). The latter observation and the above abnormalities indicate failure of reinnervation in the second type of unusual muscle fibres.

CHAPTER 7: Effects of temporary ischaemia on the muscle spindle: general observations

Devascularization of soleus muscle produces ischaemic effects in the muscle spindle. The intrafusal fibres degenerate during the first three days after the operation. Between 3-5 days, when revascularization occurs, degeneration and regeneration take place simultaneously. Regeneration continues alone after day 5. Reinnervation of muscle spindles occurs at 21-28 days. Not all spindles are affected to the same degree at any particular stage in any one muscle. The following observations are thus generalized for each stage (see also Fig. 7.1).

7.1 0-3 days:

The first ischaemic effects were seen in a few spindles as early as 30 minutes after devascularization (Fig. 7.2), when the space between capsule layers appears dilated and may contain erythrocytes. The inner capsule layer is often collapsed and occasional macrophages were observed in the periaxial space.

By 3 hours, degenerative changes are evident and may be severe in the intrafusal fibres (Fig. 7.3). At 6 hours, hyaline degeneration has already occurred in the intrafusal fibres (Fig. 7.4). Bag fibres degenerate more rapidly than chain fibres.

By 1-2 days (Figs. 7.5, 7.6), large numbers of blood and inflammatory cells have invaded the muscle tissue. The capsule is often packed with erythrocytes and the intrafusal fibres are infiltrated by macrophages and granular leucocytes. Inflammatory cells are also found in the periaxial space and within the axial

sheath. Satellite cells are occasionally seen and satellite myoblasts become numerous from day 2.

By day 2, the myofibrils of the bag fibres are converted to hyaline material in most regions. The chain fibres are also affected, but to a less extent. Some pyknotic nuclei were seen at 3 hours. By day 2, all of the nuclei are affected and only remnants of the chromatin material and nuclear membrane are present. Bags and chains of nuclei are no longer evident.

The spindle nerve terminals and axons are also affected. Sensory terminals degenerate and their remnants eventually mix with the necrotic sarcoplasm. Motor terminals contract and withdraw from their sites on the intrafusal fibres. They become engulfed by Schwann cells, as do the axons.

The identification of intrafusal fibre types becomes difficult as degeneration proceeds. However, distinction between bag and chain fibres is still possible since the chain fibres are thinner and shorter than the bag fibres. In the next stages therefore, it is appropriate to designate the bag and chain fibres as thick and thin fibres, respectively.

7.2 3-5 days:

Signs of revascularization were first seen in one muscle removed at day 2. Other muscles did not show these signs until day 3 or 4. Full revascularization was not seen in any muscle until day 5. The degeneration process continues up to day 5, and mainly involves the removal of necrotic debris and any persisting contractile elements from the intrafusal fibres by macrophages.

The regeneration process begins at day 2, when presumptive

myoblasts were first seen in some intrafusal fibres (Fig. 7.6A). Myoblasts and nascent myotubes however are more common at day 3, particularly in the thick intrafusal fibres. Therefore, spindles examined at 3-4 days show signs of both degeneration and regeneration (Fig. 7.7). The basal lamina survives temporary ischaemia and acts as a scaffold for the regeneration of the intrafusal fibres in the same way as it does in extrafusal fibres.

7.3 5-21 days:

This stage is characterized by the maturation of the regenerating muscle cells. At day 5, the regeneration of the intrafusal fibres is well advanced, particularly in the thick fibres (Fig. 7.8), which consist of nascent myotubes with occasional myoblasts. The thin fibres consist of a bundle of myoblasts contained within the basal lamina.

At day 14 (Figs. 7.9,7.10), the intrafusal fibres appear as immature muscle fibres. They contain well-developed myofibrils and central nuclei in all regions. At this stage, additional muscle fibres were seen for the first time. They are found in the polar and equatorial regions. The capsule and axial sheath are often thickened and the periaxial space is reduced.

By day 21, the intrafusal fibres are well-formed and most of the nuclei are peripheral in position (Fig. 7.11). The number of satellite myoblasts is highly reduced. Several additional fibres are present in the spindle. They are generally short and thin and found in the axial bundle, in the periaxial space and between the capsule layers. There is a noticeable increase in the number of capillaries at this stage. They are present around the capsule, between its layers and in the periaxial space.

7.4 21-28 days:

Reinnervation of muscle spindles takes place during this stage. Motor terminals were first seen on both intrafusal and extrafusal fibres at day 21, when some restoration of the sensory innervation had occurred. The spindles are varied in the distribution of their sensory terminals and in the development of equatorial nucleation. In some spindles (Fig. 7.11), the sensory terminals are mostly confined to the thin fibres. The myotube region in all fibres is either poorly-developed or absent and no bags or chains of nuclei are present. In other spindles, the sensory terminals are distributed to both thin and thick fibres. The thin fibres contain almost normal chains of nuclei The thick fibres have poorly-developed nuclear bags that contain fewer nuclei than normal.

At this stage, the contractile elements of all intrafusal fibres are well-formed in all regions of the spindle. Additional fibres are common and lack any kind of nerve terminals. Most of them are short, very thin and found in the equatorial and juxta-equatorial region. Only a few are thick and extend for a short distance into the polar region. The capsule, periaxial space and axial sheath are normal.

7.5 28-182 days:

Eighteen spindles at this stage were serially sectioned for most of their length. They can be categorized into four groups according to their sensory innervation and equatorial nucleation (Fig. 7.12). The first group consists of three spindles of normal structure and innervation (Fig. 7.13). In the second group of three spindles, the sensory terminals are distributed to all intrafusal fibres, and the thin fibres contain a nuclear chain in the equatorial region (Fig. 7.14).

The thick fibres have small bags of nuclei. In the third group of eight spindles, the sensory terminals are mostly confined to the thin fibres (Fig. 7.15). These fibres contain poorly-developed nuclear chains, whereas the thick fibres lack any organized central nucleation. Spindles of the second and third groups have a normal motor innervation. Apart from the abnormalities in sensory innervation and equatorial nucleation, the capsule, periaxial space, axial sheath and intrafusal fibres are normal in these spindles.

The fourth group consists of four spindles that lack any sensory innervation and equatorial nucleation. They can be divided into two subgroups (each consists of two spindles), that differ with respect to their motor innervation. In the first subgroup, well-developed motor terminals were seen (Fig. 8.53A). Most intrafusal fibres in this subgroup are thicker than normal, particularly in the polar region, where the capsule tightly encloses them (Fig. 7.16B). In the equator, they are slightly thinner and the periaxial space is almost absent (Fig. 7.16A). Motor terminals were not seen in the two spindles of the second subgroup. Each spindle contains only two intrafusal fibres (Fig. 7.17), which are thinner than normal and show signs of denervation atrophy. The capsule is flat and the periaxial space is reduced. The majority of the spindles, from all groups, contain a number of additional fibres.

Regenerated spindles clearly vary considerably in the structure of the components of the axial bundle and in their innervation. As a general rule, however, most are deficient in terms of the complexity of their sensory innervation and in their equatorial nucleation, and contain additional short thin muscle fibres.

CHAPTER 8: Effects of temporary ischaemia on the muscle spindle: detailed observations

8.1 Degeneration:

8.1.1 Intrafusal muscle fibres:

As a general rule, changes in intrafusal fibres following devascularization are similar to those described in extrafusal fibres (chapter 6). The early changes are not related to any particular intrafusal fibre type, but from 6 hours onward, degeneration of the bag fibres is more advanced than that of the chain fibres. Amongst the bag fibres, bag_2 fibres degenerate more rapidly than bag_1 fibres.

Hyaline degeneration (see e.g. Moore et al., 1956) is the usual type observed in ischaemic intrafusal fibres. It involves the conversion of the contractile and sarcoplasmic components of the fibres into a homogeneous hyaline material. Eventually, this material and any persisting elements are removed by macrophages. The basal lamina and satellite cells survive the injury, and following revascularization they form the scaffold for the regeneration of intrafusal fibres.

Mitochondria:

During temporary ischaemia, the earliest changes seen in the intrafusal fibres occur in the mitochondria. These changes are also the earliest ultrastructural sign of extrafusal-fibre damage in ischaemic muscle (Hanzliková & Schiaffino, 1977).

Two hours after devascularization, mitochondria are usually unchanged. By three hours however, several enlarged mitochondria were seen either singly or in clusters, mainly at the periphery of the intrafusal fibres. They contain tubular and vesicular cristae with a

few osmiophilic bodies. Rod-like structures may be found in the tubular cristae and occasionally between the outer mitochondrial membranes (Fig. 8.1).

At 6 hours, the enlarged mitochondria have increased in number and contain rod-like structures and osmiophilic bodies. Occasional giant mitochondria are observed and others contain a granular material (Fig. 8.2).

Similar changes were observed at 12 hours and day 1, with an increasing number of mitochondria involved at each stage. By day 2, mitochondria are of three sizes; normal, enlarged and giant (Fig. 8.3). Vesiculation of the cristae is common at this stage, and the number of mitochondria containing rod-like structures is reduced.

Basal lamina:

The basal lamina usually survives the ischaemia. In one affected spindle examined at 3 hours, two intrafusal fibres had already degenerated together with their basal laminae (Fig. 8.4). A few remnants of the latter were observed in some regions. As reported for extrafusal fibres (Fig. 6.14), ruptures in the basal lamina of intrafusal fibres are also observed, and are probably produced by invading phagocytes (Fig. 8.35).

At the end of intrafusal-fibre degeneration, the only element that persists is a tube of folded basal lamina. This tube is either empty or contains macrophages, activated satellite cells and remnants of degenerated sarcoplasm (Fig. 8.15,8.16). The basal lamina plays an important role in regeneration by acting as a support for the regenerating intrafusal fibre.

Sarcolemma:

Changes in the sarcolemma are usually confined to those regions

of intrafusal fibres undergoing degeneration. These changes were first seen at 3 hours, when fragmented sarcolemma was observed in a degenerating fibre (Fig. 8.5). In a neighbouring unaffected fibre the sarcolemma was intact. However, this is not always the case in the later stages of degeneration. For instance, at 6 hours, an occasional strand of sarcolemma can be seen in muscle fibres undergoing hyaline degeneration (Fig. 8.6). Similarly at 12 hours, the sarcolemma is usually fragmented or absent in the well-preserved chain fibres and in some regions of the bag fibres (Fig. 8.9), but in severely-affected regions of the bag fibres, where only an empty basal lamina tube is left, an intact sarcolemma may still underlie the basal lamina (Fig. 8.10). In general, however, few remnants of sarcolemma are present in degenerating fibres at 1-2 days.

Dissolution of the sarcolemma in closely-opposed intrafusal fibres, leads to a direct mixing of their sarcoplasm (Fig. 8.11).

Nuclei:

Changes in the subsarcolemmal and equatorial nuclei of ischaemic intrafusal fibres are similar to those described in extrafusal fibres (chapter 6; Stenger et al., 1962; Jirmanova & Thesleff, 1972; Karpati et al., 1974; Mäkitie & Teräväinen, 1977b).

At 3 hours, pyknotic nuclei were seen in affected regions of intrafusal fibres (Fig. 8.7). The nuclear chromatin is condensed and accumulates at the periphery. The space between the two nuclear membranes is enlarged. At 6-12 hours, most nuclei are pyknotic (Fig. 8.8,8.9). They have condensed chromatin and the nuclear membranes appear as vesicles and tubules.

By day 2, all nuclei have degenerated, so that the characteristic nucleation of the equatorial region is lost, and only remnants of the

nuclei may be present (Fig. 8.17). The chromatin appears as a dense granular substance, and the nuclear membranes are reduced to tubules, vesicles and vacuoles. As described by Milburn (1976), occasional large vacuoles derived from the nuclear membrane were seen.

Contractile material:

The contractile elements of ischaemic intrafusal fibres undergo degenerative changes, which are generally similar to those described in bupivacaine-treated muscle spindles (Milburn, 1976) and ischaemic extrafusal fibres (see e.g. Moore et al., 1956).

The first changes were seen at 3 hours in some (Fig. 8.1) or all of the intrafusal fibres of the spindle (Fig. 8.4). Myofibrils are disorganized and the Z-line is disintegrated. The M-line is usually preserved in these fibrils. In some regions, the intrafusal fibres are severely affected and only folded tubes of basal lamina are present (Fig. 7.3). These early changes are not related to any particular fibre type at this stage, but in the later stages, degeneration of the bag fibres usually precedes that of the chain fibres and between the bag fibres bag_2 fibres usually degenerate more rapidly than bag_1 fibres.

At 6-12 hours, all of the intrafusal fibres show degenerative changes, in all regions of the spindle. The appearance of lipid droplets, altered mitochondria, dilated sarcoplasmic reticulum and a reduction in the amount of glycogen granules are common. As in extrafusal fibres, hyaline degeneration has already appeared in most regions of bag fibres at 6 hours (Fig. 7.4). In chain fibres, although hyaline degeneration is abundant, bundles of myofilaments are still present in some regions. Less-affected areas may also be seen in bag_1 fibres, and occasionally in bag_2 fibres.

At 1-2 days, the whole contractile apparatus of the intrafusal

fibres is converted to hyaline material (Fig. 7.6). A hyalinized intrafusal fibre appears as a mass of homogeneous material enclosed by the preserved basal lamina (Fig. 8.18,8.19). The hyaline mass is interrupted here and there by degenerating mitochondria, sarcoplasmic vacuoles, lipid droplets and macrophage processes. In some regions of a degenerating fibre, it is possible to recognize the original architecture of the myofilaments (Fig. 8.12). However, less-affected areas are still present in a few regions of the chain fibres (Fig. 8.13) and occasionally in bag_1 fibres (Fig. 8.14).

At day 2, the removal of the necrotic debris by macrophages is well underway. Spaces are usually seen in the centre and at the periphery of the degenerating fibres (Fig. 8.3). In some regions, only a folded tube of basal lamina is left. This tube is either empty or contains varying amounts of necrotic debris, macrophages and satellite myoblasts (Fig. 8.15,8.16). In some rapidly-affected spindles, however, basal-lamina tubes were also observed at 3, 6 and 12 hours (Figs. 7.3, 8.10).

At 3-4 days, the intrafusal fibres show signs of both degeneration and regeneration, and these vary along the length of the fibres. In some regions of spindles at this stage, some intrafusal fibres contain hyaline material that is surrounded or invaded by myoblasts and inflammatory cells (Figs. 7.7, 8.20). In other regions, the necrotic sarcoplasm has been removed and replaced by myoblasts and macrophages all enclosed by a folded basal lamina. Signs of degeneration are absent at day 5.

Sarcotubular system:

Following devascularization, the sarcotubular system shows similar changes to those described by other investigators (Jirmanová &

Thesleff, 1972; Karpati et al., 1974; Milburn, 1976; Mäkitie & Teräväinen, 1977b).

Dilated sarcoplasmic reticulum, transverse tubules and junctional couplings were observed at 3 hours (Fig. 8.1B). Subsarcolemmal micropinocytotic vesicles are also enlarged in affected regions of the intrafusal fibres (Fig. 8.5).

At 6 hours, most of the sarcotubular elements appear as vesicles or vacuoles of varying size, and many are filled with a granular material (Fig. 8.2). Similar structures are present at 1-2 days, and all of the sarcotubular elements are converted to vesicles and vacuoles scattered in the hyaline material (Fig. 8.18,19).

8.1.2 Satellite cells:

Satellite cells are commonly seen associated with normal intrafusal fibres. A typical satellite cell is oval or spindle-shaped and contains a relatively large dense nucleus enclosed in a thin layer of cytoplasm with few other organelles. It lies between the sarcolemma and basal lamina of the intrafusal fibre (Landon, 1966a).

Satellite cells survive temporary ischaemia and during the early stages of degeneration they are easily identified. At these stages, satellite cells may contain one or two lipid droplets in their relatively increased cytoplasm.

At day 2, activated satellite cells are present. They differ from normal satellite cells in the increased volume of their cytoplasm which contains more organelles. These cells are similar to the mononuclear cells described in ischaemic extrafusal fibres (see e.g. Karpati, et al., 1974). By this time, the invasion of intrafusal fibres by macrophages is common, and they may occupy a sublaminar position. Activated satellite cells also have rough endoplasmic

reticulum, clusters of free ribosomes and cell processes (Nichols & Shafiq, 1979). However, they could be distinguished from the macrophages by the usually greater prominence of rough endoplasmic reticulum and phagocytic vacuoles in the latter cells, as suggested by Nichols & Shafiq (1979). The above observations are illustrated in Fig. 8.21.

8.1.3 Sensory terminals:

At 3 hours, a fragmented axolemma is usually observed at the junctional region between the sensory terminal and the muscle fibre. Mitochondria are swollen and with disrupted cristae. Only few terminals contain myelin figures and autophagic vacuoles (Fig. 8.22).

At 6 hours, the sensory terminals are considerably changed (Fig. 8.23). Besides the fragmented axolemma, the axoplasm of the terminal is clumped and contains autophagic vacuoles, myelin figures and dense mitochondria that contain osmiophilic bodies.

It seems that the degenerative changes of the sensory terminals are not comparable to those in the intrafusal fibres. For example in a spindle examined at 12 hours (Fig. 8.24), the chain fibres are less affected than the bag fibres, but their sensory terminals are more severely affected than those of the bag fibres. As a general rule, however, the sensory terminals begin to show ultrastructural changes at 3 hours until they are completely removed by day 3. The mitochondrial changes are generally similar to those described in degenerating intrafusal fibres. Swollen mitochondria with dense tubular, vesicular, filamentous or granular contents are common. Dense osmiophilic bodies are also present, but giant mitochondria and rod-like structures were not seen. Degenerating sensory terminals also contain myelin figures and autophagic vacuoles of varying size.

Vesicles and vacuoles containing granular material, similar to those described in the sarcotubular system, may also be present. The axoplasm is sparse, flocculent and clumped. Fragmentation of the axolemma is common. In the junctional region, the sarcolemma is usually absent and the axolemma occasionally, so that the contents of the sensory terminal and muscle fibre mix together. Invading macrophages may occupy the site of the sensory terminals by pushing them toward the centre of the intrafusal fibre (Fig. 8.13). In the late degenerative stages, remnants of both sensory terminals and muscle fibres are removed by macrophages.

8.1.4 Motor terminals:

Degenerative changes were first seen at 6 hours in some motor terminals (Fig. 8.25). As well as normal synaptic vesicles, the axon terminals may contain lipid droplets, autophagic vacuoles and enlarged mitochondria with dense osmiophilic bodies. The covering Schwann cells may also contain lipid droplets.

At 12 hours, some normal axon terminals are completely encircled by Schwann-cell processes and the basal lamina may be absent from the junctional region (Fig. 8.26).

Fig. 8.27 shows two degenerating adjacent terminals in a spindle examined at day 1. They are enwrapped by thin processes of Schwann cells and contain normal vesicles with autophagic vacuoles and enlarged mitochondria. The latter contain dense osmiophilic bodies and tubular, vesicular or granular contents. The Schwann cell also encloses two nearby spaces which presumably housed degenerated preterminal axons.

A variety of changes were observed at day 2. Some axon terminals are of normal ultrastructure, although completely enclosed by Schwann

cells. Some of these terminals had withdrawn for a distance from their site on the muscle fibre. Others remain, but Schwann-cell or macrophage processes occupy the junctional region which may be devoid of basal lamina (Fig. 8.28). Some axon terminals are completely degenerated by this stage. They appear as a dense material enclosed by Schwann-cell processes (Fig. 8.29). This type of damage resembles the so-called honeycomb structures of degenerated terminals in denervated extrafusal fibres (see e.g. Miledi & Slater, 1970).

The effect of temporary ischaemia on the spindle motor terminals is different from that on the sensory terminals. They retract and withdraw from the intrafusal fibres, and become invested by Schwann cells.

8.1.5 Nerve axons:

At 3 hours, the nerve axons are usually normal, but a few covering Schwann cells contain lipid droplets and vacuoles. At 6-12 hours, changes were observed in some axons of the spindle nerve trunk and in those associated with the axial bundle. In Fig. 8.27, only a few mitochondria and remnants of clumped axoplasm are left from a preterminal axon which is enclosed by a Schwann cell. Degenerated axons were also observed and their myelin layers were disorganized.

At 1-2 days, the cytoplasm of the Schwann cells increases in volume and contains lipid droplets and numerous cytoplasmic organelles. As in degenerating motor terminals (8.1.3), the Schwann cells also play a role in the degeneration of nerve axons. Engulfment of axons by Schwann cells is common. The myelin layers are disorganized and often collapsed. The axoplasm is clumped, contracted and resembles a honeycomb structure (Fig. 7.6). As early as 12 hours, changes were also observed in the perineurial cells of the spindle and intramuscular

nerve trunks (Fig. 8.30,8.31),and are similar to those occurring in the capsule cells. Lipid droplets are usually seen in their cytoplasm, which increases in volume and shows some vacuolation.

At the later stages, Schwann cells and their processes may contain myelin remnants. This was seen even after the restoration of the blood supply. It seems that the degeneration continues until the removal of the necrotic debris is complete. In this way, the endoneurial tubes are prepared for the subsequent reinnervation.

8.1.6 Capsule, periaxial space and axial sheath:

Despite some changes, the capsule and axial-sheath cells survive the temporary ischaemia. Changes in the capsule may be seen as early as 30 minutes (Fig. 7.2). The space between the capsule layers appears dilated and may contain erythrocytes. The inner capsule layer is often collapsed and occasional macrophages were observed in the periaxial space. At 3 hours, occasional lipid droplets and enlarged mitochondria were seen in capsule cells. At 6-12 hours, signs of pyknosis were observed in some nuclei of the axial-sheath cells and, occasionally, of the capsule cells. Condensed chromatin accumulates at the periphery of these nuclei, and the nuclear membrane may be vesicularized. However, such pyknotic nuclei are absent in later stages. From 6 hours onward, lipid droplets become common in the capsule and axial-sheath cells. At 12 hours, vacuolation of the capsule cells, and to a less extent in the axial sheath, is evident (Figs. 8.32,8.33). At this time, flocculent material, contained in vacuole-like structures, is found clustered in the periaxial space (Fig. 8.34). Some of these are also observed at later stages and may be attached to the axial sheath.

At day 2, the capsule is usually packed with erythrocytes (Fig. 7.5), some of which may lie free in the periaxial space. Inflammatory cells

are also found between the capsule layers, in the periaxial space and in the axial sheath. In some regions, the cytoplasm of capsule and axial-sheath cells has increased in volume and their nuclei appear denser than normal. Lipid droplets are now more common and are found in all regions of the spindle. Spaces between the capsule layers are usually enlarged and the inner layer is collapsed. Vacuolation of the capsule cells is common.

8.1.7 Haemorrhage and inflammation:

Erythrocytes and inflammatory cells are numerous in the interstitial space as early as 6 hours. Following devascularization, capillaries, arterioles, venules and large blood vessels are usually blocked by erythrocytes and other blood cells. Granular leucocytes are the most common of the inflammatory cells. They are characterized by their dense cytoplasm, lobulated nuclei, and the numerous granules that vary in size and density (Figs. 7.6B; 8.35). The cytoplasm is rich in ribosomes and glycogen particles, and contains few mitochondria, poor endoplasmic reticulum and occasional lipid droplets. Small vesicles and vacuoles may also be present. Macrophages are relatively less numerous than leucocytes. They have large nuclei and several cytoplasmic extensions. The cytoplasm contains a well-developed endoplasmic reticulum, Golgi system, autophagic vacuoles, free ribosomes, mitochondria and lipid droplets.

Occasional fibrocytes, lymphocytes, mast cells and blood platelets are present. Fibrin deposition is abundant at day 2. Bundles of fibrin threads are commonly observed in the interstitial space, around the spindle capsule and around the extracapsular region of intrafusal fibres (Figs. 8.19, 8.35).

Infiltration of the extrafusal fibres by macrophages and granular

leucocytes was first seen at 6 hours. At 6-12 hours, many inflammatory cells are present around, and occasionally inside, the spindle capsule. However, infiltration of the intrafusal fibres was not seen until day 1, but is common at day 2. Blood cells and inflammatory cells may be observed in the periaxial space and between the capsule layers, but only macrophages and granular leucocytes invade the intrafusal fibres, probably by rupturing the basal lamina (Fig. 8.35). As described before, erythrocytes may be seen between the capsule layers as early as 30 minutes, and by day 2, the capsule is usually packed with them.

8.2 Regeneration:

The first signs of muscle regeneration were seen at day 2, when presumptive myoblasts were observed at the periphery of intrafusal fibres (Fig. 8.21D). By day 3, myoblasts had increased in number. At this time, the phagocytosis of degenerated sarcoplasm and any persisting material is well in progress. By day 4, most necrotic debris has been removed. As described by Karpati et al. (1974) in ischaemic extrafusal fibres, phagocytes begin to withdraw from the already-regenerating intrafusal fibres through the basal lamina, in the same manner as they enter (Fig. 8.36). Therefore, spindles examined at 2-4 days show signs of both degeneration and regeneration.

8.2.1. Intrafusal muscle fibres:

Regenerating intrafusal fibres can be classified, according to their diameter, as thin or thick fibres (usually two of each in a spindle). They probably represent the original chain and bag fibres of the spindle.

In general, regeneration proceeds more rapidly in the thick fibres than in the thin fibres. At 3-4 days, the thin fibres are

composed of a chain or bundles of myoblasts contained within the original lamina. The thick fibres usually contain one or two nascent myotubes in addition to myoblasts (Fig. 8.37). A gap is usually present between these muscle cells and the enclosing basal lamina, which is folded in many places. No new basal lamina has formed by this stage.

At day 5, the thick fibres generally consist of a maturing myotube with occasional myoblasts associated with its walls (Fig. 8.38). In some regions, the thin fibres are similarly composed, but more generally they consist of a bundle of nascent myotubes and myoblasts contained within the preserved basal lamina (Fig. 8.39). The axial bundle also includes proliferating Schwann cells with bands of Büngner. Cellular processes, similar to the bands of Büngner are occasionally observed beneath the basal lamina of regenerating intrafusal fibres.

Regenerating myotubes contain central nuclei, with diffuse nucleoli, embedded in a core of sarcoplasm rich in mitochondria, ribosomes, glycogen granules, vesicles, and occasional microtubules. Peripheral myofibrils are at varying stages of assembly depending on the maturity of the myotube. In nascent myotubes, the myofilaments are scattered in the sarcoplasm. As the myotube develops, myofibrils assemble at its periphery and increase in number. Z- and M-lines may be observed in the newly-formed sarcomeres (Fig. 8.40). The sarcotubular system assembles at the same time and appears more prolific in immature myotubes than in normal fibres (Karpati et al., 1974).

In one spindle examined at day 5, only three (two thick; one thin) intrafusal fibres were present, all of which were at the same stage of regeneration.

At day 6, thin fibres have increased in diameter (Fig. 8.41). The regenerated fibres are now of about the same diameter in most

regions of the spindle, but the original bag and chain fibres are still distinguishable because of differences in the stage of regeneration. Two intrafusal fibres consist of well-formed myotubes with central nuclei. The myotube is packed with well-orientated myofibrils separated by mitochondria and sarcoplasmic reticulum. The sarcoplasm is more concentrated around the nuclei and at the periphery of the myotube. The other two intrafusal fibres consist of nascent myotubes with peripheral myoblasts. At this stage, macrophages are rarely encountered inside regenerating intrafusal fibres, but they are usually present in the periaxial space.

By day 14, all intrafusal fibres have the same diameter and appear at the same stage of regeneration. The intrafusal basal-lamina tubes now contain maturing muscle fibres with well-formed myofibrils and central or peripheral nuclei (Figs. 7.9, 8.42). The size and distribution of the mitochondria are generally similar in all fibres. Lipid droplets are also present, but in smaller numbers than in the early stages of degeneration. Occasionally, two regenerating intrafusal fibres were observed laterally fused for a short distance (Figs. 8.42, 8.43).

At this stage, a new basal lamina has formed in places where the regenerating fibre is not in contact with old basal lamina. Two leaflets of basal lamina surrounding a regenerating fibre were rarely seen.

All intrafusal fibres exhibit a prominent M-line in all regions of the spindle (Fig. 8.42). Nuclear bags or chains and nerve terminals are absent. Additional muscle fibres are common at this stage (see 8.2.2). At day 21, the regenerated intrafusal fibres have an irregular shape, but their structure is normal. They are packed with well-formed myofibrils and contain peripheral, or occasionally central,

nuclei. The sarcotubular system is well-developed and distributed normally in most regions. Beside the abnormally-shaped intrafusal fibres, the axial bundle also includes several additional muscle fibres (Fig. 7.11). This makes the identification of the original fibres difficult, particularly at the equator. In this region (Fig. 8.44), a common basal lamina (presumably the old one) usually encloses a satellite cell, myotube and one or more muscle fibres. A new basal lamina delineates each of these cells in those places where they are not closely associated with another cell or with the original common basal lamina.

At this stage, reinnervation of the axial bundle has started. Sensory terminals are few and distributed only to some fibres (Fig. 7.11). Motor terminals are also present (Fig. 8.45). Bags and chains of nuclei are still absent. As at day 14, an M-line is present in all regions of the intrafusal fibres.

At 28-182 days, spindles vary with respect to their innervation and equatorial nucleation (see 8.3). In general however, in those spindles that are reinnervated by sensory axons, the intrafusal fibres are normal with respect to the structure of their contractile elements and sarcoplasm. The development of the equatorial nucleation is related to the extent of sensory reinnervation (Figs. 7.12-7.17). The intrafusal fibre types can only be identified in spindles with well-restored sensory innervation and equatorial nucleation.

Spindles that lack sensory terminals are of two types. Firstly, spindles that receive motor axons contain thick intrafusal fibres, all of which exhibit a distinct M-line in all regions (Fig. 7.16). Secondly, in those spindles that lack motor innervation the intrafusal fibres are thinner than normal, some showing signs of denervation atrophy and all exhibit an M-line (Fig. 7.17).

Various abnormalities were observed in regenerated intrafusal fibres. A number of muscle fibres are enclosed by a common basal lamina. Some are separated by narrow clefts without interposed basal lamina. Others are laterally fused and a strand of mitochondria marks the site of the fusion (Fig. 8.46). Others appear as fragments of one muscle fibre that had split longitudinally (Figs. 8.47, 8.48). The common basal lamina may also contain satellite cells, Schwann cells, sensory terminals and myelinated axons, each with its own basal lamina. The clefts between them may be wide and contain collagen fibrils, strands of basal lamina, and cellular extensions from the axial sheath.

8.2.2 Additional muscle fibres:

In serial transverse sections of regenerated spindles, a muscle fibre which extended through most of the capsule length was defined as an "original" fibre. In contrast to this, an "additional" fibre is short and unusually located. Using these definitions, eighteen spindles were examined at 14-182 days (Table 8.1). Amongst them; two spindles contained no additional fibres, six contained 1 fibre, and the rest (12 spindles) contained varied numbers (from 2-18) of additional fibres. The number of original intrafusal fibres in these spindles was: 5 in four spindles, 4 in thirteen, and 2 in one spindle. Spindles sectioned longitudinally are not included in these counts.

Several short thin muscle fibres were first seen at day 14. At the equator, they are usually associated with the regenerating intrafusal fibres and the axial-sheath cells. Some are also found in the periaxial space and others between the capsule layers, where their delineating basal lamina is continuous with that of the capsule cells. Those in the axial bundle are less developed at this stage, and

Table 8.1

Number of additional muscle fibres in muscle spindles examined 14-182 days after devascularization

Number of additional muscle fibres	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Number of muscle spindles	2	6	1	1	1	1	1	1	1	0	1	0	1	0	0	0	0	0	1

consist of nascent or well-developed myotubes. Some are attached to axial-sheath cells and lack a basal lamina. In the spindle pole, the additional fibres are usually thicker and appear to form part of the capsule and may be relatively long.

At day 21, some additional fibres are well-developed and have the same structure as the regenerated intrafusal fibres. Others are poorly-developed. The additional fibres are more numerous and thinner at the equator of the spindle than in its poles. Some of those in the axial bundle still lack a basal lamina and are closely associated with the axial sheath cells.

At 28-182 days, most spindles contain additional muscle fibres. Some of these fibres are well-developed, but are undergoing fragmentation and cellular invasion, as seen in some regenerated intrafusal and extrafusal fibres. Others appear as fragments scattered in the axial bundle. Some are poorly-developed and enclosed by axial-sheath cells.

In general, the additional fibres are short and thin. They are either free or closely associated with intrafusal fibres, axial-sheath and capsule cells. They first appear in the spindle before reinnervation occurs, and are also found in spindles that lack any innervation. Additional muscle fibres usually lack any kind of nerve terminals. However, some of those associated with regenerated intrafusal fibres have sensory terminals. These observations are shown in Figs. 8.44 & 8.46-8.50.

8.2.3 Nerve terminals:

Following devascularization, all sensory terminals degenerate, and motor terminals either degenerate or at least withdraw from their sites on the degenerating intrafusal fibres. The regeneration of

intrafusal fibres occurs in the absence of any innervation. Regenerating nerve terminals were not seen until day 21. Some intrafusal fibres and muscle spindles may not receive any sensory or, to a lesser extent, motor terminals even after 182 days.

The ultrastructure of regenerated sensory terminals appears similar to that of normal spindles, although myelin figures are more numerous (Fig. 8.51). Regenerated motor terminals also appear normal (Fig. 8.52). Some neuromuscular junctions exhibit post-junctional folds and sole-plates with nuclei.

Regenerated intrafusal fibres reinnervated by motor, but not sensory axons, have a thicker diameter than normal (Fig. 7.16). Their motor end-plates appear more developed than normal with branched post-junctional folds (Fig. 8.53A). On the other hand, signs of denervation atrophy are evident in intrafusal fibres which lack both motor and sensory terminals. Degenerated motor terminals were occasionally observed (Fig. 8.53B).

Several terminals were observed lying away from the intrafusal fibres, and were more numerous in the equatorial than the polar region. At the equator (Fig. 8.54), they are found in the axial bundle, periaxial space, and between the capsule layers. These free terminals are not enclosed by Schwann cells. In the polar region (Fig. 8.55), free terminals are usually found between the capsule layers and enclosed by Schwann cells.

8.2.4 Capsule, periaxial space and axial sheath:

Degenerative changes are no longer evident at day 5. Apart from the relative increase in the cytoplasmic volume of some cells, the capsule and the axial sheath appear normal. Erythrocytes and macrophages may still be present in the capsule, periaxial space and

axial sheath.

At day 14, the capsule cells are usually changed in structure, particularly in the polar region (Fig. 7.10). Their abundant cytoplasm is rich in organelles including centrosomes. The capsule usually contains one or more broad additional muscle fibres which lie parallel to the capsule layers. Therefore, the capsule appears thicker than normal at this stage. Some of the axial-sheath cells are similarly changed. The axial bundle appears crowded and consists of intrafusal and additional fibres, axial-sheath cells, scattered collagen and elastic fibrils, loose strands of basal lamina, and, in addition, nerve axons at day 21 (Fig. 7.11).

The periaxial space appears reduced at day 5, and this reduction becomes more evident at day 14. The number of capillaries associated with the spindle has increased. They are usually present around and between the capsule layers, in the periaxial space and inside the axial bundle.

At 28-182 days, the capsule, periaxial space and axial sheath are normal in most regenerated spindles. However, in group 4 spindles (see 8.2.5), the capsule may be constricted and the periaxial space is reduced (Fig. 7.16,17).

8.3 The regenerated spindles:

It is clear from the previous sections, that the process of regeneration is almost complete by day 28. Spindles examined at 42, 56, 119 and 182 days, did not show any additional regenerative changes compared with those examined at day 28. It was therefore concluded that all spindles examined at 28-182 days had reached their maximum degree of restoration. It is convenient to classify them

into four groups, according to the extent of their sensory reinnervation and the restoration of equatorial nucleation (Fig. 7.12).

8.3.1 Group 1:

The three spindles in this group appeared normal (Fig. 7.13). The sensory terminals are distributed to all of the intrafusal fibres. Bag_2, bag_1 and chain fibre types are easily identified, and each has the features of its counterpart in the normal spindle. The nuclear chains and bags are well-developed. Motor terminals are also distributed to all of the intrafusal fibres. Additional muscle fibres are present in these spindles.

8.3.2 Group 2:

In this group of three spindles, sensory terminals are distributed to all intrafusal-fibre types, which have the ultrastructural features of bag_2, bag_1 and chain fibres of normal spindles, with the exception of deficiencies in the equatorial nucleation of the thick fibres (Fig. 7.14). Additional muscle fibres are present and motor terminals are distributed to all intrafusal fibres.

8.3.3 Group 3:

In this group of eight spindles the intrafusal-fibre types can not be clearly identified. They are classified as thick and thin fibres and probably represent the original bag and chain fibres respectively (Fig. 7.15). Sensory terminals are mostly confined to the thin fibres, which generally contain poorly-developed nuclear chains. Apart from deficiencies in their sensory innervation, the thick fibres also lack any organized equatorial nucleation, but exhibit variations in the M-line condition characteristic of normal

bag fibres. Motor terminals are distributed to all intrafusal fibres. A well-developed motor terminal is usually present on the thick fibres that lack any sensory innervation (Fig. 8.53A). The spindles of this group also contain additional muscle fibres.

8.3.4 Group 4:

The four spindles in this group are devoid of both sensory innervation and equatorial nucleation. They are classified into two subgroups (each of two spindles) according to their motor innervation.

Subgroup A:

The nerve axons in these spindles are fewer than normal and mostly confined to the polar regions. Well-developed motor terminals are found on the intrafusal fibres (Fig. 8.53A). Other terminals are associated with the capsule. The intrafusal fibres are thicker than normal (Fig. 7.16) and all of them exhibit a distinct M-line in all regions. Because of their increased diameter, the intrafusal fibres are tightly enclosed by the capsule in the polar region. At the equator, they are slightly thinner and the periaxial space is almost absent. Additional muscle fibres are also present in all regions of the spindles.

Subgroup B:

Although the spindles of this subgroup contain more nerve axons in the polar and equatorial region (Fig. 7.17), compared with those of subgroup A, their intrafusal fibres are devoid of any kind of nerve terminals. However, nerve terminals and preterminal axons are present in all regions of the capsule, periaxial space and axial sheath. Occasionally, degenerated motor terminals, similar to honeycomb structures (e.g. Miledi & Slater, 1970), were seen (Fig. 8.53B).

Each of the two spindles contains two intrafusal fibres as well as additional fibres. The intrafusal fibres are usually thin and show signs of denervation atrophy in some regions (Fig. 8.56). At the equator, the capsule is flat and the periaxial space is reduced. In the capsular polar region, the intrafusal fibres are surrounded by large amounts of collagen and elastic fibrils (Fig. 8.57).

CHAPTER 9: Control experiments

The events that occur in intrafusal fibres in all of the experimental series are illustrated in Fig. 9.1.

9.1 Effects of denervation (series Dn):

The changes seen in denervated extrafusal fibres in this study were generally similar to those described in the literature (see e.g. review by Pellegrino & Franzini-Armstrong, 1969). Following prolonged denervation, soleus muscle becomes threadlike and contains giant fat cells and abundant collagen fibrils. At day 21, extrafusal fibres show focal changes and by day 364 all of them have a reduced diameter. Intrafusal fibres are more resistant to denervation. In sections of long-term denervated muscle, spindles stand out against a pale background of severely atrophied extrafusal fibres (Fig. 9.2).

9.1.1 Extrafusal muscle fibres:

Motor endings:

Axon terminals are absent by day 21, but the subneural apparatus persists even after 364 days denervation (Fig. 9.3). Collagen fibrils occupy the site of the axon terminals. Schwann-cell processes are also found at the early stages. Axons are absent from the intramuscular nerve trunks and in their place there are varying amounts of collagen fibrils and remnants of Schwann cells.

Contractile material:

At day 21, the extrafusal fibres are generally normal. In some areas, streaming and disintegration of the Z-line is evident and is usually accompanied by dislocation of the sarcomeres,

disorganization of myofilaments and dilation of sarcotubular system. These focal changes are more frequent at day 56 when the extrafusal fibres have irregular contours due to sarcolemmal indentations and projections. At day 156, most extrafusal fibres are reduced in diameter and denervation atrophy is extensive. Some extrafusal fibres show advanced atrophy, as at day 364, but many others are still undergoing early changes (Fig. 9.4).

In cross sections of whole muscle taken at day 364, all extrafusal fibres show a significant reduction in diameter; the majority are small and some are flat and very thin. Various stages of denervation atrophy coexist in the same muscle. Firstly, a few muscle fibres appear normal but are small in diameter. Secondly, myofibrils are absent around the nuclei and at the periphery of some thin fibres but persist in other areas. Thirdly, other fibres contain preserved myofilaments which, in cross-section, show varying orientations. In some, the entire section is occupied by randomly-orientated filaments. In others, the peripheral myofibrils are disorganized but their filaments retain a longitudinal orientation whereas the central zone consists of transversely-orientated myofibrils that show Z-line streaming. The two zones have the contrary position in other fibres. Lastly, severely atrophied fibres, which have the thinnest diameter, contain central nuclei and remnants of degenerated sarcoplasm. The above observations are shown in Figs. 9.2, 9.7.

Nuclei:

At 56-156 days, most of the nuclei retain their peripheral position but are indented with dense nucleoli. Some nuclei, encircled by a small amount of sarcoplasm, project above the muscle-fibre

surface. This is probably caused by indentation of the sarcolemma around these nuclei (Fig. 9.4).

By day 364, the nuclei are dense, irregularly-shaped and central in position. Many are indented and some show nuclear projections which appear to engulf contractile material and necrotic debris. There is a general increase in the number of nuclei which, together with the reduction in diameter, gives the denervated extrafusal fibres a high nucleo-cytoplasmic ratio (Fig. 9.8, 9.9).

Sarcoplasm:

The sarcotubular system is absent from severely-atrophied areas and converted to dense tubules and vesicles in less-affected areas where the dense tubules usually encircle persisting myofibrils (Fig. 9.4). Abnormal arrangements are present at the few persisting junctions between the T-tubules and sarcoplasmic reticulum (Fig. 9.10). Mitochondria are reduced in size and number and usually confined to less-affected areas. Numerous ribosomes are usually observed in atrophied fibres.

Other observations:

Sarcolemmal invaginations are occasionally observed at the periphery of extrafusal fibres. Vacuoles of varying size and shape are also seen deep inside other fibres (Fig. 9.7B). Projections of the basal lamina with or without the sarcolemma are common. Helical complexes similar to those described by Miledi & Slater (1969) are frequently observed in long-term denervated extrafusal fibres. They usually lie close to a nucleus, at the periphery of the fibre. Occasionally, an oval mass of dense granular material surrounded by a more translucent zone is observed. Duchen (1971) described similar structures in mice gastrocnemius muscle injected with botulinum toxin.

Figs. 9.11-9.14 illustrate the above observations.

Satellite cells seem to be more resistant to denervation than muscle fibres and are regularly observed even after 364 days (Fig. 9.15). Unusual small cells are observed in the interstitial space. These cells are more numerous in series Isch/Dn and Isch/Rn/Dn. Therefore they will be described in detail later (9.3.1).

9.1.2 Muscle spindles:

In denervated spindles, the nerve terminals degenerate and the intrafusal fibres lose their typical equatorial nucleation which is replaced by contractile material. Intrafusal fibres are more resistant to denervation atrophy compared to extrafusal fibres (Fig. 9.2). Additional muscle fibres are also observed in denervated spindles. The capsule and axial sheath show more changes than in ischaemic spindles.

Innervation:

By day 21, all sensory and motor terminals have degenerated. The remnants of a degenerated sensory terminal are shown in Fig. 9.16. The site of motor axon terminals is occupied by Schwann-cell processes that disappear at later stages. Subneural specializations (which are not always present in normal motor end-plates) appear normal (Fig. 9.17). At day 21, the spindle nerve trunk contains Schwann-cell processes (bands of Büngner) and collagen fibrils. In addition it may contain remnants of degenerated axons when sectioned at a distance from the spindle (Fig. 9.18).

Intrafusal muscle fibres:

At day 21, the number of nuclei in chain and bag fibres is reduced (Fig. 9.19). By day 56, the typical equatorial nucleation is no

longer present. Instead, the myotube region is filled with contractile material (Fig. 9.20). In some sections, it is possible to observe a distinct M-line at the equatorial region of the bag fibres where it is absent in normal spindles (Fig. 9.21). Because of the loss of characteristic features, the identification of intrafusal-fibre types becomes difficult particularly in long-term denervated spindles.

Focal atrophy in intrafusal fibres is observed as early as day 21 and does not seem to increase significantly even after 364 days denervation. The earliest changes are streaming and disintegration of the Z-line and disorganization of the myofibrils. In severely-atrophied areas, myofilaments are replaced by large amounts of ribosomes in which mitochondria, Golgi bodies, rough endoplasmic tubules and a few granular membrane-bound structures are embedded. These areas occasionally contain tubular structures and concentric membranes with dense granules. The concentric membranes may show irregular configurations. Accumulations of small vesicles may also be observed at the periphery. They resemble the subsarcolemmal pinocytotic vesicles of normal intrafusal fibres but are much more numerous. These changes are shown in Figs. 9.22-9.25 & 9.28B.

The subsarcolemmal nuclei of denervated intrafusal fibres usually retain their position but are irregular in shape with processes of varying lengths. These nuclear processes may enclose sarcoplasmic material. Whorls of densely-packed membranes are occasionally observed in a perinuclear position or enclosed by nuclear processes (Fig. 9.26). They are similar to the heterogeneous bodies described in cold-injured mouse gastrocnemius muscle (Reznik, 1973).

After denervation, the close apposition between the sarcolemma and basal lamina is maintained around the fibre's circumference, but in some places this relationship is altered by invagination of the

sarcolemma without the basal lamina. The space formed between the two membranes usually contains collagen fibrils, occasional degenerating mitochondria and cellular processes which may resemble the bands of Büngner formed by Schwann-cell proliferation. The invagination begins by retraction of the sarcolemma and vacuoles are formed by subsequent fusion of its lips. At 21-56 days, these vacuoles are usually small, round, lack basal lamina and are peripheral in position. At 156-364 days, they are larger, irregular in shape, lined with basal lamina and are located deep inside the intrafusal fibre. At the later stages, a double basal lamina is observed in some places around intrafusal fibres and the space between may contain collagen fibrils. Various stages of sarcolemmal invagination are shown in Fig. 9.27.

Additional muscle fibres:

At day 21, undifferentiated cells, similar to those described in series Isch (chapter 8: 8.2.2), are found at the equatorial region. Additional muscle fibres are few and usually found within the axial bundle. At 21-56 days, they are short and not associated with the original intrafusal fibres (Fig. 9.28). At 156-364 days, they are longer and seem to be formed by splitting of the original intrafusal fibres. Occasionally a cell can be seen to interpose between additional and original intrafusal fibres. This cell resembles both the satellite and axial-sheath cells (Fig. 9.22A).

Capsule, periaxial space and axial sheath:

At day 21, the axial sheath contains numerous Schwann-cell processes and increased amounts of loose basal lamina. At 156-364 days, Schwann-cell processes are fewer but there is a significant increase in collagen and elastic fibrils. At these later stages, the axial

sheath is thicker than normal and consists of 3-5 elaborate cellular layers which may be enclosed by a basal lamina. The capsule appears shrunk and thickened. Shrinkage is caused by separation and widening of the space between the capsule layers at the expense of the periaxial space. Thickening is more evident at 21-56 days when some capsule cells are enlarged, but in long-term denervation an apparent thickening is caused by accumulation of small atrophied extrafusal fibres, capillaries and bundles of collagen fibrils around the capsule.

Capillaries are increased in number and found in the capsule, periaxial space and within the axial sheath. Macrophages are occasionally observed in the periaxial space. The above observations are shown in Figs. 9.19, 9.22A.

9.2 Effects of combined devascularization and denervation (series Isch/Dn):

Muscles in this series were permanently denervated at the time of devascularization. Following this, both the extrafusal and intrafusal fibres undergo degeneration followed by regeneration. Subsequently, because reinnervation is prevented, regenerated muscle fibres undergo a slow process of denervation atrophy. This process is comparable to that observed in some muscle fibres of series Isch which failed to be reinnervated (chapter 6: 6.5; chapter 8: 8.3.4).

The processes of degeneration and regeneration are generally similar to those described in series Isch (chapters 6, 7 & 8). The time-course of degeneration is also similar, but regeneration is more rapid in this series compared with series Isch. Therefore the detailed observations that follow are confined to highlighting these differences.

9.2.1 Extrafusal muscle fibres:

Degeneration:

During the first three postoperative days, extrafusal fibres show degenerative changes similar to those described in series Isch. Autolysis and phagocytosis of the muscle fibres leave tubes of basal lamina that contain occasional satellite cells. Motor terminals also degenerate in the same way as described in series Isch (Fig. 6.18).

Regeneration:

As in series Isch, presumptive myoblasts were first seen at day 2, but regeneration proceeds more rapidly than in series Isch. At day 5, regenerating fibres are well-formed myotubes (Fig. 9.29). By day 8, they are mature muscle fibres comparable to those at the day-14 stage in series Isch (Fig. 9.30).

The sole plate also regenerates and was regularly observed despite the absence of reinnervating axon terminals (Fig. 9.31). Postjunctional folds, aggregated mitochondria, sole-plate nuclei and other subneural organelles are present as early as day 8. The presynaptic site is empty at day 8, but in later stages it is usually occupied by collagen fibrils and occasionally by cellular processes probably derived from Schwann cells. The sole-plate nuclei are irregular in shape at these later stages. The nerve trunks are packed with collagen fibrils and degenerating Schwann cells. By day 56, they have collapsed and contain fewer Schwann cells (Fig. 9.32).

Denervation atrophy:

Signs of denervation atrophy were first seen at day 21 in some extrafusal fibres. By day 56, the number of degenerating fibres has increased. The degenerative changes are similar to those described in

series Dn (9.1.1).

9.2.2 Muscle spindles:

Degeneration:

Following the combined operation, intrafusal fibres degenerate in the same way as in series Isch. The effects on sensory and motor terminals, capsule, periaxial space and axial sheath are also similar.

In one spindle examined at day 2, only two degenerating bag fibres are present. The chain fibres, together with their basal laminae, have degenerated and only a few remnants are left.

Regeneration:

As in extrafusal fibres, regeneration of intrafusal fibres proceeds more rapidly than in series Isch. The regenerating fibres are classified as thick and thin fibres according to their diameter. At day 5, the thick fibres consist of myotubes with occasional satellite myoblasts. In general, the thin fibres are similarly composed, but in some regions they consist of myoblasts contained within the preserved basal lamina. Collagen fibrils and basal-laminal strands have increased in density in the axial sheath (Fig. 9.33).

At day 8, both the thick and thin fibres are usually at the same stage of regeneration. They are round maturing muscle fibres with well-formed myofibrils and central nuclei (Fig. 9.34). This stage is comparable to the day-14 stage in series Isch.

At 14-56 days, regenerated intrafusal fibres become thinner with an irregular contour and lack equatorial nucleation. A regenerated fibre may show lateral extensions which often attach to an axial-sheath or capsule cell or another muscle fibre (Fig. 9.35).

Undifferentiated mononuclear cells are found in the axial bundle at day 5. They are enclosed by a basal lamina and have a high nucleo-cytoplasmic ratio. They are more numerous at the equator than the poles (Fig. 9.36). Additional muscle fibres were first seen at day 8 and are regularly observed at later stages (Fig. 9.37, 9.38). In some spindles, they have the same diameter as regenerated intrafusal fibres and it was difficult to distinguish between them without serial sectioning.

A regenerated subneural apparatus at the sites of former motor endplates was regularly observed. Schwann cells or their processes are usually present nearby but fail to contact the muscle fibres.

Bands of Büngner, which indicate Schwann-cell proliferation (see e.g. Schmalbruch, 1977), are present in the intramuscular and spindle nerve trunks and in all regions of the spindle. They are more numerous than in series Isch. They are also found beneath the basal lamina of the regenerated intrafusal fibres. They seem to enter through breaks in the basal lamina and are occasionally accompanied by processes of axial-sheath cells. These structures are more numerous at day 8 than day 5, and are greatly reduced at later stages particularly those associated with the intrafusal fibres (Fig. 9.39).

Denervation atrophy:

Because reinnervation is prevented, regenerated intrafusal fibres show signs of denervation atrophy which are similar to those described in series Dn (9.2.2) with the exception that these fibres lack equatorial nucleation.

One spindle examined at day 56 is strikingly similar to those of group 4B in series Isch (Fig. 9.40).

9.3 Effects of denervation following temporary ischaemia (series Isch/Rn/Dn):

Following the initial operation (devascularization), the muscle fibres degenerate and then regenerate (see chapters 6, 7 & 8). The second operation (denervation) was carried out at day 28 when reinnervation of the regenerated muscle fibres had started. Muscles were examined 14, 28 and 56 days after denervation.

Since the observations are generally similar to those described in series Dn and Isch/Dn, only the differences between this and the other studies are highlighted here.

9.3.1 Extrafusal muscle fibres:

At day 14, the extrafusal fibres are generally smaller than normal, some have normal structure whilst others are undergoing denervation atrophy. At 28-56 days, the atrophy is extensive.

As in series Dn and Isch/Dn, satellite cells are more prominent than normal and may be separated by a gap from the muscle fibre (Fig. 9.41).

Unusual small cells are observed in the interstitial space between the extrafusal fibres. They are more numerous in this and series Isch/Dn than in the Dn series. Some resemble satellite cells but lack any association with extrafusal fibres. Others are muscle cells at various stages of regeneration, but are much smaller than the ordinary extrafusal fibres. These unusual cells are shown in Fig. 9.42. They are similar to those reported by Schultz (1978) in denervated mouse muscle.

A compact thread of double basal lamina is observed to run around a number of extrafusal fibres and to have, in places, a close contact with their basal laminae. Small granules are interposed and produce

swellings at intervals along the thread (Fig. 9.43).

9.3.2 Muscle spindles:

As in series Dn and Isch/Dn, regenerated spindles of this series are more resistant to denervation atrophy than regenerated extrafusal fibres. These observations are generally similar to those described in the other control studies (this chapter; 9.1; 9.2). Axon terminals are absent, but subneural specializations are occasionally observed. All intrafusal fibres lack the typical equatorial nucleation and exhibit a distinct M-line in all regions of the spindle. They all show focal denervation atrophy.

Free mononucleated cells and additional muscle fibres are common and usually found within the axial bundle. Combinations of additional and original intrafusal fibres, satellite cells and Schwann cells with their processes are frequently found to be enclosed by a common basal lamina. Processes of axial sheath cells occasionally extend into these clusters. It is common to observe satellite cells well-separated from the intrafusal fibres but still enclosed by its basal lamina. Other satellite cells appear to be leaving the basal-laminal sheath (Fig. 9.44).

The changes in the capsule, periaxial space and axial sheath are similar to those in the regenerated spindles of series Isch/Dn (9.2.2). In one spindle examined at day 56, the periaxial space contained numerous erythrocytes, a few platelets and fibrin deposits (Fig. 9.45).

P A R T I V D I S C U S S I O N

Chapter 10: Discussion

The main purpose of this work was to study the effects of temporary ischaemia on rat muscle spindles. The effects of this lesion on extrafusal muscle fibres were also examined to estimate the efficiency of the technique used and as a guideline for the comparison of the changes between extrafusal and intrafusal fibres.

The process of extrafusal-fibre degeneration and regeneration that are produced by terminal devascularization are in general agreement with those reported by other investigators who used different techniques to achieve the temporary ischaemia and, to a lesser extent, to the studies that involved grafting techniques (for ref. see chapter 2; 2.2). The nature of extrafusal-fibre degeneration and regeneration was also analysed by these investigators. Therefore, only changes of particular importance to this study and those which have not been reported elsewhere will be highlighted in this discussion. Other changes are also mentioned in order to compare them with the changes in intrafusal muscle fibres.

10.1 Effectiveness of devascularization:

Serial transverse sections of soleus muscle 4 days after devascularization (Fig. 6.1) showed that the majority of extrafusal fibres were affected. The survival of peripheral fibres does not necessarily invalidate the use of this technique to achieve complete devascularization as superficial fibres commonly survive in grafted muscles (see e.g. Carlson, 1976). These fibres escape injury presumably because of the diffusion of oxygen and other substrates

from the tissues surrounding the operated muscle (Hanzlíková & Gutmann, 1979). Elimination of surviving fibres by myotoxic injections (Hall-Craggs, 1978) was not used in this study for two reasons: firstly, muscle spindles were not seen in the peripheral unaffected zone, and secondly, an extra lesion may be introduced using this technique in addition to the temporary ischaemia. The effects would not then be entirely ischaemic in origin.

10.2 Degeneration:

At 30 minutes after devascularization, enlarged mitochondria are aggregated at the periphery of extrafusal fibres. Their hypertrophy is regarded as a sign of the onset of degeneration. By 3 hours, mitochondrial changes are usually accompanied by changes in the contractile material, nuclei, sarcolemma and sarcotubular system.

A striking observation (particularly at 3-24 hours), is that extrafusal fibres vary in the extent of their degeneration; normal or almost normal fibres appear contiguous with those showing advanced degeneration. By the end of the first day however, all extrafusal fibres are affected, although not to the same degree, with the exception of the peripheral normal fibres discussed earlier. This lack of correlation between the degree of degeneration and the interval of time after injury may be explained by the following. Under normal conditions, only a small percentage of the muscle capillaries is open for circulation (Moore et al., 1956). Thus the devascularization initially affects muscle fibres which vary with respect to their oxygen reserves. In addition, extrafusal muscle fibres are also known to differ in the predominance of enzymes that control aerobic respiration (Yellin, 1969; Karpati et al., 1974).

Some fibres would therefore be better able to withstand anaerobic conditions compared with others.

10.2.1 Degeneration of extrafusal muscle fibres:

During the first five days after devascularization, extrafusal fibres undergo a process of degeneration that begins as early as 30 minutes with enlargement and accumulation of mitochondria at the periphery. At 3 hours, irreversible changes are observed which include: (1) formation of inclusions in mitochondria, (2) disruption of the sarcolemma, (3) loss of the Z-line and disorganization of myofibrils, (4) dilation of the sarcotubular system, and (5) nuclear pyknosis. These ultrastructural changes are regarded as definite early indications of the process of necrosis (Karpati et al., 1974).

Invasion of necrotic fibres by phagocytes was first seen at 6 hours when inflammation and haemorrhage is evident in the interstitial spaces. By day 3, the process of phagocytosis is completed within some extrafusal fibres and the phagocytes withdraw through the basal lamina. The timetable is variable since at the same time other muscle fibres, particularly those located at the centre of the muscle, are undergoing the initial stages of phagocytosis. This presumably reflects the variation in the onset of necrosis and degree of revascularization of different parts of the muscle. By day 4 however, the maximum degree of muscle damage has developed.

Two observations in this part of the study have not been reported elsewhere. Firstly, rod-like structures were occasionally found in tubules, probably sarcoplasmic in origin, at the periphery of degenerating extrafusal fibres. They are similar to those noted in the mitochondria and presumably form in the same way. Secondly, there is general agreement in the literature concerning the survival of the

basal lamina in degenerating muscle fibres (see e.g. Carlson, 1973). Destruction of the basal lamina is only reported by Stuart, McComas, Goldspink & Elder (1981) in their muscle-crush study. The present study confirms that the basal lamina of the majority of degenerating extrafusal fibres survives the ischaemia but in a few fibres it was fenestrated or absent as a result of which degenerating sarcoplasm spilled into the interstitial space. This may be caused by the retraction of the basal lamina from regions ruptured by the invasion of phagocytes.

Changes in motor end-plates are similar to those seen after denervation (e.g. Miledi & Slater, 1970). The axon terminals become enclosed by Schwann-cell processes, retract from the junction site and undergo ultrastructural changes following which they resemble "honeycomb" structures. The subneural apparatus degenerates in the same way as other regions of the muscle fibre. The basal lamina underlying the axon terminal remains intact and reflects the original junctional folds.

10.2.2 The nature of degenerative changes in the muscle spindle:

The effects of devascularization on the muscle spindle are generally similar to those described by Milburn (1975, 1976) following the administration of bupivacaine. The difference is the total involvement of individual muscle fibres rather than the focal necrosis of fibres noted in Milburn's study.

Degeneration of the intrafusal fibres is in parallel with that of extrafusal fibres and usually ends with empty tubes of preserved basal lamina. The rate of degeneration varies between different types of intrafusal fibres. The effects on the various components of the spindle during the course of degeneration will be discussed separately.

Intrafusal muscle fibres:

According to Karpati et al. (1974), ischaemia results in a shortage of substances needed by the muscle, in particular oxygen, glucose, fatty acids and other compounds. In addition, it results in a build up of metabolic end-products such as carbon dioxide and lactate within the muscle fibre. The lack of oxygen presumably has its affect on the muscle fibre by the reduction in the amount of high-energy phosphate compounds available to the muscle cell.

Mitochondria:

Three types of mitochondrial changes were observed; (1) increase in size of many mitochondria, (2) appearance of inclusions in the intermembranous space, and (3) modifications of the mitochondrial cristae. These changes were seen in both intrafusal and extrafusal fibres and are similar to those reported in ischaemic extrafusal fibres (e.g. Reznik & Hansen, 1969; Hanzliková & Schiaffino, 1977).

Enlargement and accumulation of peripheral mitochondria were seen as early as 30 minutes, but the appearance of dense osmiophilic bodies and rod-like structures was not seen until 3 hours. This probably reflects a temporal sequence in mitochondrial degeneration. A regional sequence may also be indicated by the restriction of the early degenerative changes to the peripheral mitochondria, whereas at a later stage almost all of the mitochondria are altered in severely-affected fibres.

Tubular cristae are known to be a common feature of enlarged mitochondria in the early stages of degeneration (Reznik & Hansen, 1969). Vesiculation of the cristae, which is common at day 2, is regarded by Hanzliková & Schiaffino (1977) to be a sign of advanced mitochondrial degeneration. These authors also described other signs such as clearing of the matrix and loss of the outer membrane. These

signs, however, were rarely encountered in this study.

By day 2, fewer mitochondria contain rod-like structures. This is consistent with the general reduction in the number of mitochondria, probably caused by autolysis and phagocytosis.

Sarcolemma:

Changes in the sarcolemmal complex of intrafusal fibres are generally similar to those described in extrafusal fibres in this and previous studies (Jirmanová & Thesleff, 1972; Karpati et al., 1974; Mäkitie & Teräväinen, 1977b).

Fragmentation and disappearance of the sarcolemma was seen as early as 3 hours in affected regions of intrafusal fibres. Up to day 1, the changes in the sarcolemma appear not to be correlated to the degree of muscle-fibre degeneration. Sarcolemma of less-affected fibres is occasionally absent, while it may be well-preserved in severely-affected ones. After day 1 however, the sarcolemma is usually absent and only remnants may be seen.

At both ends of the myotube region where the bag_1 fibre is usually closely associated with the bag_2 fibre, and the two chain fibres are similarly associated, dissolution of the sarcolemma leads to a direct mixing of their sarcoplasm.

As in extrafusal fibres, the basal lamina of the intrafusal fibres usually survives the degeneration process and subsequently plays an important role in the future regeneration. This is not always the case however, since in some spindles examined at 3-6 hours, the basal lamina of degenerated intrafusal fibres had also disintegrated prior to phagocytic invasion. This probably leads to the reduction in the number of intrafusal fibres in some regenerated spindles (see 10.4.4). Ruptures in the basal lamina were also seen at the later

stages of degeneration, and are presumably caused by phagocytic invasion.

Sarcotubular system:

The changes in the sarcotubular system of both intrafusal and extrafusal fibres are similar and were first observed at 3 hours. Dilation and fragmentation of the sarcotubular system results in numerous vesicular profiles of different size and shape. Many of these profiles are filled with a granular material. Similar structures are reported by Jirmanová & Thesleff (1972) in extrafusal fibres treated with methyl-bupivacaine. Moore et al. (1956) suggested that the dilation of sarcotubules and enlargement of mitochondria are probably caused by hypotonicity of the fluid entering the muscle fibre.

Contractile material:

In general, similar changes are observed in the contractile material of both intrafusal and extrafusal fibres. They were observed as early as 3 hours. The loss of Z- and I- bands is probably the earliest change and is followed by disorganization of the myofibrils. A-bands, with or without M-lines, may persist even after two days particularly in chain fibres and some extrafusal fibres. Previous reports (Moore et al., 1956; Stenger et al., 1962) also suggest that the A-bands are more resistant to the damage caused by ischaemia.

Hyaline degeneration, first seen at 6 hours, is more abundant in bag than in chain fibres. It is also reported in bupivacaine-treated intrafusal (Milburn, 1976) and extrafusal fibres (Hall-Craggs, 1974), in ischaemic muscle (e.g. Allbrook & Aitken, 1951), and in diseased muscle (e.g. Hudgson & Field, 1973).

Mitochondria are bigger and more numerous in the chain than in bag fibres. Moore et al. (1956) suggest that extrafusal fibres containing many mitochondria undergo "granular" degeneration, whilst

fibres with few mitochondria appear homogeneous or hyaline in the later stages of degeneration. However, the difference in the rate of degeneration between the bag and chain fibres is probably a reflection of the morphological and physiological differences between the intrafusal-fibre types.

Nuclei:

The nuclei of intrafusal and extrafusal fibres undergo a process of pyknosis similar to that reported in myotoxin-treated and ischaemic muscle fibres (Stenger et al., 1962; Jirmanová & Thesleff, 1972; Karpati et al., 1974; Milburn, 1976; Mäkitie & Teräväinen, 1977b). This process consists of changes in the chromatin and nuclear membrane. The chromatin becomes condensed and usually accumulates at the periphery. Later it dissolves and mixes with the sarcoplasm. The nuclear membrane dilates and breaks into short tubules, vesicles and vacuoles. By day 2, all of the nuclei in the intrafusal fibre have degenerated.

The loss of the equatorial nucleation of the intrafusal fibres cannot be attributed to the degeneration of the sensory terminals but is a direct ischaemic affect on the muscle fibre. This is suggested by the fact that not only the equatorial nuclei degenerate, but also all of the muscle-fibre components. If one assumes it is due to sensory-terminal degeneration, then pyknosis should take weeks rather than days as is the case in denervated non-ischaemic spindles (series Dn).

Innervation:

Changes in the sensory terminals first appear at 3 hours. These include enlargement of mitochondria and disruption of their cristae, appearance of myelin figures and autophagic vacuoles,

clumping of the axoplasm and fragmentation of axolemma. Fragmentation and dissolution of the axolemma and sarcolemma results in the mixing of the contents of the nerve and muscle cells. The changes in sensory terminals are not necessarily comparable with those of the intrafusal fibres they innervate. By day 3 however, all of the sensory terminals have degenerated. Similar observations are reported by Milburn (1976) in bupivacaine-treated muscle spindles.

The effects of ischaemia on the motor terminals appear to be different from those on the sensory terminals. Initially, some motor axon terminals are changed. As well as normal synaptic vesicles, they contain enlarged mitochondria, autophagic vacuoles and occasional lipid droplets. Other terminals are of normal structure even at day 2. Engulfment of motor terminals by Schwann cells was first seen at 6 hours and is widespread at the later stages. Engulfment is followed by the conversion of the axon terminal to a dense granular material similar to the "honeycomb" structure described in denervated muscle (e.g. Miledi & Slater, 1970). In some degenerating neuromuscular junctions, basal lamina is absent from the junctional region and only Schwann-cell or macrophage processes are present. This is seen only in muscle spindles and not in extrafusal fibres. Disintegration of basal lamina at neuromuscular junctions has not been reported elsewhere and the reason for this effect remains unclear. However, the presence of Schwann cells and macrophages in such regions may be linked with the destruction of the basal lamina.

At 3-12 hours, some preterminal (motor and sensory) axons and sensory terminals are swollen and electron-lucent. A few remnants of axoplasm may be present. Similar observations are reported following the injection of the black widow spider venom in mouse muscles (e.g. Gorio, Hurlbut & Ceccarelli, 1978; Duchen & Queiroz,

1981) and after denervation (e.g. Ohmi, 1961; Manolov, 1974; Winlow & Usherwood, 1975; Bixby, 1981). Two explanations have been given: (1) it is due to physical retraction (Bixby, 1981), which is the primary means by which synapses are removed, and (2) the change in electron-density, which is accompanied by an increase in the axonal diameter, suggests not only a degeneration of the axoplasm but also a rise in the water content (Ohmi, 1961).

Axons in the spindle and nerve trunks are surrounded by activated Schwann cells. The axoplasm is either absent or changed into a dense material similar to the honeycomb structures described earlier in degenerating motor terminals. The cytoplasm of Schwann cells has increased in volume and contains numerous organelles with occasional lipid droplets. Similar changes were seen in the perineurial cells of the nerve trunks. The ability of Schwann cells to engulf motor terminals and nerve axons is seen in ischaemic muscle and is similar to that reported in denervated muscle (e.g. Miledi & Slater, 1970).

Two observations suggest that ischaemia has a direct effect on the innervation of the spindle. Firstly, not only the nerve terminals degenerate but also all of the axons in the spindle and intramuscular nerve trunks. Secondly, reinnervation is delayed to day 21 at a time when both extrafusal and intrafusal fibres have regenerated. These observations suggest that the degeneration of the nerve terminals and axons in particular is primarily due to a direct ischaemic effect. An indirect effect mediated by the muscle fibre, as suggested by Milburn (1976) would more likely affect the axon terminals. If the latter is the only reason, then the nerve axons would be damaged only for a short length and would subsequently reinnervate the muscle fibres more rapidly.

Capsule, periaxial space and axial sheath:

During the first few days after devascularization, vacuolation of the capsule and axial-sheath cells is common. The space between the capsule layers is dilated and may contain erythrocytes and other inflammatory cells, which are also seen in the periaxial space and axial sheath. Pyknotic nuclei were only observed in a few capsule and axial-sheath cells. Clusters of vacuole-like structures, containing flocculent material, were sometimes observed in the periaxial space. The nature of this flocculent material is unknown, but it is probably formed by condensation of periaxial fluid.

Despite these changes, the capsule and axial sheath survive the temporary ischaemia. The survival of the spindle's capsule is also reported in bupivacaine-treated (Milburn, 1976) and grafted muscles (Schmalbruch, 1977; Rogers & Carlson, 1981).

10.3 Revascularization:

Observations made at the time of dissection of the muscle and from serial sections indicate that revascularization of the devascularized muscle is similar to that of freely-grafted muscles (e.g. Carlson et al., 1979).

Starting at the second and third postoperative day, large vessels were seen penetrating the muscle periphery. According to Hansen-Smith, Carlson & Irwin (1980), these vessels rapidly differentiate into capillaries, arterioles, and venules which progressively grow in to reach the centre of the muscle by about the fifth day.

The increase in the number of capillaries noted in this study is consistent with that described by Mäkitie (1977) who suggests that it is an adaptation to the high energy demands of muscle regeneration.

This might also explain the increase in number of capillaries associated with the spindle's capsule and also the presence of some capillaries in the periaxial space observed after revascularization.

As the ingrowing capillaries pass the surviving muscle fibres and enter the ischaemic region, large numbers of phagocytes penetrate the basal lamina of the necrotic fibres. This results in the removal of the old sarcoplasm. At this time, mononuclear myogenic cells become activated beneath the original basal lamina. By day 4, the zone of phagocytosis and myoblastic activation has moved into the centre of the muscle. More peripherally is a region of myotubes that displays a gradient of decreasing maturity from the periphery toward the centre. By the end of the first week, most of the muscle is filled with immature regenerating muscle fibres. As muscle spindles are located in different regions of the muscle they are presumably affected by this gradient (see 10.5.2).

10.4 Regeneration:

According to Carlson et al. (1979), there is a very close relationship between the removal of old sarcoplasm and the progression of the regenerative process, once the satellite cells are activated. At 2-3 days, activated satellite cells or presumptive myoblasts are present beneath the basal lamina of both intrafusal and extrafusal fibres when phagocytosis of old sarcoplasm may still be taking place.

Regeneration of new muscle fibres from mononucleated myoblasts has traditionally been referred to as "discontinuous" regeneration in contrast to "continuous" regeneration, which involves the outgrowth of multinucleated sarcoplasm from viable muscle fibres (e.g. Reznik, 1973). In ischaemic muscle, discontinuous regeneration is the most

common form in extrafusal fibres. Continuous regeneration was observed in a few partially damaged extrafusal fibres.

As stated by Carlson (1973), the stages of development in regenerating muscle fibres are quite similar to those which have been described during embryonic development in that myotubes are formed by the fusion of myoblasts. The main differences lie in the presence of satellite cells and preserved basal lamina in regenerating myotubes.

10.4.1 Extrafusal muscle fibres:

At 3-4 days, regenerating extrafusal fibres consist of a bundle of myoblasts and nascent myotubes enclosed in the original basal lamina. By day 5, myotubes are widespread. Well-formed myotubes, with central nuclei, together with occasional myoblasts and satellite cells are all enclosed by the old basal lamina. A tube of basal lamina may occasionally contain two separate myotubes. As described by Vracko & Benditt (1972), at about this stage a new layer of basal lamina appears along portions of the plasma membrane where it is not in apposition to the old basal lamina. In areas where plasma membrane is in close proximity to the old basal lamina, a new basal lamina does not form. The result is focal duplication of basal lamina. Regenerated muscle fibres with two leaflets of basal lamina, as described by Schmalbruch (1977), are only occasionally observed.

By day 14, the myotubes have matured into muscle fibres. These differ from normal extrafusal fibres in their irregular shape, small diameter and central nuclei. From day 21 onward, the number of extrafusal fibres of normal diameter increases, although some fibres remain small even after 182 days. Some of these small fibres may

form in the same way as some additional muscle fibres (see 10.4.3). Most nuclei become peripheral, but some remain central in position. Satellite cells lying at the periphery of extrafusal fibres were seen at all stages.

Repair of partially-damaged extrafusal fibres is accomplished in two ways: (1) from myoblasts of unknown origin (see Reznik, 1969), (2) by the extension of intact sarcoplasm into the damaged area.

Subneural specializations of the motor endplates regenerate in the absence of axon terminals and reinnervation of extrafusal fibres was first seen at day 21 (see 10.4.6).

10.4.2 Intrafusal muscle fibres:

Since they lack many of the characteristic features of normal intrafusal fibres, regenerating fibres are classified as thick or thin fibres representing the original bag and chain fibres, respectively. Regeneration proceeds more rapidly in the thick than in the thin fibres. This may well be due to the rapid degeneration and early removal of necrotic debris in the bag fibres compared with the chain fibres. This is supported by the reported close relationship between the removal of old sarcoplasm and the progression of regeneration in extrafusal fibres (Carlson et al., 1979). Activated satellite cells were seen in both types of intrafusal fibre regardless of whether phagocytosis was completed or not. Moreover, different regions of individual intrafusal fibres were seen to be at different stages (myoblast or myotube) of regeneration depending on the presence or absence of necrotic debris.

At 3-4 days, the thick fibres are composed of nascent myotubes and myoblasts contained within the original basal lamina. The thin

fibres consist of myoblasts only. At 5-6 days, the thick fibres are composed of well-formed myotubes while the thin fibres consist of nascent myotubes. The thin fibres subsequently increase in diameter and display a stage of regeneration similar to that of the thick fibres. By day 14, all intrafusal fibres are maturing muscle fibres and are similar in diameter. They all exhibit a distinct M-line in all regions of the spindle. As in extrafusal fibres, a new basal lamina has formed only in places where the regenerating fibre is not in contact with the old one. By day 21, the muscle fibres are fully regenerated, but are irregular in shape with occasional central nuclei. The development of equatorial nucleation appears to be related to the extent to which the fibre is reinnervated by the Ia axon (e.g. Zelená, 1957).

10.4.3 Additional muscle fibres:

In spindles examined before reinnervation has occurred (14-21 days), and in those (21 days onward) only reinnervated by motor axons, additional fibres are usually thicker, longer and more numerous than in the other spindles. In general, additional fibres are found in all regions of the spindle; lying free in the periaxial space, within the axial sheath, or between the capsule layers; or associated with the original fibres, axial-sheath, or capsule cells. Most additional fibres lack any kind of innervation. Sensory terminals were seen only in a few of those associated with original fibres.

The location of the additional fibres is probably related to the way in which they formed. These observations suggest that they originate from more than one source, as follows:

I Additional fibres associated with original fibres:

On the basis of different observations, three ways of additional-

fibre formation may be distinguished:

a) Detachment of myotubes:

This was first noted by Schmalbruch (1977, 1979) in regenerating rat muscle. In this study, a common basal lamina was seen enclosing an original fibre, one or more additional fibres and occasionally satellite cells. Such clusters may arise from muscle cells that failed to fuse during the regeneration process.

At the equator, Schwann cells and myelinated axons may be included in such clusters, having entered through the entry-sites of original sensory axons, or through breaks in the basal lamina where occasional cellular processes from the axial sheath and collagen fibrils had intruded.

b) Branching of muscle fibres:

Branching fibres were first reported in regenerated extrafusal fibres (e.g. Walton & Adams, 1956; Schmalbruch, 1976a, 1979; Ontell, Hughes & Bourke, 1982) as an alternative proposal to muscle-fibre splitting. In this study, a regenerated intrafusal fibre was seen to branch into two daughter fibres each with its own basal lamina. One of these fibres (the additional fibre) is shorter and usually thinner than the other (original) fibre. Further branching and recombination was occasionally observed. No accumulations of specialized organelles were seen in the branching region. The cleft in between may contain processes from axial sheath cells. Similar branching in extrafusal fibres was also seen in this study and a capillary was occasionally observed in the cleft.

c) Splitting of muscle fibres:

Muscle-fibre splitting, as described by Hall-Craggs & Lawrence (1969, 1970) and Hall-Craggs (1970, 1972), was rarely observed in regenerated spindles. In a few instances, two contiguous fibres

appeared to arise from a longitudinally-split fibre. Serial sectioning revealed that they consisted of two original intrafusal fibres laterally fused for a short distance. The fused region probably coincides with the region of close-apposition between pairs of fibres seen in normal spindles. During degeneration the sarcolemma degenerates, so that the two fibres are contained in a common basal lamina. A single myotube forms in this region at regeneration and is retained as a single fibre when regeneration is complete. Satellite cells or specialized organelles were not seen in the region of fusion.

Splitting seems to be a feature of some of the free additional fibres. A strand of mitochondria is often present in the region of the cleft that partially divides the fibres. One cytological mechanism proposed for longitudinal division of mature fibres proposes that clefts are formed by fusion of intracellular membranes and that mitochondria and other sarcoplasmic elements are assembled at the place where the fibre is to be split (Hall-Craggs, 1970; Hall-Craggs & Lawrence, 1970). The alternative explanation is that these mitochondria had been subsarcolemmal and now mark the site of lateral fusion of two fibres (e.g. Schmalbruch, 1979).

II Additional fibres lying free or associated with the capsule and axial sheath:

Additional fibres occasionally lie independently in the periaxial space. Others are found within the axial sheath, either independently, encircled by cellular processes, or attached to axial-sheath cells. Those within the capsule are found either between the capsule layers or in continuity with the capsule cells, often sharing the same basal lamina. Some of the additional fibres of this group lack a

basal-laminal sheath.

The origin of these additional fibres is uncertain. In the series Isch, they were first observed at day 14, while in series Isch/Dn they are present as early as day 8. Comparison between the two series shows that the later stages of regeneration (maturation) of the intrafusal fibres proceeds more rapidly in denervated ischaemic muscles. Maturation involves an increase in the diameter of the regenerating muscle fibres. As maturation proceeds, myotubes of a large diameter may exert pressure on the smaller muscle cells, some of which may be forced out of the basal-laminal sheath. Destruction of the old basal lamina (observed during degeneration and regeneration) would facilitate such a process.

A second possible source of the additional fibres is from the migration of satellite cells (see 10.4.5). In vitro, Bischoff (1979) has shown that some migrating cells fuse to form myotubes outside the muscle fibre. The random location and distribution of the additional fibres in regenerated spindles may reflect the random dispersal of such satellite cells.

The origin of those additional fibres closely-associated with axial-sheath and capsule cells, and those enclosed by the basal lamina of the latter, is more obscure. If they do not stem from migrating satellite cells, then is it possible they originate by the redifferentiation of circulating, endomysial or perivascular cells (see e.g. Bateson, Woodrow & Sloper, 1967; Partridge & Sloper, 1977).

Often, small muscle fragments are found next to a larger regenerated intrafusal or extrafusal fibre, enclosed by an individual basal lamina. The structure of these muscle fragments is similar to that of the large fibre. They are likely to be projections of the large fibre that appear separated in transverse sections.

Small muscle fibres (usually immature) are often found in the interstitial space between extrafusal fibres. They probably stem from the undifferentiated cells observed at day 5, which have their origin in satellite cells that migrate through broken basal lamina of extrafusal fibres during degeneration. This type of muscle fibre is also reported by Schmalbruch (1976b) in rat muscle regeneration following the injection of hot Ringer solution.

10.4.4 The role of basal lamina:

The results of this and other muscle-regeneration studies are in general agreement with respect to the persistance of the basal lamina during degeneration of the muscle fibre. The removal of the necrotic material, which is essential for regeneration, is carried out by phagocytes. These cells move inside the basal lamina tube and leave it after phagocytosis is complete. According to Vracko & Benditt (1972), phagocytes do not seem to disturb the integrity of the basal lamina. Bischoff (1979) has effected the removal of the basal lamina, in vitro, by enzyme-hydrolysis. Whether the phagocytes invade the muscle fibres in the same way is unknown. However, phagocytes were observed passing through breaks in the basal lamina (Fig. 8.35 and Karpati et al., 1974). Moreover, this study shows that the basal lamina of some fibres may be fenestrated or destroyed before phagocytic invasion. The degenerating sarcoplasm of a few extrafusal fibres is seen to extend into the interstitial space in regions where basal lamina is absent. Serial transverse sections of muscle spindles showed that some intrafusal fibres can completely degenerate together with their enclosing basal lamina. Why the basal lamina should persist in most areas and degenerate in others remains to be answered.

However, an intact tube of basal lamina survives following the degeneration of most muscle fibres. New muscle fibres regenerate within these basal-lamina tubes. As suggested by Vracko & Benditt (1972), the formation of a new basal lamina appears to be related to the distance which exists at a certain stage of regeneration between the sarcolemma of the newly-formed muscle cell and the old basal lamina. A new basal lamina forms only in those areas where the old one lies some distance from the sarcolemma. Where this occurs, the muscle fibre is delineated by two layers of basal lamina. This implies that where the old basal lamina lies next to the sarcolemma, it inhibits the formation of a new one.

In a few muscle fibres, rings of basal-lamina material are associated with the old basal lamina, where a new basal lamina has formed. The centre of each ring is occupied by electron-opaque granules or a single clear vesicle. This observation is more common in regenerated extrafusal and intrafusal fibres of series Isch/Dn. In long-term denervated extrafusal fibres (series Dn), the rings are found in a double-layered basal lamina which loosely surrounds two or more muscle cells. The significance of these observations is unknown. It may represent a mean by which the muscle fibre rejects unwanted materials. Similar observations are reported by Miledi & Slater (1969) and Schmalbruch (1976).

10.4.5 The role of satellite cells:

Satellite cells, as originally described by Mauro (1961), were frequently observed in this study associated with both normal extrafusal and intrafusal fibres. Following devascularization, changes such as the formation of lipid droplets were only seen in some satellite cells. Degenerating satellite cells were never

observed in this study. The survival of satellite cells is probably due to their separation from the muscle fibre (Church, Moronha & Allbrook, 1966). Many satellite cells appear separated by an empty cleft from the muscle fibre, sometimes as early as 30 minutes after devascularization. This seems to be the first stage in their migration. The same was seen in long-term denervated control muscles. In ischaemic muscle the destruction of the basal lamina of some fibres facilitates the next stage of satellite-cell migration, outside the muscle fibre. In vitro, Bischoff (1974; 1975; 1979) noted that satellite cells leave the muscle fibre if its basal lamina is digested by certain enzymes. Other reports (Teräväinen, 1970; Konigsberg, Lipton & Konigsberg, 1975; Schultz, 1978) also support the hypothesis that satellite cells migrate from existing muscle fibres. The undifferentiated cells observed within the axial sheath, and occasionally in the interstitial space, at day 5 probably originate from migrated satellite cells.

By day 2, the cytoplasm of satellite cells has increased in volume. No visible myofilaments are found in these activated satellite cells. Because of the controversy about the origin of the myoblasts, Carlson (1973) suggested the use of the term "presumptive myoblast" instead of activated satellite cell. According to Carlson, the main difference between the typical and presumptive myoblasts is the absence of visible myofilaments from the latter.

Satellite cells undergoing mitosis were not seen in this study. At 2-3 days, a mass of cytoplasm is found to contain two or occasionally four rounded nuclei. According to Reznik (1969), similar multi-nucleated cells have formed by fusion of myoblasts. The breaking-off of myonuclei to give rise to myogenic cells, as described by Reznik (1970), was never observed in this study. On the contrary, the

degeneration of all myonuclei, the increase in cytoplasmic volume of satellite cells, the appearance of presumptive myoblasts and then typical myoblasts, are all in support of the general concept that the satellite cell is the origin of myoblasts in regenerating muscle.

10.4.6 Reinnervation:

Proliferation of Schwann cells is evident at day 5 when numerous cellular processes are present particularly at the equatorial region. Schwann-cell processes (in the form of bands of Büngner) are found in groups enclosed by basal lamina. They are also present beneath the basal lamina of regenerating intrafusal fibres probably occupying the former sites of sensory terminals.

This study has shown that the postsynaptic elements of the motor terminals degenerate like other parts of the muscle fibre. The basal lamina remains intact at most neuromuscular-junction sites. During the regeneration of muscle fibres, the postsynaptic elements also regenerate despite the absence of nerve terminals. Other studies (e.g. Sanes, Marshall & McMahan, 1978; Burden, Sargent & McMahan, 1979; Bader, 1981) showed that the biochemical and structural organization of the subsynaptic membrane in regenerating muscle is directed by structures (namely the basal lamina and Schwann cells) that remain at synaptic sites after removal of the nerve.

Few regenerating axons were observed at day 14 inside the intramuscular nerve trunks. Reinnervation of extrafusal and intrafusal fibres were first seen at day 21. By day 28, maximal reinnervation has occurred and the number of terminals does not seem to increase later. Regenerated sensory and motor terminals exhibit normal structure. Restoration of motor terminals appears more successful than that of

sensory terminals (see 10.5.1). However, not all extrafusal and intrafusal fibres become reinnervated. A number of muscle fibres, of both types, were observed undergoing denervation atrophy. Axon terminals were absent from these fibres despite persisting postsynaptic elements.

Regenerated spindles usually contain varying numbers of abnormally-located nerve terminals (see 8.2.3). Presumably they failed, for some reason, to innervate regenerated intrafusal fibres (see 10.5.1). At the equator, they are numerous, lack Schwann cells and some lack a basal lamina. At the poles, they are few in number and enclosed by Schwann cells.

Some regenerated spindles (that lack sensory or sensory and motor nerve terminals on their intrafusal fibres) contain a larger number of axons than normal (see 10.7).

10.4.7 Capsule, periaxial space and axial sheath:

The thickening of the capsule and the reduction of the periaxial space that occurs during regeneration (see chapter 8; 8.2.4) appear to be reversible. Restoration of their normal appearance coincides, and is probably linked to, the reinnervation of the spindle (see 10.5.1). The changes in the axial-sheath cells are also reversible. The increased amounts of collagen and elastic fibrils and basal-lamina profiles, that were observed during regeneration, are retained within the axial sheath. Regenerated spindles commonly enclose larger numbers of capillaries than normal. Similar observations are reported by Schmalbruch (1977) in grafted muscle.

10.5 The regenerated spindles:

Regenerated spindles are classified into four groups according to the extent of their sensory reinnervation and the restoration of equatorial nucleation (see chapter 8; 8.3). The regeneration of intrafusal muscle fibres is mostly completed before reinnervation has occurred. A comparison of the spindle's structure before and after reinnervation, and between individual intrafusal fibres in different regenerated spindles, shows that intrafusal-fibre differentiation may be controlled by the timing and extent of their reinnervation.

10.5.1 The role of reinnervation in the restoration of characteristic features:

Before reinnervation, regenerated intrafusal fibres differ from extrafusal fibres only with respect to their small size and intracapsular location. They lack equatorial nucleation and exhibit a distinct M-line in all regions. Reinnervation results in the further differentiation of these fibres.

Differences between individual intrafusal fibres in terms of the extent of reinnervation and differentiation are probably caused by the interaction of three factors: (1) the time of the arrival of reinnervating sensory and motor axons, (2) the regeneration stage of the intrafusal fibres at the time of reinnervation, and (3) the location of the spindle in the muscle.

There appears to be a specific period during the regeneration of intrafusal fibres, after which the Ia afferent particularly is unable to exercise an influence on intrafusal-fibre differentiation. The bag fibres degenerate more rapidly than the chain fibres and they are the first to regenerate (see chapters 7 and 8). When the sensory

axons arrive at the spindle, regeneration of the bag fibres is more advanced than that of the chain fibres. Therefore, the Ia afferent may exert a greater influence on the chain fibres than on the bag fibres.

The restoration of equatorial nucleation requires all of the intrafusal fibres to be at the appropriate stage of regeneration when the Ia afferent reinnervates. If the arrival is delayed, then the axon may not be able to influence the further equatorial differentiation of some or all of the intrafusal fibres even if sensory terminals are established. The rate of regeneration is different not only between bag and chain fibres within the same spindle, but also between spindles located at different zones of the muscle (see next section). Analysis of the four groups of regenerated spindles supports the above hypothesis.

In the spindles of group 1, reinnervation and muscle-fibre regeneration are synchronized. The spindles of this group thus appear normal. In the spindles of group 2, although the sensory axons form terminals on all fibres, because of their relative delay in reaching the spindles, they influence the equatorial differentiation of the thin "chain" fibres but not the thick original bag fibres. In the spindles of group 3, the Ia afferent is further delayed in its arrival, therefore the sensory terminals are mostly confined to the thin "chain" fibres which have poorly-developed nuclear chains and the thick fibres lack equatorial nuclei. Lastly, in the spindles of group 4, sensory reinnervation occurred very late in regeneration thus preventing the establishment of sensory terminals on any intrafusal fibres, which lack equatorial nucleation.

It appears that the restoration of regional variations in the

M-line structure may also be linked to the extent to which the spindle's normal innervation is restored. In the normally innervated spindles of groups 1 and 2 the bag fibres exhibit the normal variations in the M-line condition. In group 3 spindles, although the thick original "bag" fibres usually lack sensory terminals, they exhibit the normal variations in the M-line condition. The reason for this is unclear. However, these fibres have abnormally-developed motor terminals. In the spindles of group 4 which lack sensory terminals, the "intrafusal" fibres retain a distinct M-line in all regions. In addition, their motor end-plates exhibit elaborate postjunctional folds, similar to those seen in extrafusal fibres. There may be two alternative explanations for these observations: (1) the branches of β axons that originally innervated extrafusal fibres may have reinnervated intrafusal fibres, (2) branches of α motor axons may have invaded the spindle to reinnervate intrafusal fibres (see e.g. Schröder, 1974a; Barker & Boddy, 1980).

Moreover, spindles that lack sensory innervation retain the reduced periaxial space that is characteristic of most regenerating spindles before reinnervation begins.

It appears that the failure of sensory and motor axons to innervate intrafusal fibres does not prevent them from terminating elsewhere. The "free" nerve terminals seen in ischaemic regenerated spindles may represent the endings of reinnervating axons that have failed to reach their targets.

10.5.2 The role of location in the restoration of characteristic features:

Because of the progressive nature of revascularization, the

muscle fibres are subjected to different periods of ischaemia. Muscle fibres located at the muscle centre are subjected to a longer period of ischaemia than those at the periphery. Similarly, phagocytosis of necrotic fibres begins at the periphery and then spreads toward the centre (see 10.3). As stated by Carlson et al. (1979), there is a close spatial and temporal relationship between the removal of old sarcoplasm and the regenerative process once the satellite-cell population is activated. Subsequently, the regenerating muscle cells display a gradient of decreasing maturity from the periphery toward the muscle centre. The regeneration of the muscle fibres at the centre is delayed for about two days compared with those at the periphery. The spindle population is also likely to be affected by this regeneration gradient. In addition, individual spindles have their own internal gradient since the bag fibres start to regenerate before the chain fibres.

The mode of nerve ingrowth following temporary ischaemia requires further study. However, regenerating axons were first seen at day 14, although they may have invaded the muscle earlier than this. Nerve terminals were first seen to innervate intrafusal and extrafusal fibres at day 21. Allbrook & Aitken (1951) have also reported that extrafusal motor end-plates are not visible until day 21 after devascularization. They also doubted further recovery after day 28 owing to the development of fibrous tissue between muscle fibres.

At the time of sensory reinnervation, the thick original bag fibres are generally more mature than the thin original chain fibres in individual spindles. Similarly, the intrafusal fibres of peripheral spindles are generally more mature than those in the

centre, with a range in between. If the suggestion that the ability of regenerating muscle fibres to receive nerve terminals decreases with time is true, then the Ia afferent will not form terminals on the more mature intrafusal fibres of those spindles located most peripherally. Spindles of group 4 would result from these conditions. In contrast, the fibres of spindles located at the muscle centre would be less mature at the time of sensory reinnervation allowing the formation of nerve terminals and the subsequent differentiation of all intrafusal fibres. The result would be the fully restored spindles of group 1. Incomplete differentiation of "bag" fibres in group 2 spindles may be related to their advanced maturity compared with the "chain" fibres of the same spindle. Spindles of group 3 are probably located more peripherally than those of group 2. Consequently, their "bag" fibres are more mature and prevent the formation of sensory terminals. In the same way, their "chain" fibres are more mature than those of the group 2 spindles, and are thus incompletely differentiated (Fig. 10.1).

The number of spindles in group 3 is higher than those of other groups (8 out of 18 spindles). The reason for this is probably related to the distribution of the spindles in the muscle core.

10.6 Comparison with control experiments:

10.6.1 General degenerative changes:

Comparison of series Isch and Isch/Dn show that the process of degeneration are similar in both in all respects, indicating that the rapid degeneration of muscle fibres is a result of devascularization and not denervation. On the other hand, the degeneration

of nerve terminals is not enhanced by denervation suggesting that the changes observed in series Isch are purely ischaemic. However, the degeneration of nerve axons is probably enhanced by denervation.

Comparison between series Isch and series Dn shows that their effects on muscle fibres differ. In ischaemic muscle, degeneration develops within a few hours and ends by the fifth day at most, while in denervated muscle, muscle-fibre atrophy is first seen at the end of the third week and proceeds slowly and gradually for a very long period (a year or so).

Ischaemia causes necrosis and fragmentation of the sarcoplasm followed by a process of phagocytosis which results in the removal of necrotic debris. Denervation causes atrophy and gradual disintegration of the myofibrils resulting in a decrease in the diameter of the muscle fibres. The basal lamina survives following both types of lesion, as do the satellite cells. The sarcolemma degenerates in ischaemic muscle fibres whereas it persists following denervation where it shows indentations and invaginations into the sarcoplasm of the denervated fibre. The nuclei of ischaemic fibres undergo rapid pyknosis which leads to their complete disintegration. Following denervation they become indented and often appear to engulf sarcoplasmic material. In long-term denervated extrafusal muscle fibres the nuclei become more prominent, centrally-placed and appear to increase in number.

The changes in denervated intrafusal fibres differ from those in extrafusal fibres. They lose the normal features of their equatorial region (see next section). Only focal atrophy was noted in some intrafusal fibres, even one year after denervation.

10.6.2 The role of sensory terminals:

The role of the sensory reinnervation in the restoration of the normal equatorial region in regenerating spindles is discussed earlier (10.5.1). The events that follow the degeneration of the sensory terminals in denervated spindles are the reverse of those following reinnervation in regenerating spindles. Following denervation, the nuclei of the equatorial region undergo a gradual decrease in number and are no longer present at day 56. This process is accompanied by a progressive development of myofibrils, which fill the fibre. The bag fibres, in particular, show a distinct M-line in the newly-formed myofibrils, as in regenerated spindles prior to reinnervation.

These observations indicate: (1) that the rapid degeneration of the nuclear bags and chains following devascularization is primarily due to a direct ischaemic effect. If it was a result of a secondary effect mediated by the degeneration of nerve terminals then the nuclear degeneration would have a longer time course; (2) that the maintenance of the normal ultrastructural features of the equator, including M-line conditions of bag fibres, requires the permanent presence of nerve terminals.

10.6.3 The nature of the increase in number of intrafusal fibres:

In all of the experimental series (Isch, Isch/Dn, Isch/Rn/Dn & Dn), the spindles contain varying numbers of muscle fibres. Some of these are regenerated intrafusal fibres while others are added as a result of regeneration or denervation. In all of the series, the additional fibres are usually short and thin. Some may attain the diameter of the original regenerated intrafusal fibres but never attain the same length.

As discussed before (10.4.3), the additional fibres in series Isch spindles may have originated by the branching of regenerating intrafusal fibres, by the detachment of myotubes, or the maturation of migrated satellite cells.

In spindles of series Dn, two types of additional fibre were observed that may have a different origin. Firstly, in short-term denervation (21-56 days) small additional fibres are seen within the axial bundle separate from the intrafusal fibres. Undifferentiated mononucleated cells were also found at day 21 but not later. These cells may have originated from migrated satellite cells. Therefore, the additional fibres probably formed by maturation of such cells. Secondly, in long-term denervation (156-364 days), additional fibres are longer, thicker and appear to form by the splitting of the intrafusal fibres. Cells were often observed interposed between additional and intrafusal fibres. They resemble the satellite cells and are probably involved in the process of muscle-fibre splitting as suggested by Miledi & Slater (1969).

Similar kinds of additional fibres are reported by Schröder (1974a) in denervated and reinnervated rat lumbrical muscles. The present study also supports Schröder's suggestion that the increase in the number of intrafusal fibres is due to denervation rather than reinnervation. On the other hand, Arendt & Asmussen (1976), in a light-microscope study of rat soleus, reported that the number of the bag fibres (but not the chain fibres) increases significantly in long-term denervated spindles. In a histochemical study, Kucera (1977) also observed the two kinds of additional fibres in denervated rat spindles. He attributed the formation of the second kind of fibre exclusively to the splitting of the bag_1 fibre.

In this ultrastructural study, it was not possible to identify the intrafusal-fibre types in long-term denervated spindles because of the loss of many characteristic features (see 10.6.2). However, the additional fibres are not associated with any particular intrafusal fibre. For instance, a single transverse section may contain two or more additional fibres each associated with different intrafusal fibres.

In his study of grafted muscle, Schmalbruch (1977) also observed an increase in the number of intrafusal fibres in regenerated spindles. On the other hand, Milburn (1976) found no evidence of such an increase following the administration of bupivacaine, although she noted the presence of "myogenic fragments" within the axial bundle which may correspond to the thin and short additional fibres observed in this study. An increase in number of intrafusal fibres is also reported in rat denervated spindles (Gutmann & Zelená, 1962; Maynard & Tipton, 1971) and in human spindles of diseased muscle (e.g. Swash, 1972).

This raises the question of how different investigators carried out the intrafusal-fibre counts in regenerated or denervated spindles. For Milburn (1976), the myogenic fragments were not included in the count, but only those fibres continuous throughout the length of the axial bundle. By transverse serial sectioning of the spindle, it was possible in this study to follow the individual fibres. By doing so, the number of fibres that run throughout the capsule length always corresponded with the average number of intrafusal fibres found in normal spindles. In contrast, additional fibres are much shorter than the original intrafusal fibres. The expression "increase in number of intrafusal fibres" may give the impression that the additional fibres have a comparable length with the original

intrafusal fibres. Therefore, the term "additional fibres" is found more appropriate in this study.

10.6.4 Denervation atrophy in intrafusal fibres:

As noted above, intrafusal fibres are more resistant to denervation atrophy than extrafusal fibres. Only focal changes were observed in some intrafusal fibres of series Dn. Because they are denervated, intrafusal fibres of series Isch/Dn and Isch/Rn/Dn also showed atrophic changes. In series Isch, focal atrophy was only seen in intrafusal fibres that failed to be reinnervated (subgroup 4B).

The resistance of the intrafusal fibres to denervation atrophy has been reported by many investigators (see chapter 2; 2.1.1). According to de Reuck et al. (1973) this resistance could be due to structural or metabolic differences between extrafusal and intrafusal fibres.

The decrease in the number of original intrafusal fibres observed in a few regenerated spindles is probably not due to failure of reinnervation, but more probably due to destruction of the basal lamina of the original fibres during the early ischaemic phase. The migration of the satellite cells from these fibres is facilitated by the destruction of the basal lamina. Therefore, the satellite cells and basal lamina, both of which are essential for regeneration, are lost. Spindles deficient in the number of original intrafusal fibres were observed during the early and later stages of regeneration and also seen in series Isch/Dn and Isch/Rn/Dn but not in series Dn. Furthermore, Maynard & Tipton (1971) did not see any reduction in the number of intrafusal fibres in denervated rat spindles.

10.7 Comparison with grafting and myotoxin techniques:

The techniques used to initiate muscle degeneration are reviewed earlier (chapters 2, 3). Muscle spindles have been observed in free grafts of entire muscles of rat (Carlson & Gutmann, 1975; Schmalbruch, 1976b, 1977; Rogers & Carlson, 1981) and cat (Hakelius, Nyström & Stalberg, 1975), but not in regenerated minced muscle (Zelená & Sobotková, 1971; Carlson, 1973). Milburn (1975, 1976) studied the effects of the local anaesthetic (bupivacaine) on rat spindles.

The cause of the initial degeneration on which the grafting model, and possibly the local-anaesthetic model, depends is ischaemic in origin and these models are mimicked by devascularization of the muscle (Hall-Craggs, 1974). The advantages of the devascularization technique for those who wish to observe the processes involved in intrafusal fibre degeneration, regeneration and reinnervation are two-fold. Firstly, unlike the grafting technique, with devascularization the innervation of the experimental muscle is left intact apart from some temporary peripheral damage. Predenervation and reinnervation of the spindle is therefore more easily controlled. Secondly, the whole of the muscle can be induced to degenerate, especially if the muscle is temporarily bathed in a local anaesthetic at the time of devascularization (Hall-Craggs, 1978).

The overall pattern and time course of the degeneration-regeneration cycle is similar following ischaemia and myotoxin administration. However, the effects of devascularization on the intrafusal fibres are more uniform than those of the myotoxin. The uniformity of the effects attained by devascularization probably requires multiple injections of the myotoxin instead of the single injection used by Milburn (1976).

The pattern of regeneration described by Schmalbruch (1976b, 1977) in grafted soleus muscle is generally similar to that observed in this study. In Schmalbruch's study, half of the grafts examined after 30 and 60 days contained reinnervating axons, whereas the two muscles examined after 250 days both lacked innervation. By definition, the grafting model involves sectioning of the nerve supply to the muscle, therefore, reinnervation can not be ensured. In the present study, all muscles of series Isch successfully reinnervated. This highlights the advantage of the ischaemic model.

Schmalbruch (1977) found muscle spindles in almost all muscle regenerates, but none contained sensory or motor innervation. Bag and chain fibres could not be identified. Observations from the present study showed that the differentiation of intrafusal-fibre types requires properly timed sensory reinnervation. The muscle spindles observed by Schmalbruch are equivalent to those of series Isch/Dn where reinnervation was prevented.

In reinnervated grafts, Rogers & Carlson (1981) reported that muscle spindles are often surrounded by a large number of axons. In the present study, large numbers of axons were only observed in spindles of subgroup 4B. The intrafusal fibres of these spindles lacked any kind of axon terminals. The reason for this increase in the number of axons is unclear. The incidence of such hyperneurotization is common in reinnervated spindles following nerve injury (see e.g. Barker & Boddy, 1980).

10.8 General implications of the pattern of spindle regeneration:

In normal muscle, specific patterns of innervation, both sensory and motor, are critical for the proper functioning of the

spindle. Muscle spindles in rat lower hindlimb muscles begin to form during the late fetal period when sensory axons contact developing myotubes. This initial afferent innervation is critical to differentiation of intrafusal fibres in terms of both their morphological and histochemical characteristics (Zelená, 1957; Landon, 1972; Milburn, 1973; Werner, 1973; Zelená & Soukup, 1973, 1974; Schiaffino & Pierobon Bormioli, 1976). Muscles deprived of sensory innervation during a critical period of development were found to contain no spindles at all or a few atypical small spindles (Zelená, 1957; Zelená & Hnik, 1960; Hnik & Zelená, 1961).

As with other lesions (Milburn, 1976; Schmalbruch, 1977; Rogers & Carlson, 1981), this study demonstrates that muscle-spindle regeneration can occur, to some extent, in the absence of innervation. This contrasts markedly with their development (Zelená, 1957). Similarly, series Isch/Rn/Dn shows that denervation of the regenerating muscle at the time of reinnervation does not cause the degeneration of intrafusal muscle fibres as is the case during development (e.g. Zelená, 1957).

Following devascularization, many of the spindle components survive; namely the capsule, axial sheath and the basal lamina and satellite cells of the original intrafusal fibres. The importance of the capsule and axial sheath in providing a framework for intrafusal -fibre regeneration is suggested by the absence of spindles from minced muscle regenerates (Zelená & Sobotková, 1971; Carlson, 1973) where it is likely that the mincing procedure disrupts these capsules to a considerable degree.

It is widely agreed that an intact basal lamina containing viable satellite cells is essential for muscle-fibre regeneration (see Mauro, 1979). The present study suggests that the destruction

of the basal lamina causes the loss of satellite cells and results in failure of the muscle fibre to regenerate. However, all these factors are recently challenged in a light-microscope study by Mackenson-Dean et al. (1981). They reported that muscle spindles form de novo in a pigeon muscle, which normally lacks spindles, grafted into sites of muscles that normally contain spindles. The main difference, however, lies in the choice of animal. Avian muscle spindles are considerably simpler than those of rat (see Barker, 1974). Nevertheless, this avian study reopens the possibility of muscle spindles developing de novo in mammalian regenerating muscle under certain conditions.

Finally, apart from their contribution towards a better understanding of the structure and physiology of the muscle spindle in the normal animal, the results presented in this study may be of practical use in the development of surgical techniques for nerve and muscle repair.

The free autografting of human skeletal muscle has come into increasingly widespread use as a clinical treatment for conditions in which there is a functional deficit in a muscle or group of muscles (e.g. Thompson, 1974; Hakelius, 1974). Little is known of the extent to which muscle-spindle structure and function is restored in grafted muscles of higher mammals (Mufti, Carlson, Maxwell & Faulkner, 1977), or of the factors that are relevant to the survival and functioning of the muscle spindle in regenerated muscle grafts.

It is widely known that following nerve injury sensory systems are always less-successfully restored than the gross motor control of the muscle. This study indicates that treatment of the muscle itself might improve the restoration of the muscle-spindle sensory system.

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