The development of muscle spindles in the rat

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THE DEVELOPMENT OF MUSCLE SPINDLES

IN THE RAT.

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ABSTRACT

The morphogenesis of muscle spindles in rat lower hindlimb muscles has been investigated using the electron microscope. The earliest detectable spindles are seen in the 19.5-day foetus and consist of a single myotube bearing simple nerve terminals of the large primary afferent axon from the nearby unmyelinated spindle nerve trunk. The capsule forms by an extension of the perineural epithelium of the supplying nerve fasciculus, and is confined initially to the innervated zone. Myonuclei accumulate in this region, so that the first intrafusal muscle fibre to develop is a nuclear-bag fibre. Myoblasts, that are present within the axial bundle throughout its development, fuse to form a smaller, less-differentiated myotube by the 20-day feotal stage. This matures in close association with the initial fibre, and by birth (21-22 days gestation) has formed the smaller intermediate bag fibre that has been identified histochemically and ultrastructurally in the adult. Nuclear-chain fibres develop in the same way; myoblasts fuse to form satellite myotubes that mature in apposition to one or more of the other fibres, lying within a common basement membrane.

By the 4-day postnatal stage the full adult complement of 4 intrafusal muscle fibres is present, although the ultrastructural and histochemical variations, seen in the adult, are not present. The fusimotor innervation begins to arrive by birth, but is not fully established until the 3rd postnatal week, when the ultrastructural and histochemical maturation of the axial bundle is complete. The

maturation of the capsule and periaxial space occurs at the same time.

It is suggested that the sequential development of the intrafusal fibres is a reflection of the decreasing morphogenetic effect of the afferent innervation, whereas the role of the fusimotor innervation is in ultrastructural and histochemical differentiation.

PREAMBLE

The early histological studies of muscle spindles, culminating in those of Ruffini (1898), and the interest aroused in these structures by the recognition of their function as intramuscular end-organs of afferent nerves (Sherrington, 1894), led a number of histologists to study the normal morphogenesis of muscle spindles in a variety of animal species. Subsequent advances in the understanding of their role in the control of movement, and a realization of the practical importance of all of this research for clinical neurology have led, in turn, to a series of investigations into the fine structure of adult spindles, but none of foetal spindles at the time of the outset of this study.

The development of muscle spindles in shank muscles of the rat has been studied, employing mainly electron-microscopic techniques. The findings are presented in four parts. Part I introduces the results of previous investigations into muscle-spindle morphogenesis and neuromuscular development in general. It is obvious that any study of spindle development must include a monitoring of events extrafusally, so that any similarities or differences in these two processes can be compared. The literature is therefore reviewed and discussed in depth. As with most anatomical studies, the problems of nomenclature have led to confusion. The semantics of the terms used in Parts III and IV are therefore pre-defined in this section.

It was not considered within the scope of this thesis

to review the past and current findings of investigations into the mature spindle, as this has been carried out recently, in great detail by Matthews (1972) and Barker (1973). Only those investigations immediately relevant to the present study, and therefore concerning the fine structure and histochemistry of adult rat spindles are included here.

The final chapter of the Introduction presents a justification and the aims of the present study. Parts II and III deal with the materials and methods used and the results obtained. Finally Part IV is an assessment and discussion of the findings in relation to previous and concurrent work in this and other fields of neuromuscular development.

PART I - INTRODUCTION

Chapter 1. The development of muscle spindles.

1.1 Historical review.

At a time when the sensory nature of adult muscle spindles was becoming established (Sherrington, 1894), the examination of these organs in foetal muscle was also contributing to an understanding of the adult structure. Forster (1902) noted in immature spindles of man, a distinct development and enlargement of the intrafusal muscle fibres, capsule and "lymph space", which led her to confirm the view "that the spindle is of physiological importance for the fully developed muscle, and not the vestige of an embryonic structure" (p. 203). No thorough attempts were made to study the embryology of the spindle until 1915, when Sutton traced its development in the extrinsic eye muscles of pig. His findings were later confirmed in the tongue musculature by Langworthy (1924). Both studies however, were concerned exclusively with the growth pattern of nerve terminals. It was not until the investigations of Tello (1917, 1922) mainly in chick, that the development of the non-nervous components of the spindle was recorded.

As the morphological picture of the adult spindle became more complete, and with the results of investigations into neuromuscular relations during development (eg. Harrison, 1904), embryological studies turned towards a correlation of the time of arrival of the nerve terminals and the differentiation of other spindle structures. The first study of this type was carried out by Cuajunco in biceps muscle of pig (1927) and man (1940).

With the exception of one of the first fundamental studies in chick and a more recent one in lizard (Lui & Maneely, 1969) most of the research into the development of the spindle has been confined to mammals, namely rat (Kalugina, 1956; Zelená, 1957, 1959; Marchand & Eldred, 1969); pig (Sutton, 1915; Langworthy, 1924; Cuajunco, 1927); sheep (Dickson, 1940) and man (Forster, 1902; Tello, 1917, 1922; Hewer, 1935; Cuajunco, 1940; Bowden, 1963; Mavrinskaya, 1967), as reviewed recently by Smith & Ovalle (1972) and Werner (1972).

A variety of staining techniques was utilized in these studies, but the majority relied primarily on silver impregnation to show the developing nerve clearly. The counterstains used to bring out the myofibrillar, nuclear and capsular components of the muscle spindles constituted the major difference in technique, and all of these investigations were made with the light microscope, with particular emphasis on the temporal sequence of development. As with most developmental processes the morphology of the spindle at birth can be correlated to gestation length and the maturity of the animal at this time (Dickson, 1940). The differentiation of the spindle does not even begin or advance at the same time or rate in any single muscle (Zelená, 1957). A pattern in the order of development of the spindle can, however, be extracted from these studies. The sequence described is based on the timing in rat, because of its pertinence to the present study, but reference is made to other species.

1.2 The pattern of development of the spindle components.

1.21 Innervation. Before the onset of differentiation of the muscle spindle, which occurs for example between the 9th and 11th week of foetal life in man (Cuajunco, 1940; Bowden, 1963; Mavrinskaya, 1967) and at the 19th-20th day of gestation in rat (Zelená, 1957), there are apparently no differences between those myotubes destined to become intrafusal muscle fibres and those that will mature into extrafusal types (Tello, 1917, 1922; Cuajunco, 1940; Zelená, 1957; Bowden, 1963). The first signs of its formation are seen in the branching of nerve terminals of sensory fibres, (Tello, 1917, 1922; Cuajunco, 1927, 1940; Hewer, 1935; Zelená, 1957, 1964; Bowden, 1963) around primitive muscle cells, indistinguishable from neighbouring cell types. Tello suggests that the thick exploratory fibres contact a group of myoblasts. Subsequent research in rat suggests that the early contacts are made with myotubes (Zelená, 1957). In the vicinity of this nerve branching, nuclei of the muscle cells proliferate. The source of these new nuclei remains obscure. It has been suggested that it is the result of amitotic divisions of the original myonuclei (Tello, 1917, 1922; Mavrinskaya, 1967), but there is no evidence to confirm this.

According to Cuajunco (1940) it is not until after the formation of the nuclear bag that the motor nerve supply reaches the developing spindle. Zelená (1957) found that this occurs in rat after the 5th postnatal day. Mavrinskaya (1967) suggests however that in man both sensory and motor components of the spindle nerve supply arrive simultaneously. The exact temporal sequence of the development of the nerve terminals

remains uncertain, especially the time of arrival of the secondary sensory innervation; Zelená (1964) suggests this occurs postnatally in rat.

1.22 Intrafusal muscle fibres. Cuajunco showed in pig (1927) and in man (1940) that the number of intrafusal muscle fibres increases during the development of the spindle. He suggested that this occurs by either the encapsulation of extrafusal muscle fibres, the development of associated myotubes or in addition, by the splitting of existing intrafusal fibres, in man. The process by which intrafusal fibres increase in number has subsequently been disputed. Latyshev (1955) suggested that in cat and man, "supplementary fibres" develop from elements of the inner layer of the spindle capsule. Couteaux (1941) suggested their origin lies in myoblasts associated with existing intrafusal myotubes. Marchand & Eldred (1969) found that the neonatal intrafusal-fibre complement of rat consists of one nuclear bag and one nuclear chain, that subsequently split longitudinally, so that by the 6th postnatal day the adult number is attained. The results of this and a subsequent study (Bravo-Rey, Yamasaki, Eldred & Maier, 1969) have presented evidence that the total complement of nuclei is present in the two "parent" intrafusal fibres at birth and at the time of longitudinal splitting, the original nuclei are merely re-distributed to the "daughter" fibres, without further replication.

1.23 Capsule and periaxial space. The development of the capsule is initiated at the future equatorial zone of the spindle (Cuajunco, 1927, 1940; Zelená, 1957; Mavrinskaya, 1967) and does not extend into the polar regions until,

in rat, the 5th postnatal day. Both Cuajunco and Tello (1917, 1922) suggested that the capsule forms from cells of the internal perimysium. More recent investigations into the structure of the adult capsule (Shantha, Golarz & Bourne, 1968) suggest however, a continuity of structure between the capsule and perineural epithelium of the supplying nerve trunk.

The periaxial space is the last component of the spindle to develop, directly following capsule formation in the latter part of the 1st postnatal week, in rat (Zelená, 1957). Subsequent development of the spindle includes an increase in size and extent of both capsule and periaxial space, an increase in diameter and a peripheral shift of nuclei in the polar zones of intrafusal muscle fibres, accompanied by overall longitudinal growth of the spindle. Morphological maturity of the muscle spindle is reached in rat by the 25th postnatal day (Zelená, 1957); in pig (length at birth 250-300 mm) by the 190 mm stage (Cuajunco, 1927) and in man (gestation period 40 weeks), by the 24th week of intrauterine development (Cuajunco, 1940).

1.24 Histochemical and ultrastructural differences in intrafusal muscle fibres. Histochemically, 3 types of intrafusal muscle fibre may be recognized in the adult spindle (see review by Barker, 1973), corresponding to types A, B and C extrafusally (Table 1). In rat, Yellin (1969) found that nuclear-chain fibres show high oxidative and glycolytic enzyme activity, whereas nuclear-bag fibres can resemble type A or B, or, occasionally have low activity in both classes of enzyme.

In rabbit, the histochemical differences between intrafusal muscle fibres can be correlated with their ultrastructural morphology (Barker & Stacey, 1970; Barker, Harker, Stacey & Smith, 1972). On the basis of this and other studies, Barker recognizes the typical bag fibre as possessing little interfibrillar sarcoplasm or glycogen, a poorly developed SR network, and mitochondria that are shorter and fewer than in nuclear-chain fibres. An M line may be absent or present, as in rat lumbucal muscle (Ovalle, 1971, 1972 a), as a faint double line. Intermediate forms of bag fibre may vary in their ultrastructure in one or more ways from typical bag fibres; this may be reflected histochemically by a higher oxidative-enzyme activity.

Few attempts have been made to trace the histochemical differentiation of intrafusal muscle fibres. Wirsen & Larsson (1964) suggest that in mouse the phosphorylase content of the successive intrafusal fibres becomes progressively less during development. In rat spindles at the 1-5th day postnatal stage (Ostenda & Strugalska, 1971), these fibres can be separated into two types on the basis of diameter and phosphorylase content; the large-diameter nuclear-bag fibres show intense phosphorylase activity compared to the lower activity of the smaller nuclear-chain fibres.

The ultrastructural differentiation of intrafusal muscle fibres had not been recorded at the outset of this study.

1.3 Developmental physiology of the muscle spindle.

All of the research into the physiology of immature

spindles has been carried out in cats by Skoglund (1960 b, c). He found, in newborn kittens the activity of both spindle receptors and tendon organs to be quickly adapting, the response to constant stretch becoming maintained only later in development. Skoglund found two reasons for this. Firstly, the refractory period of immature axons is longer than in the adult and the after-potential is longer lasting, summating during repetitive stimulation. Secondly, stretch of the immature muscle cannot be maintained as the muscle slowly lengthens. If the tension on the muscle is then increased, the receptors again fire in a phasic fashion, indicating that the first explanation is the more important.

This conclusion was further supported by the effect of succinylcholine on the spindle discharge, which causes, in spindles of older kittens, a maintained discharge. Muscle spindles of the newborn animal, however, always discharge phasically. Another way of causing intrafusal-fibre contraction is by γ excitation. Skoglund achieved this, comparing the spindle discharge following a shock to the ventral root maximal for α axons, with that following a shock ten times as great. It had been shown that γ axons are present in the muscle nerve of 10 day old kittens (Skoglund, 1960 a). However γ excitation of spindles could not be demonstrated until the 17th to 20th postnatal day.

Shocks to the ventral root, subthreshold for γ axon excitation or tetanic stimulation at voltages adequate only for α axon excitation also causes a "filling in" of the silent period of the spindle receptors during extrafusal contraction, even before the 17th postnatal day. This leads

Skoglund (1960 b, c) to conclude that "mixed" extrafusal-intrafusal innervation is responsible and that this innervation develops before the ɣ.

Chapter 2. Experimentally-induced abnormal development of muscle spindles.

The question of the degree of dependency of intrafusal fibres on their nerve supply during differentiation and maturation has been the subject of extensive studies by Zelená (1957, 1959, 1962, 1964, 1965), Hník (1964), Zelená & Hník (1960 a, b, 1963 a, b, c), Hník & Zelená (1961) and Zelená & Sąbotková (1971). The role of muscle stretch in this developmental process has also been studied (Zelená, 1963; Gladden, 1971). From this series of experiments, a picture can be built of the morphogenetic influences acting on the spindle during its formation. The lesions used included sectioning the muscle nerve or ventral roots, extirpation of spinal cord, crushing of the muscle nerve and tenotomy of the muscle. Rats were used in all of these experiments as well as rabbits in the nerve severance series; all observations were made with the light microscope.

2.1 The effects of nerve section.

In one of her first studies, Zelená (1957, 1959, 1962) excised part of the sciatic nerve in the mid-thigh region, unilaterally in rats on the 19th to 20th days of the intrauterine life (60 to 70 hr before birth). The same surgical procedure was performed in rabbits at the 21st day of foetal life (7 days before birth). In both species, Zelená noted that at the time of excision, the muscle is at the myotube stage of development and "differentiation into intrafusal fibres has begun" (1957, p. 287). In two other series of animals, the same surgery was performed on newborn

and 20-day postnatal rats. Muscles were examined 3, 4, 5, 7 and 10 days after intrauterine operation and after 5 and 10 days in the other two series.

In both rats and rabbits operated in utero, no distinct spindles are seen in calf muscles 3 and 7 days postdenervation, and none thereafter. In those muscles denervated at birth, discrete equatorial zones are present 5 days later but by 10 days, the intrafusal fibres undergo considerable atrophy and the nuclear bag disappears. Only occasional spindle remnants can be found (Zelená, 1957; Hník & Zelená, 1961). In those rats, in which sciatic section was performed at 20 days of age, there is only a slight atrophy of both nuclei and intrafusal fibres 10 days later.

Zelená (1964) concluded from these experiments that "since future muscle spindles receive only sensory innervation when they begin to differentiate (Zelená, 1959), the arrest of their development is evidently the result of loss of sensory innervation" (p. 195).

2.2 The effects of ventral root section.

The role of the fusimotor innervation in the postnatal development and maturation of spindles was the subject of a later investigation by Zelená (1964, 1965). She eliminated motor and fusimotor nerve fibres from the muscle of newborn rats by unilateral sectioning of ventral roots or by the extirpation of spinal cord. Ten days after surgery, the de-efferented muscles contain well developed spindles with distinct nuclear-bag and nuclear-chain fibres. No degeneration was observed even 30 days after the operation, except for a reduction in the mean diameters of the spindle

and its muscle fibres. These experiments suggest that Zelená's initial premise was correct in that it is the sensory innervation that is of primary importance for the postnatal development of muscle spindles.

2.3 The effects of nerve crush.

Further experiments by Zelená & Hník (1960 a, b; 1963 a, b, c) investigated the possibility of muscle-spindle formation following re-innervation in the early postnatal period, after crushing the nerve at birth. By doing this, they aimed to discover whether the morphogenetic influence of afferent nerve fibres is restricted to the perinatal period only or whether, under experimental conditions, they could induce differentiation during a later period of muscle growth.

In the first of this series (Zelená & Hník, 1960 a, b; Hník & Zelená, 1961), a crush lesion was applied to the sciatic nerve of newborn rats and the tibialis anterior, soleus and extensor digitorum longus muscles examined 5 and 10 months later. Following re-innervation, which begins by the 10th postdenervation day, muscle spindles do not differentiate anew, even after 10 months, although extrafusal muscle fibres fully recover from the denervation atrophy and the sensory nerve supply is reduced by only 15-25% (Zelená & Hník 1960 a, 1963 c; Zelená, 1964). Only occasional atypical spindles are found in the re-innervated muscles. These structures contain, on average, 1-2 intrafusal fibres that lack a nuclear bag and which may approach extrafusal-fibre morphology in the polar zones (Hník & Zelená, 1961). They have a resting discharge and a slowly-adapting response

to muscle stretch. The number of spindle remnants seen 10 days after nerve section at birth was found to correspond closely to the occurrence of atypical spindles in re-innervated muscles 5 months later (Hník & Zelená, 1961). It seems therefore that atypical spindles originate from re-innervated spindle remnants that survive the transitory denervation period. The regenerating sensory nerve fibres are apparently unable to induce the transformation of extrafusal muscle fibres into intrafusal fibres. They terminate in the muscle as free endings.

Under certain conditions however, it was found that regenerating nerve fibres do induce the formation of muscle spindles (Zelená & Hník, 1963 a; Zelená, 1964). The medial gastrocnemius muscle is re-innervated shortly (6 days) after crushing the sciatic nerve at birth, because of the short distance between the muscle and nerve lesion. At the onset of re-innervation, myotubes are still present in the muscle, as well as muscle-spindle remnants. The regenerating nerve may contain 60% more sensory nerve fibres than in controls. Five months after crushing the nerve, this muscle contains up to 70% more spindles than the contralateral control, suggesting that apart from the re-innervation of disintegrating spindles, differentiation of new muscle spindles has occurred.

When the re-innervation of gastrocnemius was examined following a prolonged denervation interval (by repeated crushing of the nerve) no spindles were found in the muscle 5 months after the initial crush, although the regenerated nerves still contain an increased number of fibres. Muscle histogenesis is completed before the start of nerve

regeneration, suggesting that the morphogenetic effect of sensory nerve fibres can be exerted only when the muscle cells are capable of differentiation.

Sensory neurons do not, however, induce the differentiation of muscle spindles in regenerating minced muscle of adult rat (Zelená & Sábotková, 1971), although new motor end-plates form.

2.4 The effect of tenotomy.

Following intrauterine and neonatal tenotomy of gastrocnemius and plantaris muscles of the rat, by sectioning of the Achilles tendon, muscle spindles continue to differentiate (Zelená, 1963). Four days after intrauterine operation in 1-day postnatal rats, spindles consist of two well-developed intrafusal fibres enclosed by a distinct capsule; by the 7th postoperative day, no further differentiation is observed and the nuclear bag appears to atrophy. Following neonatal tenotomy, muscle spindles develop at a normal rate, although they occur less frequently than in control muscles. Longitudinal growth is reduced however by 50%.

A later study by Gladden (1971) suggests that these spindles differ from normal in other ways. She found 6-12 weeks after neonatal tenotomy of the intertransverse caudal muscles of rat, that spindle response to muscle stretch is abnormal. The afferent terminals of some spindles appear less complex than in control spindles, although the fusimotor innervation is apparently unaffected. The sensitivity of the afferent fibres to stretching is reduced in tenotomized spindles, reflected by an elevation of their threshold levels.

It seems therefore that muscle tension and stretch are not

necessary for the formation of spindle receptors, although they do appear to play a role in their growth and physiological development.

Chapter 3. The development of extrafusal vertebrate striated muscle and its motor innervation.

3.1 Myogenesis.

Tello (1917, 1922) described 4 stages in the embryology of muscle fibres from mesodermal cells in avian muscle. He applied the term myoblast to the primordial muscle cell that is indistinguishable from associated fibroblasts. The myocyte, which can be single or multinucleate, represents an elongation of the myoblast, but lacks the cytoplasmic structure and "transverse striations" of the more mature muscle cell. Further elongation and the formation of striations at the periphery indicates the myotube stage of development, in which the nuclei still adopt a central position. The mature muscle fibre is well-striated with peripheral nuclei. Boyd (1960) suggested two amendments to this scheme; the histogenetic stages of the muscle fibres are now accepted as the premyoblast, an undifferentiated mesenchymal cell; the myoblast, a uninucleate cell now committed to myogenesis; the multinucleate myotube and the muscle fibre.

Although all muscle fibres pass through each of these stages during their development, the progression is not synchronized within the complete muscle. For a considerable period, cells corresponding to a variety of developmental stages can be observed together in close association, although at later stages the proportion of differentiated cells increases (eg. Kelly & Zacks, 1969 a). The temporal sequence of the course of this process also differs in different muscle-forming regions of the embryo. Kitiyakara (1959) found

columns of differentiated muscle fibres in the somites of chick at the 5th to 6th day of development, when the limb musculature shows only islands of myogenesis. Variations in the time of onset and duration of this process can occur even among muscles of the same area (Zelená, 1959, 1962).

With the advent of the electron microscope, research in the past decade has produced further morphological characteristics of these myogenic cells and thrown light on their possible roles in the process of differentiation. With the increased resolving powers, a new cell was discovered in muscle tissue. First identified in frog skeletal muscle by Mauro (1961), the satellite cell has also been implicated in myogenesis.

3.11 Myogenic cells. A schematic diagram (Fig. 1) illustrates the morphological characteristics of muscle cells, as used in this study. Fischman (1970), in his review of myogenesis, lists several characteristics of the myoblast. It typically contains a large, ovoid nucleus with prominent nucleoli and diffuse chromatin. Within its cytoplasm are numerous free ribosomes, little endoplasmic reticulum (ER), a poorly developed Golgi complex and some glycogen granules. Developing endomysial cells (fibroblasts) differ, in rat by a more profuse presence of rough ER (Bergman, 1962) and their lateral cytoplasmic extensions. The most positive identification of myoblasts is by their position, in direct apposition to a more mature muscle cell, without any intervening basement membrane. Investigations of cultured chick muscle suggest that the myoblast is a postmitotic cell, capable of synthesizing myofibrils (Okazaki & Holtzer, 1965, 1966;

Bischoff & Holtzer, 1968), whereas the pre-myoblast actively divides, but contains no detectable myofibrils (see also Marchok & Herrman, 1967). As the morphological distinction of premyoblast and myoblast is so tenuous, all uninucleate myogenic cells in this study are termed myoblasts.

The myotube is a long, cylindrical syncytium with pseudopodia at each end (Fischman, 1970). The nuclei are central in position, contain a more diffuse nucleolus and are separated by areas of sarcoplasm rich in mitochondria. Myofibrils at varying stages of assembly are most prominent beneath the plasma membrane. Glycogen is usually present, but in varying amounts depending, in rat, on the age of the animal (Schiaffino & Hanzlíková, 1972 a). The sarcotubular (SR) and transverse-tubule (TT) systems are incompletely developed in the myotube (Ezerman & Ishikawa, 1967; Schiaffino & Margreth, 1969; Walker & Edge, 1970, 1971; Kelly, 1971; Luff & Atwood, 1971). Basement membrane forms at this stage (Kelly & Zacks, 1969 a), except at the intervening membrane surfaces of adjacent myotubes and myoblasts.

Nuclei at the muscle fibre stage are peripheral in position. Myofibrils are closely packed and occupy the whole of the fibre core. It is at this stage that the positive identification of satellite cells can be made (see review by Muir, 1970). They, like the myoblasts of immature muscle, lie between the plasma membrane and basement membrane of the parent fibre and can only be distinguished from myonuclei in electron micrographs. Identification is made by the paucity of small mitochondria, numerous free ribosomes and little rough ER. Paired centrioles may be present, one of which, in

bat (Muir, Kanji & Allbrook, 1965; Church, 1969) gives rise to an atypical cilium. Glycogen and myofilaments are always absent.

3.12 Fusion and development. The mechanism of muscle-fibre formation has been a subject of debate for most of this century. For many years it has been recognized that the number of fibres in a muscle increases progressively during foetal life. Couteaux (1941), Cuajunco (1940, 1942) and Tello (1922) observed primary generations of muscle cells differentiating in close proximity to each other and the subsequent appearance of secondary and tertiary generations around their walls. Many investigations (see review by Boyd, 1960) described these later generations as originating as buds from the walls of primary cells, from which they subsequently separate by longitudinal fission. The new nuclei were thought to be provided by the central nuclei of myotubes which constantly replicated by amitosis. A conflicting view (Morpugo, 1898; Bardeen, 1900; Couteaux, 1941) suggested that secondary and tertiary generations of muscle cells develop from myoblasts associated with primary myotubes.

Recent investigations using tissue culture techniques have shown that neither mitotic nor amitotic nuclear division occurs in myogenic cells containing myofibrils. By the employment of radioautographic techniques, after the incorporation of a labelled DNA precursor within a short period of time, Stockdale & Holtzer (1961) found that only the nuclei of myoblasts show labelling. After a longer incorporation time however, they found many labelled nuclei in myotubes. They therefore concluded that it is by the fusion

of myoblasts that myotubes are formed, as had also been suggested in regenerating muscle (Lash, Holtzer & Swift, 1957). The fusion process is not confined to myoblasts, but can occur between myoblasts and myotubes, and between young and older myotubes (Okazaki & Holtzer, 1965, 1966).

Although fusion has been recorded cinematographically in culture (eg. Cooper & Konigsberg, 1961), its morphological identification is still not certain. Hay (1963) suggested that, in regenerating muscle, it is represented by rows of vesicles, like those usually associated with the plasmalemma of muscle fibres, seen traversing coalescing muscle units. Lipton & Konigsberg (1972) have described the fine structure of cells apparently fixed in the process of fusion, joined by a single cytoplasmic bridge.

Few electron-microscopic studies have been made of the cellular relationships between successive generations of muscle primordia *in vivo*, due probably to the immense success of tissue culture techniques. This has been done in the intercostal muscle of rat (Kelly & Zacks, 1968, 1969 *a*; Kelly & Schotland, 1972), based on the earlier observations of Bergman (1962).

In 16-day foetal (DF) rat, the muscle consists of groups of 2-4 primary myotubes that show areas of contact, represented by close and tight junctions. Undifferentiated cells, presumably myoblasts, surround and separate the myotubes. Basement membrane does not form until the 18 DF stage, when it encloses groups of myogenic cells each of which is dominated by a primary myotube containing large glycogen deposits. Primary myotubes are presumed to have formed by

the fusion of small myotubes and myoblasts seen earlier. Kelly and his co-workers suggest that fusion may be represented by areas of contact between pseudopodial processes of myotubes and the plasma membrane of muscle cells nearby. However Landon (1970, 1971) suggests that pseudopodial attachment is a constant but transitory feature of the development of all secondary and subsequent generations of myotubes.

By birth, almost all muscle cells have matured to muscle fibres, in intercostal muscle of rat, with individual basement membranes; complete separation is not acquired until the 5th postnatal day. The number of small muscle fibres increases greatly at birth, at the expense of the smaller immature muscle cells seen at the 18 DF stage. Myoblasts, seen in association with smaller, secondary fibres were presumed to have one of three fates: to fuse with secondary fibres, to form subsequent generations of myotubes or to form satellite cells in the adult. It appears therefore that in rat, developing muscle originates from a few primary generations of cells and is then progressively built up from secondary orders that develop around the walls of the primary generation. Once a certain degree of differentiation is attained, the new orders of cells diverge from the primary generation to become new, independent muscle fibres.

3.13 Myofibrillogenesis. Most of the studies on the synthesis and assembly of myocytic cytofilaments into myofibrils (eg. Przybylski & Blumberg, 1966; Fischman, 1967; Ishikawa, Bischoff & Holtzer, 1968; Kelly, 1969)

are based on the assumption that the chemical identification of these filaments can be based on morphological criteria. It is therefore assumed that thin filaments of 60μm diameter are actin and those of 160μm, myosin. However, an intermediate-diameter filament, of around 100μm diameter has been identified in myotubes and some non-myogenic cells (Ishikawa et al, 1968; Kelly, 1969; Read, Takeda & Kirkaldy-Willis, 1971), without a morphological correlate in mature myofibrils.

Myofibrils form predominantly beneath the sarcolemma of non-mitotic myogenic cells. Fischman (1970) suggests this may be related to the large numbers of thin filaments, seen by Kelly (1969), and thick filaments (Fischman, 1967) that are found in this area. Kelly also suggests that Z-band material is formed from structures associated with or derived from the plasma membrane. There is evidence, however, that it is the sarcoplasmic reticulum that is involved in Z-band formation (Walker & Edge, 1970, 1971).

These early myofibrils according to Fischman's study of embryonic chick muscle (1967), consist of aggregations of thick and thin filaments in a hexagonal lattice (see also Allen & Pepe, 1965), without any visible Z-band material or M-line cross-bridges. Allen & Grisnick (1971) suggest, however, that M lines are detectable before any banding pattern becomes visible, Myofibrils grow in length by the addition of new sarcomeric units (eg. Williams & Goldspink, 1971).

3.14 Development of the membrane systems. Both the sarcotubular (SR) and transverse tubular (TT) systems undergo important modifications during development before the complex

and regular organization of the adult differentiated state (Porter & Palade, 1957) is attained. In the psoas muscle of newborn rat, contacts between the TT and SR systems are sparse and mostly peripheral in location (Schiaffino & Margreth, 1969), which can be attributed to the incomplete penetration of the TT system from the sarcolemma (Walker & Schrodt, 1965), into the sarcoplasm. These triadic junctions have a longitudinal orientation in immature muscle (Walker & Schrodt, 1968; Schiaffino & Margreth, 1969; Edge, 1970; Kelly, 1971; Luff & Atwood, 1971); the typical transverse orientation is not attained, at least in rat hindlimb muscle, until after the 5th postnatal week (Edge, 1970).

The SR network of immature muscle consists of diverging and converging tubules that bud from the cisternae of granular ER (Ezerman & Ishikawa, 1967) orientated both transversely and longitudinally around the myofibril. Encirclement of the myofibril is at first incomplete and junctional cisternae rare (Edge, 1970). In their earliest formation, there is evidence (Kelly, 1971) that SR tubules couple with the plasma membrane before forming junctions with the TT system. The later development of the SR system separates it into two portions in register with the A and I bands of the myofibril (Schiaffino & Margreth, 1969), accompanied by a measurable increase in volume (Luff & Atwood, 1971).

Most mature muscle fibres have a particular distribution of mitochondria. They tend to aggregate beneath the sarcolemma and form pairs at the I-band level of the

myofibril. Immature diaphragm muscle of the rat lacks this organization (Bunting, 1969 quoted from Gauthier, 1970); the mitochondra are few in number and irregularly distributed.

3.15 Role of satellite cells. Since its discovery in adult muscle, several roles have been attributed to the satellite cell, including a possible myogenic function. Electron-microscopic studies have shown that the fine structure of the myoblast is very similar to that of the satellite cell (see 3.11), with the exception of glycogen and myofilament distribution, confined, apparently to the myoblast. Some early studies, however, suggested that myofibrils can appear in cells similar to satellite cells (eg. Muir et al, 1965).

The most comprehensive study of the involvement of these cells in myogenesis has been made by Church (1969, 1970 b), in the fruit bat. He found towards the end of gestation only two cell types present in the muscle primordia: maturing muscle fibres and satellite cells, which, by their mitotic divisions, provide myonuclei during the early post-natal period. He suggests that in adult muscle satellite cells are dormant premyoblasts. They are formed, as are myoblasts (Church, 1970 b) by the mitotic divisions of other premyoblasts and, although the morphological profiles are similar, their functions differ. Premyoblasts are the stem cells of developing muscle and myoblasts postmitotic cells from which other myogenic cells develop, whereas satellite cells are the stem cells of adult muscle. This suggestion correlates well with observations of regenerating muscle (Church, Noronha & Allbrook, 1966; Shafiq, Gorycki & Milhorat, 1967; Reznick, 1970; Shafiq, 1970; Jirmanová &

Thesleff, 1972), where, it is supposed, new muscle fibres form by the division and subsequent fusion of cells that adopt a satellite-cell position.

3.2 Muscle growth.

Once the differentiation of muscle has reached the muscle-fibre stage, the whole muscle undergoes a period of growth, involving both muscle fibres and connective tissue components (eg. Enesco & Puddy, 1964). Although the number of muscle fibres may increase during this period (Montgomery, 1962; Chiakulas & Pauly, 1965; Bridge & Allbrook, 1970), this is probably best considered as part of the differentiation process and not muscle growth. The two main features of muscle growth are an increase in the number of myonuclei and in the size of individual muscle fibres.

Macconnachie, Enesco & Leblond (1964) showed, by the use of colchicine and radioautographic techniques that the number of myonuclei increases by mitosis. Subsequent investigations, using the same basic methods but combined with electron-microscopy (Shafiq, Gorycki & Mauro, 1968; Moss & Leblond, 1970, 1971; Allbrook, Han & Hellmuth, 1971) have shown that it is the mitotic divisions of satellite cells that are responsible for this increase. Half of the daughter cells thus produced are incorporated into the muscle fibres, while the rest remain as new satellite cells.

Each muscle fibre increases both in length and diameter during postnatal growth. Lengthening takes place by appositional rather than interstitial growth (Kitiyakara & Angevine, 1963), probably by the addition of new sarcomeres to both ends of existing fibres (Williams & Goldspink, 1971).

The extent of the increase in fibre diameter is largely dependent on the work-load of the muscle (Joubert, 1955).

3.3 Histochemistry

3.31 Classification of adult muscle fibres. Mammalian skeletal muscle contains fibres of two major types, slow and fast twitch fibres, that contract at different speeds (eg. Denny-Brown, 1929) and contain different amounts of myoglobin (Mcpherson & Tokunaga, 1967) and enzymes. The proportions of slow and fast fibres vary in different muscles. The classical distinction into red (slow) and white (fast) fibres was first demonstrated histochemically by Dubowitz & Pearse (1960 a, b), which they designated as Type I and II respectively. Type I fibres, in man, show a high concentration of enzymes involved in oxidative metabolism, whereas glycolytic enzymes have a low activity. Type II fibres have an exactly opposite enzyme profile.

Subsequent histochemical investigations, beginning with those of Stein & Padykula (1962) in rat, revealed that at least three distinct types of muscle fibre may be present. They found that the large, fast, white fibre, re-designated as type A, shows the least reaction product when staining for succinic dehydrogenase (SDH) and that slow, red fibres are of two types, based mainly on the distribution of diformazan granules. Type B fibres have an overall darker staining pattern than A fibres but lack the strong subsarcolemmal activity typical of type C fibres. Sections stained, unfixed, for adenosine triphosphatase (ATPase) show a positive reaction in type C fibres. The suggestion from this and later studies (eg. Romanul, 1964; Nystrom, 1968;

Samaha, Guth & Albers, 1970) is that a third histochemical type of muscle fibre may be present, that can utilize both oxidative and glycolytic pathways in respiration.

There is no universally accepted classification of histochemical fibre-types (eg. Brooke & Kaiser, 1970), although the division into 3 broad types is recognized. The classification used in this study (Table 1) is similar to that of Stein & Padykula as described, but is based mainly on a physiological-histochemical correlation of fibre types in cat gastrocnemius (Burke, Levine, Zajac, Tsairis & Engel, 1971).

3.32 Developing muscle. Most of the research into the histochemical differentiation of muscle fibres has been concerned with the profile of the "pre-differentiated" fibre and the subsequent sequence of development of the fibre types. This temporal sequence is directly related to the length of gestation and the degree of maturity attained by the animal at birth (Dubowitz, 1965). Newborn guinea-pig muscle, for example, shows full histochemical differentiation, whereas neonatal rat muscle fibres are usually uniform in activity. The onset of this process also varies according to the topography of the muscle (Nystrom, 1968).

At the myotube stage of development in rat, histochemical variations are not evident (Engel & Karpati, 1968; Zamieniecka & Ostenda, 1969; Kelly & Schotland, 1972; Shafiq, Asiedu & Milhorat, 1972). Myotubes and young myofibres show a positive reaction for both oxidative and glycolytic enzyme stains, as also noted in man (Zamieniecka, 1968), chick (Dubale & Muraldiharan, 1970), pig (Cooper,

Table 1.

Histochemical classification of muscle fibres in mammalian skeletal muscle.

Type	A	C	B
Contraction speed	Fast	Fast	Slow
Fatigue speed	Fast	Slow	Slow
SDH activity	Low	Positive	Positive
ATPase (alkali) activity	Positive	Positive	Low
P'ase activity	Positive	Positive	Low

Based on Stein & Padykula (1962)
Nystrom (1968)
Burke *et al* (1971)

Cassens, Kastenschmidt & Briskey, 1970) and Ox (Ommer, 1971). It seems therefore that the profile of the immature muscle cell approaches that of the adult type C fibre.

Some enzymes show differentiation before others. Wirsen & Larsson (1964) describe differences in the phosphorylase content of successive generations of myotubes, which, they suggest, is responsible for the ckeckerboard pattern of adult muscle. The most significant histochemical change in maturing muscle is an increase in the proportion of ATPase-low (type B) fibres (Karpati & Engel, 1967 a, Engel & Karpati, 1968; Cardinet, Wallace, Fedde, Guffy & Bardens, 1969; Kelly & Schotland, 1972; Davies, 1972 a, b). The first fibres to show this differentiation are only a small proportion but of a larger diameter than the surrounding muscle fibres. Wohlfart (1937), in his histological investigation of human foetal muscle, described the present of 'b' fibres that differ from the surrounding 'a' fibres by their large size. Later studies (Fenichel, 1963, 1966; Zamieniecka, 1968) showed that these 'b' fibres have little myosin ATPase activity and represent either part, or the entire (Dubowitz, 1970) B fibre population early in differentiation.

As maturation proceeds, the actual number of type B fibres increases by the transformation of fibres staining darkly for ATPase to light fibres (Kelly & Schotland, 1972; Davies, 1972 a, b). The differentiation of types A and C fibres requires the emergence of a predominantly anaerobic fibre from the homogenous population. This may occur after

the distinction of the B fibre population is obvious (eg. Dubowitz, 1965) or at the same time (Davies, 1972 a, b).

3.4 Motor end-plate development.

Some of the early studies (East, 1931; Straus & Weddell, 1940; Dickson, 1940; Cuajunco, 1942) suggested that neuromuscular contact occurs very early in development. A subsequent view (Kupfer & Koelle, 1951; Couteaux, 1960) was that it does not occur until the muscle-fibre stage. More recent histochemical (Zelená, 1959, 1962; Wake, 1964; Hirano, 1967; Teräväinen, 1968 a) and ultrastructural (Teräväinen, 1968 b; Kelly & Zacks, 1968, 1969 b; Fidziańska, 1971) studies have proved, however, that motor end-plate formation begins on myotubes.

In the intercostal muscle of rat (Kelly and Zacks, 1968, 1969 b) definite axon terminals are present by the 18 DF stage and may contain dense-cored vesicles that have also been identified in cultured end-plates (Schimada, Fischman & Moscona, 1969; Pappas, Peterson, Masurovsky & Crain 1971). The subsarcolemmal sarcoplasm shows no differentiation at this stage; the sole-plate develops postnatally. At birth, a distinct basement membrane separates the networks of axon sprouts, which lie in saucer-like depressions (primary clefts) of the more differentiated muscle fibres. Post-junctional folds are mostly absent. This is represented histochemically by a plate-like area of acetylcholinesterase activity (Teräväinen, 1968 a). Large and small muscle fibres may be innervated by adjacent terminal axon sprouts, within a single Schwann cell. Separation, which is accompanied by the myelination of the peripheral nerve (Peters & Muir, 1959;

Friede & Samorajski, 1968) and the development of deep primary synaptic clefts, occurs by the second postnatal week.

Developing sole-plate is indicated in rat neonates by the presence of 1-2 nuclei within aggregations of sarcoplasm, either beneath or to one side of the post-synaptic membrane. Secondary synaptic clefts form by the infolding of the plasma membrane of the primary cleft. By the 10th postnatal day their development is complete. This is represented by a ramification and segmentation of the area of acetylcholinesterase activity (Teräväinen, 1968 a).

Chapter 4. Experimentally-induced changes in extrafusal neuromuscular development.

Although, as J. A. Moore (1949) suggests, "every morphological characteristic is the result of physiological processes" (p. 23), static, fixed samples of normal, developing muscle are difficult to interpret in terms of the factors influencing this process. Because of its close association with muscle, and the pathological effect when this association is withdrawn, the obvious potential effector during development is the nervous system. This has been tested in many investigations by the use of denervation techniques.

4.1 Changes in myogenesis.

The controlling factor in the conversion of myoblasts into myotubes is unknown. For many years, however, it has been appreciated that this stage of differentiation can occur independently of nerve fibres (Harrison, 1904). This classical study involved the removal of spinal cord and neural crest from frog embryos before the histological differentiation of nerve or muscle tissue, and the examination of these tissues 1-6 days later. Harrison found that the differentiation of muscle occurs in the normal manner.

The maturation of myotube into muscle fibre, which occurs much later in development than the stage studied by Harrison, is a separate consideration, and has been studied extensively in rat (Zelená, 1959, 1962). Zelená found that denervated developing muscles at first contain higher proportions of myotubes than normal muscles. This percentage is reduced later, for example by the 20th postdenervation day,

but still remains higher than in normal controls. This could mean that denervation preferentially affects the mature fibres already present and that subsequently there is a loss of both myotubes and mature fibres. However, in muscles composed almost entirely of myotubes at the time of denervation, the percentage of mature muscle fibres may be as high as 50%, 20 days later; it seems therefore, that the conclusion that differentiation occurs independently of innervation is valid. The persistence of myotubes in neonatally denervated muscle has subsequently been noted (Engel & Karpati, 1968; Boëthius, 1971; Shafiq, 1972).

Dissociated myoblasts from 12-day chick embryos differentiate *in vitro* into muscle fibres, in the absence of any functional nerve elements (Schimada, Fischman & Moscona, 1967; Askanas, Shafiq & Milhorat, 1972) suggesting again a non-neural influence.

The idea that not all muscle-cell components undergo atrophic changes following denervation, some structures being apparently less affected, or even exhibiting a definite overdevelopment, was especially emphasised by Muscatello, Margreth & Aloisi (1965), on the basis of a morphological and biochemical investigation of denervated frog muscle. Observations of developing muscle, both *in vitro* (Schimada *et al*, 1967) and *in vivo* (Schiaffino & Settembrini, 1970), have shown that the differentiation of the SR and TT systems proceeds as well in the absence of innervation as in its presence. However, the distribution and quantity of the membrane systems is abnormal.

Three distinct types of muscle fibre have been described

by ultrastructural criteria as well as by differences in their enzyme content. In the diaphragm muscle (Gauthier & Padykula, 1966) and semitendinosus (Gauthier, 1969) of rat, for example, red, white and intermediate fibres (corresponding to histochemical types B, A & C respectively) can be distinguished by differences in fibre diameter, mitochondrial content, width of Z band and form of SR. These differences do not develop in rat soleus or extensor digitorum longus muscle, following denervation at birth (Shafiq et al, 1972). It seems therefore that the nervous system does play a role in the ultrastructural differentiation of muscle fibres.

4.2 Changes in muscle growth.

In very young animals, denervated muscles not only fail to lose initial weight but may show an absolute weight increase, although at a slower rate than in normal muscle (Zelená & Hník, 1957). Zelená (1962) therefore distinguishes between absolute atrophy, which does not occur in young animals and relative atrophy, which is characterized by growth, although reduced by comparison to normal growth.

A later study by Stewart (1968), in which gastrocnemius, plantaris, soleus and hemidiaphragm muscles were denervated in rats from birth to old age, showed that the ability to gain weight following denervation is gradually lost with increasing age in all muscles except the hemidiaphragm, where an increase over initial weight occurs at all ages. The limb muscles of young animals that were tenotomized as well as denervated, atrophy to an extent approaching the sum of atrophy due to denervation and tenotomy, when carried out

separately. In older animals, the atrophy produced by the combined operation equals that obtained by denervation or tenotomy alone. Tenotomy appears therefore to prevent some, if not all of the growth that occurs in denervated muscle of newborn rat. The greater rhythmical stretch of the hemidiaphragm muscle following denervation accommodates the growth of muscle fibres. Stewart also found a close correlation between the extent of growth of denervated muscle and the elongation of the tibia, which increases the tension of the developing muscle. It appears, therefore, that tension is the cause of growth of denervated muscle in young animals.

Following intrauterine and neonatal tenotomy of limb muscles in the rat, Zelená (1963) found a rapid relative atrophy of the affected muscles represented by a very small growth increment and a reduction in both the cross-sectional area of the muscles and muscle-fibre diameter.

Denervation causes the withdrawal of two influences from innervated muscle; the possible influence of the neuron itself and the elimination of tension on the muscle fibre by its resulting disuse. The fact that denervated muscle retains the ability to grow when the limb bones are rapidly elongating, and that tenotomy enhances this atrophy only at the time of skeletal elongation suggests that it is muscle tension the primarily influences muscle growth.

4.3 Changes in motor end-plate morphology.

The sequence of post-synaptic events during the morphogenesis of the motor end-plate suggested to Couteaux (1960, 1963) that the structural changes in the sub-neural

membrane result from an inductive action of the motor axon. At the same time, Zelená (1959, 1962) found that intrauterine denervation causes the disappearance of acetylcholinesterase activity from the nerve plexus and the arrest and dedifferentiation of the developing end-plate. The differentiation of the sub-neural apparatus of the myoneural junction is therefore directly dependent on the influence of axon terminals.

Recent investigations (Terävänen, 1968 c) have indicated that this influence is long-lasting, as compression of the muscle nerve in newborn rats causes a delay in the further differentiation of the end-plate, which otherwise proceeds normally (Terävänen & Juntunen, 1968). The ability of the motoneuron to induce the formation of new end-plates decreases however with age (Juntunen & Terävänen, 1969; Saito & Zacks, 1969).

4.4 Changes in muscle physiology.

As was first appreciated by Banu (1922), at the time of birth all limb muscles of the kitten contract and relax slowly, the twitch contraction having a time-course that resembles that of adult "slow" muscle. Extensor digitorum longus and soleus muscles of newborn rat and mouse show a similar anomaly (Close, 1964), although the speed of contraction of these muscles is considerably slower than is found in adult rat "slow" muscle. After birth both in cat (Buller, Eccles & Eccles, 1960 a; Buller & Lewis, 1965) and rat (Close, 1964; Gutmann & Melichna, 1972) a progressive shortening of the contraction time of those muscles destined to become "fast" occurs over a period of 6 weeks, by which time the adult speeds of contraction are fully established.

It has been shown that this change is influenced by the motor nerves supplying the muscles (Buller, Eccles & Eccles, 1960 a, b; Close, 1969). Thus in newborn kitten when the nerves supplying soleus and a muscle that would normally become rapidly contracting are cut and cross-united, soleus develops into a "fast" muscle, and the muscle receiving the soleus nerve remains slow. Similar results can be obtained in adult cat, suggesting that the neural influence is long-lasting. A recent investigation by Lewis (1973) suggests however that the differentiation process in kitten can occur in the absence of any immediate nerve influence. It may be, as Lewis proposes, that the nerves act on muscle at an age when there are only small differences between the contractions of potential "fast" and "slow" twitch muscles and initiate a process of change that has a very slow time course.

4.5 Changes in histochemical differentiation.

The results of random re-innervation experiments in adult rats (Yellin, 1967 a, b) and cross-innervation experiments in adult rabbits (Dubowitz, 1967), guinea pig (Karpati & Engel, 1967 b), cat and rat (Guth, Samaha & Albers, 1970; Brooke, Williamson & Kaiser, 1971), where a change in the predominance of histochemical fibre types resulted, suggest that the type of innervation has a direct influence on both the contractile properties (see 4.4) and related histochemistry (Edgerton & Simpson, 1969) of a muscle.

By inference, the removal of this nervous influence at the onset of differentiation should prevent the histochemical maturation of muscle fibres. That this process

is impaired has been successfully shown (Karpati & Engel, 1967 a; Engel & Karpati, 1968; Shafiq et al, 1972). Cultures of breast and leg muscle of chick (Askanas et al, 1972) also fail to show any histochemical variations in maturing fibres in the absence of innervation.

It seems however that some fibres in rat soleus mature into type B fibres despite the removal of the nerve influence (Engel & Karpati, 1968) suggesting that this histochemical type is less dependent on a neural influence than others. The random re-innervation of neonatal rat limb muscles produces an atypical distribution of B and C type fibres in soleus muscle, in the form of islands of muscle fibres of one histochemical type (Yellin, 1967 a, b). No foreign (type A) fibres differentiate, suggesting an attraction of the appropriate muscle nerve by soleus muscle.

Chapter 5. The present study.

5.1 Justification.

The understanding and treatment of normal and abnormal motor function are dependent on the realization of the importance of the balance of influences necessary, not only for normal development, but also for the maintenance of this function in the adult. The evidence presented in Chapters 2 and 4 indicate that this balance may be quite different during development than in maturity (also Zelená, 1964). Knowledge of adult and developmental morphology can provide a firm foundation for diagnostic and therapeutic procedures in muscle disease. Knowledge of the adult spindle morphology has increased greatly in the past decade, due chiefly to the recent use of electron-microscopic and histochemical techniques (see Barker, 1973). During the course of this investigation, observations of the adult rat spindle (Ovalle, 1971, 1972 a; James, 1971 a, b), revealed the presence of ultrastructural and histochemical differences between intrafusal muscle fibres, the development of which had not been traced.

Several events in the development of the muscle spindle remain vague due to conflicts in experimental results or lack of study. Although it is generally accepted that before the onset of spindle differentiation muscles consist of a homogeneous mass of myotubes, the possibility of fine structural differences between potential intrafusal and extrafusal fibres cannot be excluded. Mavrinskaya's (1967) recent finding that afferent and efferent nerve fibres arrive at the future spindle site simultaneously, re-opens

the question of whether the myogenic pathway of myotubes is induced by sensory or motor nerve fibres, by neither, or by both. The ultrastructural morphology of developing sensory and fusimotor terminals is not documented. It was thought that evidence of the nature of the mechanisms through which these endings may exert a morphogenetic effect may be found in their ultrastructure.

The sequence and origin of the increment of intrafusal muscle fibres has been disputed since the very first studies on spindle morphogenesis. It was thought that the increased resolving power of the electron microscope might throw some light on this controversy. Similarly, although some operative procedures, such as ventral-root section (Zelená, 1964, 1965) do not apparently affect spindle morphogenesis, the subsequent ultrastructural maturation has not been traced.

When the examination of developmental and adult material is carried out at the same time, it is inevitable that the knowledge of one will contribute to the other. It was therefore considered that an investigation into the fine structure of foetal spindles might elucidate some of the features of the adult spindle.

At the outset of this study (October, 1970) there were no published accounts of the fine structure of developing muscle spindles. During its course, however, the results of a similar investigation were reported by Landon (1972 a, b) and a passing reference made to an immature spindle in cat (Scalzi & Price, 1971). Because of the concurrence of these investigations with this study, the results are presented

and discussed in Parts III and IV, with those of this study. Some of the results of this investigation have been published (Barker & Milburn, 1972; Milburn, 1973).

5.2 The animal used.

An essential requirement for studies of muscle-spindle development is a plentiful supply of accurately aged foetal material, and so this has restricted previous investigations to mainly the embryos of man, pig and rat. One of the advantages of studying the rat is that, unlike the other species, the later stages of development are completed post-natally, which lends itself to easier experimental interference of the developmental process. The present study was carried out in rat for these reasons. The fact that the conventional chronological development was already known, and that the musculature of foetal and neonatal rats is of a suitable size for electron microscopy, also contributed to its selection. The relative wealth of information on the adult spindle and on the ultrastructure of extrafusal neuromuscular development were also contributing factors.

5.3 Plan of study.

The fine structure of muscle spindles was studied in developing lower hindlimb muscles of the rat under normal and experimentally-induced abnormal conditions. For clarity, these conditions are treated separately in both Parts II and III, and the results discussed jointly in Part IV. This separation also reflects their treatment and actual sequence during the study.

Muscle spindles of newborn guinea pig and mouse were also inspected as a comparison with rat, to confirm that

gestation length and degree of maturity at birth are reflected in the ultrastructure of the spindle at this time.

From the ultrastructural observations of developing and adult rat spindles it was postulated that the ultrastructural variations of rat intrafusal muscle fibres may be an expression of their variable motor innervation. The experimental model needed to test this, involves the prevention of the arrival and establishment of the fusimotor innervation in neonatal rat spindles. Because of the operative difficulties in ventral-root section and spinal cord extirpation (Zelená, 1965 and personal communication), as well as the problems in the survival of such operated animals, an alternative method of selectively destroying motoneurons was tested.

Barker (1962) found that the interosseous nerve of adult cat distributes 150-200 myelinated nerve fibres to flexor hallucis longus (FHL) muscle and the interosseous membrane. The fibres supply, on average, 60 Pacinian corpuscles, 20 tendon organs, 3 muscle spindles and a number of free endings. The nerve contains no motor nerve fibres. The spindles that are innervated by this nerve receive their motor component from a separate source, namely the FHL muscle nerve (Barker, personal communication).

An exploratory investigation into the innervation of FHL muscle of adult rat, by osmium teasing and silver impregnation (Barker & Ip, 1963) showed that the topography of the interosseons nerve (Fig. 2) is similar to that in cat. It supplies tendon organs and Pacinian corpuscles, but contains no motor nerve fibres. Whether some spindles

receive their sensory nerve supply from this nerve remained unclear, due largely to incomplete silver impregnation and the difficulties involved in teasing out a whole nerve and all of its end-organs. It was thought justified however, to assume that there may be a peripheral separation of the sensory and motor components of the nerve supply to some spindles.

If this assumption is correct, then sectioning of the FHL muscle nerve at birth in rat, would deprive most of the developing spindles in this muscle of both their sensory and immature motor supply (Fig. 3). Those supposed spindles that have their innervation from separate nerve trunks, would therefore be effectively de-efferented. From Zelená's (1957) previous observations, the denervated spindles would degenerate almost entirely by the 10th postnatal day whereas those lacking a motor component only would develop fully except for a slight reduction in size. The de-efferented spindles would be the only normal spindles present by the 2nd postnatal week.

By extensive sectioning of the FHL muscle nerve in rat neonates, this model was examined. In the same series of operations, the degeneration of denervated spindles and the structure of atypical spincles was examined by electron microscopy.

PART II - MATERIALS AND METHODS

Chapter 6. The normal material.

6.1 Choice of muscles in rat.

The development of muscle spindles begins at different times in the various muscles of a given species, depending essentially on the topography of the muscle. It is known, for example, that its onset is earlier in the thoracic musculature (Kalugina, 1956) than in hindlimb muscles (Zelená, 1957) of rat. Variations can occur however between muscles of the same area and even within the same muscle (Zelená, 1957), although the order of appearance of the spindle components, being the main requirement of this study, is similar in the different muscles of all species studied.

Using as a basis the findings of Zelená (1957), an electron-microscopic study was made of the development of muscle spindles in lower hindlimb shank muscles of Carworth-Europe, Strain CF - HB rats. Because of the initial difficulties encountered in identifying muscles in this area, due to their small size in foetal and neonatal rats (compare Figs. 4 & 5), linked to the speed of dissection necessary for successful electron-microscopic fixation, individual muscles are not named. At least 3 separate muscles were used, from the left limb of each animal at each developmental stage.

To check the feasibility of using a selection of muscles in such a developmental study, the sequence of events leading to muscle-spindle formation was monitored in a single identified muscle of postnatal rats. Flexor hallucis longus

was selected because of its pertinence to the experimental part of this study.

The fine structure of the more mature spindle was examined in peroneus longus (PL) of 16-day postnatal (DPN) and adult rats. This muscle was selected for its small size, which makes it suitable for electron microscopy.

In developing rats, whole lower hindlimbs were sectioned for histochemical and routine histological study; individual muscles could therefore be identified (Fig. 4B). The histochemical profiles of extrafusal muscle fibres were studied in extensor digitorum longus (EDL) of developing and adult rat, as this muscle shows the typical "checker-board" distribution of histochemical fibre-types in each fascicle (Yellin, 1969). Any single fascicle at each developmental stage could therefore be treated as representative of the whole muscle and a comparison made with the adult. The developmental and adult profiles of intrafusal muscle fibres were studied in PL, so that a comparison could be made with their fine structure in the adult.

6.2 Ages of rats used.

Unidentified shank muscles were obtained for electron microscopy from rats at each of the following intervals: 18.5, 19.5, 20 and 20.5 DF, newborn, 1, 2, 4, 8 and 12 DPN. Peroneus longus was examined in 16 DPN and adult rats.

The fine structure of muscle spindles was examined in FHL of newborn, 1, 3, 5 and 12 DPN rats, and in FHL of newborn guinea pig and mouse. Whole lower hindlimbs were used for histochemical and histological survey from rats of the following ages: 18.5, 19.5 and 20.5 DF, newborn, 4, 8 and

12 DPN. Additional histochemical studies were carried out in 5 and 17 DPN rats and in PL and EDL of adult animals.

Foetal rats were aged in days from the time of conception, which is assumed to coincide with the appearance of the vaginal plug. A series of mating pairs were introduced for a maximum period of 3hr and the time of the formation of the plug noted. The normal inaccuracy in estimating foetal age was therefore ± 3hr. Each foetus was checked at sacrifice against the drawings of Long and Burlingame (1938) for gross inaccuracies. Gestation lasts 21-22 days in this strain of rat.

Newborn rats were never more than 3 hr old and all came from litters of normal gestation length. Postnatal material was aged in days from the moment of birth. Adult rats were always more than 25 days old, when the spindle is morphologically mature (Zelená, 1957).

All animals in this developmental study came from previously undisturbed litters of more than 8 pups, to prevent accelerated maturity due to small litter size (Dubowitz, 1965).

6.3 Number of rats used.

Table 2 gives the details of the numbers of rats, for each technique in the normal study, and their litter derivation. Of the 45 animals used for electron microscopy, specific muscles were dissected from 15, 10 of which were used for the control study in FHL. The remaining 30 animals were derived from 15 litters at the developmental stages indicated in Table 2 and provided unidentified limb muscles (UM). Histochemical assays were carried out on sections of

whole lower hindlimbs of 19 animals from litters at 8 separate developmental stages in addition to muscles of 2 adult rats. These immature rats were litter-mates of some of those animals used for electron microscopy and of the 7 animals used for routine histological survey.

6.4 The guinea-pig and mouse material.

A fine-structural analysis was made of muscle spindles in FHL of 1 newborn guinea pig (Dunkin Hartley strain), where gestation lasts 61-63 days. A similar investigation was made of spindles in FHL of 2 newborn mice (A_2 G strain) from a single litter. The gestation period is 19-21 days in this strain of mouse.

Table 2.

Number of rats used for each technique in the normal study, and their litter derivation.

AGE	LITTER	NUMBER OF ANIMALS			TOTAL NUMBER		
		EM	HC	HL	EM	HC	HL
18.5 DF	A	2(UM)	2(WL)	1(WL)	2	2	1
19.5 DF	A B	2(UM) 2(UM)	2(WL)	1(WL)	4	2	1
20 DF	A	2(UM)			2		
20.5 DF	A B	2(UM) 2(UM)	2(WL)	1(WL)	4	2	1
Newborn	A B C	2(UM) 2(UM) 2(FHL)	2(WL)	1(WL)	6	2	1
1 DPN	A B	2(UM) 2(FHL)			4		
2 DPN	A	2(UM)			2		
3 DPN	A	2(FHL)			2		
4 DPN	A B	2(UM) 2(UM)	2(WL)	1(WL)	4	2	1
5 DPN	A	2(FHL)	3(WL)		2	3	
8 DPN	A	2(UM)	2(WL)	1(WL)	2	2	1
12 DPN	A B C	2(UM) 2(UM) 2(FHL)	2(WL)	1(WL)	6	2	1
16 DPN	A	2(PL)			2		
17 DPN	A		2(WL)			2	
Adult		3(PL)	2(EDL+PL)		3	2	
TOTAL	22				45	21	7

DF days foetal
DPN days postnatal
EDL extensor digitorum longus m.
EM electron microscopy
FHL flexor hallucis longus m.
HC histochemistry
HL histology
PL peroneus longus m.
UM unidentified shank m.
WL whole lower hindlimb

Chapter 7. The operated rat material.

7.1 Muscle used.

Peripheral de-efferentation of muscle spindles in FHL of newborn rats was attempted on the basis of a previous study in cat (Barker, 1962; 5.3). Figure 2 illustrates the gross anatomy and innervation of this muscle in adult rat. Flexor hallucis longus arises from the head and medial surface of the shaft of the fibula, the interosseous membrane and the flexor surface of the tibia. Its tendon passes beneath that of flexor digitorum brevis, to divide into 5 slips that insert on to the digits (Green, 1955). The FHL muscle nerve is a branch of the tibial nerve and adopts a course parallel to those nerves innervating tibialis posterior (TP) and flexor digitorum longus (FDL), before entering the muscle (Fig. 2). A branch of the FDL muscle nerve gives rise to the interosseous nerve.

7.2 Age of rats at operation and sacrifice.

The denervation surgery was carried out on rats within 6hr after birth. The operated animals came from 13 litters, each containing at least 9 pups except for litter (Lt) 8 (Table 3). At least 3 of the newborn rats and especially all small, weak animals were left unoperated in each litter. In Lt 8 only 1 animal was not operated. Surviving animals were sacrificed 12 days later, except for those of Lt 12, sacrificed at the 16 DPN stage, and Lt 13, that were sacrificed in pairs, sequentially, at the 1, 3, 5, 7 and 9 DPN stages. In addition to operated rats, one normal animal was sacrificed in all litters, except Lt 2, 3, 7 and 8. Flexor hallucis longus of the left side served as a control

in these animals, as well as the contralateral muscle of all operated rats.

7.3 Number of rats used.

Table 3 summarizes the number of animals used in the experimental study. Thirteen litters of newborn rats were used. Of a total of 87 operated animals, only 52 survived after surgery due, mainly, to cannibalism of the whole litter by the mother (Table 3, Lt 2, 3, 7 & 8). An attempt was made to avoid this by preventing direct contact between the operated animals and the handler. Rubber gloves were worn when the rat pups were removed from and replaced back into the nest.

In litters 1-6, the newborn animals were removed from the mother in pairs and returned after surgery before the removal of the next pair. The survival rate of operated animals using this method was 37%. In litters 7-13, all of the rats to be operated were removed together and replaced after surgery in one batch; the survival rate subsequently rose to 74%. It appears therefore that the frequency of nest disturbance contributes to the occurrence of cannibalism. All of the litters were born to rats in at least their second pregnancy.

In those litters where only some animals survived (Lt 1, 5, 6, 9, 11 & 12), there was no evidence that surgery and handling contributed to the death of the other animals, as the percentage survival rate was higher in operated (64%) than in unoperated (53%) animals. Death in these cases could be attributed to the natural cause of under-nutrition and to cannibalism due to disturbance. The higher survival

rate of operated animals is caused by the selection procedure adopted at birth. Each of these litters (1, 5, 6, 9, 11 and 12) contained at least one unhealthy pup that was always left unoperated and would, under normal circumstances have died shortly after birth. Operated animals were therefore selected for their better chance of survival.

At sacrifice, the FHL muscle nerve of each operated animal was examined with a Zeiss operating and examination microscope. Where nerve section was incomplete or re-innervation had obviously occurred, the operated animal was discarded. This examination reduced the number of apparently successful operations to 22 (Table 3).

Table 3.

Number of animals used in the experimental study.

Litter No.	No. in litter at birth	No. operated	No. unoperated	No. in litter at sacrifice	No. of operated surviving	No. of unoperated surviving	No. of operated discarded at sacrifice	No. of apparently successful operations
Lt 1	13	6	7	7	5	2	3	2
Lt 2	10	6	4	0	0	0	-	0
Lt 3	14	6	8	0	0	0	-	0
Lt 4	9	5	4	9	5	4	5	0
Lt 5	12	6	6	2	1	1	0	1
Lt 6	10	6	4	3	2	1	0	2
Lt 7	14	6	8	0	0	0	-	0
Lt 8	3	2	1	0	0	0	-	0
Lt 9	13	10	3	12	9	3	6	3
Lt 10	16	10	6	16	10	6	7	3
Lt 11	14	8	6	11	6	5	5	1
Lt 12	14	6	8	10	4	6	1	3
Lt 13	13	10	3	13	10	3	3	7
TOTAL	155	87	68	83	52	31	30	22

Chapter 8. Operating Procedure

8.1 The anaesthetic.

Newborn rats were anaesthetized by whole-body cooling. Stainless steel dishes, lined with soft paper, were pre-cooled for 15 min at -5°C. Individual rats were placed in the dishes for an average of 10 min. Hypothermia is indicated by a fading of skin colour from red to white and an insensitivity of the animal to tactile stimulation Deep anaesthesia using this method lasts more than 15 min. The operation was carried out at room temperature in aseptic conditions.

8.2 The operation.

A single incision was made on the medial surface of the lower hindlimb of the left leg, beginning below the knee and extending over a length of 1 cm. The great saphenous vein, which lies just below the skin, acted as a guide. The incision was made between this blood vessel and the tibia. The gracilis and semitendinosus muscles were sectioned close to the tibia, exposing the proximal portion of FHL. Lateral deflection of gastrocnemius and plantaris further exposed the tibial nerve and its branches (Fig. 2). The FHL muscle nerve was then sectioned several times using fine tenotomy scissors. Bleeding was minimal under these conditions. The incision required a single suture. The wound was sprayed with a surgical plastic dressing (Nobecutane, BDH Ltd).

8.3 Recovery.

Each animal was left at room temperature for a maximum of 30 min after surgery. Recovery from anaesthesia always began within this time. It was then transferred to a padded

hotplate at 37-40°C, until normal skin colour had returned (usually within 15 min) and then returned to the nest. At the time of sacrifice, the wound had healed well, with very little scar tissue. It was therefore found necessary to mark the operated animals. This was carried out at the time of surgery, by snipping the tip of the tail.

Chapter 9. Methods for histochemistry.

9.1 Preparation of fresh-frozen sections.

Fresh-frozen sections were used for the demonstration of enzyme activities of intrafusal and extrafusal muscle fibres both in developing and mature muscle.

9.11 Adult material. Peroneus longus and EDL were excised from adult rats, cut into suitable pieces and frozen by immersion, for at least 1 min into a slurry of liquid nitrogen and isopentane at approx. -160°C (Maxwell, Ward & Nairn, 1966). The muscles were frozen directly on to metal chucks using 5% gum tragacanth (Engel & Cunningham, 1963) or, more often, to pieces of stiff card and later attached to the chucks using gum tragacanth and a similar freezing mixture as before. If not immediately required for sectioning, the muscles were stored in sealed containers at -30°C. Before sectioning, all frozen material was placed in the cryostat (Slee Ltd) to equilibrate at -20°C.

Serial 10μm transverse sections were cut from the middle portion of the muscle. The exact orientation and position of spindles in the muscle was checked initially with the light microscope, using frozen sections stained with a histological stain, toluidine blue (TB). Sections were then picked up in pairs on clean microscope slides and stored in the cryostat for further use. In this way, successive pairs of sections could be processed for each of the histochemical methods and one for the histological stain. Whenever possible, sections were taken of muscle spindles in the polar and equatorial regions, so that the identification of nuclear-bag and nuclear-chain fibres could be made.

9.12 Immature material. Foetal rats were dissected quickly from the mother. Lower hindlimbs of the left side were excised from knee to ankle. Similar whole lower hindlimb (WL) preparations were made from neonatal and postnatal rats. All were frozen and sectioned as described for adult material.

Spindles are not easily identified in foetal and neonatal rat muscle using these techniques, because of their small size and the thickness of the sections. The histochemical study at these developmental stages was therefore confined to extrafusal muscle fibres. The first histochemical profile of intrafusal muscle fibres was obtained at the 5 DPN stage and was compared to a similar study at the 17 DPN stage. As with adult muscle, sample serial sections were taken, where possible, at the polar and equatorial levels of each spindle.

9.2 Staining techniques.

Fresh-frozen sections were incubated to demonstrate the activity of succinic dehydrogenase (SDH), alkaline-stable actinomyosin, adenosine tri-phosphatase (ATPase) and phosphorylase (Pase), so that the histochemical profiles of both intrafusal and extrafusal muscle fibres could be determined. Histochemical staining was carried out on 3 separate occasions, using frozen sections of muscles at the following developmental stages:-

a. 18.5, 19.5 & 20.5 DF and adult rat

b. newborn, 4, 8 and 12 DPN and adult rat

c. 5 and 17 DPN rat.

On each occasion the sections were passed through the same batch of incubation media, but in different vessels for each developmental stage, to reduce any variation in treatment. The incubation media were freshly-prepared from stock solutions on the day of staining for (a). It was found, however, that the media for all of these enzymes stay active for at least 2 weeks, if kept at a temperature of -30°C and allowed to thaw at room temperature on the day of staining. Sections of (b) and (c) were treated with such pre-frozen incubation media. The details of the histochemical techniques are briefly described in the following sections.

9.21 Succinic dehydrogenase. Nitroblue tetrazolium was used as a hydrogen ion acceptor according to the method of Nachlas, Tsou, de Souza, Cheng & Seligman (1957) as modified by Pearse (1961). Incubation was carried out at 37°C and pH 7.6. The optimum incubation time was 25 minutes. The sites of activity of SDH are defined by purple diformazan granules scattered in the sarcoplasm of intrafusal and extrafusal muscle fibres.

9.22 Alkaline-stable actinomyosin adenosine tri-phosphatase. The ATPase activity of developing and adult muscle fibres was demonstrated by the method of Guth & Samaha (1970). The alkaline pre-incubation solution was adjusted to a predetermined cptimum pH of between 9.6 and 9.8. Incubation was carried out at 37°C for 45 minutes.

9.23 Phosphorylase. The method of Eränkö & Palkama (1961) was used with the single modification that incubated sections were mounted in D.P.X. and not iodine-glycerol. This gives a better contrast between different intensities

of staining for photographic purposes. Sections were incubated at room temperature for 25 minutes.

9.3 Examination of preparations.

Intrafusal and extrafusal muscle fibres in both developing and adult muscle were classified on the activity of all 3 enzymes wherever possible, as it is only in this way that true profiles of developing fibres can be obtained (Dubowitz, 1970). Variations in a positive reaction of each enzyme were not recorded in developing muscle fibres; each fibre was simply recorded as low or positive. Low activity of SDH is represented by a fine scattering of small diformazan granules, of ATPase by colourless fibres and of P'ase by fibres of a golden-yellow colour. Positive reactions for SDH include larger, closely-packed diformazan granules and thus an overall darker staining pattern; for ATPase by various shades of brown fibre and for P'ase by brown and blue-black fibres.

The positive reactions of adult muscle fibres for ATPase and P'ase could usually be sub-divided into intermediate and high activities. Intermediate activity for ATPase and P'ase is represented by brown fibres. High activity is represented by dark brown staining for ATPase and a blue-black colour for P'ase.

Sections stained for the activity of each enzyme were examined using a Zeiss GFI microscope. Each extrafusal muscle fibre within selected fascicles of EDL was identified (Table 1) and its enzyme profile recorded. The histochemical differentiation of intrafusal muscle fibres was monitored in PL of developing and adult rats. The most valuable observa-

tions were made at the 5 and 17 DPN and adult stages. Intrafusal muscle fibres were identified as nuclear bag or nuclear chain by the examination of sections stained with TB at the equatorial level of muscle spindles. Individual enzyme profiles of their polar regions were then recorded.

Representative preparations of both intrafusal and extrafusal muscle fibres, selected on the basis of their clarity were photographed using the Zeiss Ultraphot II microscope. All photographs were produced to a standard enlargement.

Chapter 10. Methods for wax sections.

The gross anatomy of the limb and conventional histology of the muscle spindle were examined at several developmental stages (Table 2), using serial wax sections of whole lower hindlimb preparations (Figs. 4 & 5), excised as before (see 9.12).

The preparations were fixed in Bouin solution, dehydrated in ethyl alcohol, cleared in chloroform and embedded in plasticized paraffin wax (Paramat, George T. Gurr Ltd). Serial transverse section (10μm) were cut on a Spencer A20 microtome, and stained with haemalum and eosin (H & E). Representative sections were examined, and photographed as described before at the level of insertion of the semitendinosus muscle (Figs. 4 & 5). Since the conventional histology of the muscle and muscle spindle does not constitute a major part of this study, it is not treated separately but used simply as a reference point in Parts III and IV.

Chapter 11. Methods for electron microscopy.

The examination of the fine structure of immature muscle spindles in rat constituted the main part of this study. Spindles in the adult PL muscle were also examined so that the mature structure could be assessed. Operated muscles of 12 DPN rats were treated in a similar manner to normal postnatal muscle.

11.1 Fixation and embedding techniques.

11.11 Fixation of foetal material. Foetal rats were removed quickly from the mother. Their lower hindlimbs were then skinned, excised from above the knee and immersed into buffered fixative at 4°C. The initial fixation was for 30 min, after which individual, unidentified shank muscles were dissected out and fixed for a further 2 hr in fresh solutions at 4°C.

The fixative used varied according to the age of the material, as recommended by Kelly & Zacks (1969 a, b). The muscles of 18.5 and 19.5 DF rats were fixed in a solution of 6% gluteraldehyde in 0.1M phosphate buffer at pH 7.2. For those of 20 and 20.5 DF rats a solution of 2.5% gluteraldehyde and 1% paraformaldehyde in 0.1M phosphate buffer at pH 7.2 was used. All fixative solutions were made immediately before use.

The muscles were post-fixed using the following solutions, at 4°C: 0.2M sucrose in 0.1M phosphate buffer, pH 7.2 (1 hr); 1% osmium tetroxide in 0.1M phosphate buffer, pH 7.2 (2 hr); 0.1M phosphate buffer, pH 7.2 (30 min).

11.12 Fixation of postnatal and operated material. Muscles of postnatal and operated rats and FHL of neonatal guinea pig and mouse were all, as with foetal material, fixed initially, for 30 min, in position within the whole limb. The required muscle (see 6.1 & 7.1) was then excised using the Zeiss examination microscope, and fixed for a further 2 hr in fresh solutions at 4°C. In operated rats, the complete FHL muscle was precisely excised from origin to insertion with the interosseous nerve and membrane. In 16 DPN and adult rats, PL was removed and immersed in fixative while slightly stretched. After preliminary fixation the undamaged muscle was cut into smaller pieces and re-immersed into fresh fixative for a further 2 hours. Severe stretching of the muscle was avoided, as this is known to affect the configuration of the myofibril (Knappeis & Carlsen, 1968). The fixative used was 3% gluteraldehyde in 0.1M sodium cacodylate buffer, at pH 7.3 (Mercer & Birbeck, 1966). Post-fixation was carried out as described for foetal material, using 0.1M sodium cacodylate buffer, at pH 7.2.

11.13 Dehydration and embedding. The dehydration of all material was carried out using the following solutions: 50%, 70%, 95% and absolute alcohol (1.5 hr); propylene oxide (10 min). The muscles were infiltrated with Epon using the following mixtures: 50% Epon/50% propylene oxide (3 hr); 75% Epon/25% propylene oxide (3 hr); absolute Epon (12 hr). The muscles were then embedded in fresh Epon, subsequently polymerised at 60°C for 48 hr.

11.2 Sectioning and staining techniques.

11.21 Sectioning of normal material. All material was

sectioned on a LKB or Reichert OMU2 ultra-microtome, using glass knives. Thick (approx 1μm) transverse sections, stained with TB in 1% borax were scanned with a light microscope for muscle spindles. The definite identification of muscle spindles at the 19.5 DF stage could only be made in the equatorial region, by the paleness of the spindle nerve trunk (Fig. 6). Once located, the block face was then trimmed so that the spindle lay in the centre. A series of thin sections (silver to yellow interference colour) were cut and picked up on uncoated copper grids. Thick sections, covering a length of 10-15μm were then taken, stained as before, and examined with a light microscope for any changes in spindle morphology, followed by a second series of thin sections. Using this method the position of the spindle on the block face could be gauged before viewing with the electron microscope and its location consequently, could be made more rapidly. Beyond the zone of sensory innervation, spindles at this early stage of development could not be located with the light microscope. The block was therefore uniformly trimmed to maintain the central position of the spindle, thin sections cut and their fine structure scanned for any configurations typical of muscle spindles.

At later stages of development (Figs. 7-11), the identification of spindles with the light microscope was made easier by the progressive thickening of the capsule in the equatorial region, and its extension into the polar zones. Thin transverse sections for electron microscopy were taken at progressively longer intervals in maturing

muscles, of approx. 20μm at the 20.5 DF stage and 100μm in the adult. By this technique of alternate electron- and light-microscopic examination of skip-serial transverse sections, a more complete picture of the morphology of developing spindles could be gained. The guinea-pig and mouse material was treated in the same manner.

Longitudinal sections of muscle spindles were obtained at postnatal and adult stages only. Identified spindles were sectioned transversely as described before; the block was then re-orientated through 90° and thick sections cut to check the plane of section. If necessary, adjustment was then made so that the plane was longitudinal. Thick serial sections were then examined until the axial bundle became visible. The intrafusal muscle fibres were usually serially-sectioned for electron-microscopic study.

11.22 Sectioning of operated material. The FHL muscle of operated animals was skip-serially sectioned for possible de-efferented muscle spindles. Beginning at the level of entry of the interosseous nerve (Figs. 2, 167, 168), thick sections at approx. 100μm intervals were examined by TB staining, to the terminations of the nerve in Pacinian corpuscles (Figs. 2, 169). The distance sectioned in this manner was less than 0.5 cm. Sample transverse sections for electron-microscopic observation were taken of any structures with muscle-spindle characteristics.

The effects of denervation on the development of the spindle were studied in the operated animals of Lt 13, at successive postoperative stages (see 7.2). The denervated spindles were sectioned as for normal material, in the

transverse plane only.

11.23 Staining and examination. Thin sections were stained with uranyl acetate followed by lead citrate (Reynolds, 1963) and subsequently examined and photographed with an AEI 801 electron microscope. This sections stained with TB were photographed using the Zeiss Ultraphot II microscope.

PART III - RESULTS

Chapter 12. Muscle spindles of adult rat.

12.1 General morphology.

The adult complement of intrafusal muscle fibres in PL of rat was found to be on average 4, of which 2 are normally nuclear-bag fibres and the remainder nuclear-chain fibres. Of the 12 spindles examined in detail by electron-microscopic and histochemical techniques, 10 contained 4 intrafusal fibres, consisting of 2 nuclear-bag and 2 nuclear-chain fibres; one spindle contained 2 nuclear-bags and 3 nuclear-chains (Fig. 12) and another 3 nuclear-bags and 3 nuclear-chains (Figs. 31-34). The central regions of these fibres are surrounded by a well-defined capsule, which is penetrated near the equator by the spindle nerve trunk (Fig. 12).

The nuclear-bag fibres are longer than the nuclear-chain fibres (Fig. 26). The equatorial region of the intrafusal fibres is enwrapped by the coiled sensory nerve endings. Secondary sensory terminals have not been identified by electron microscopy with any certainty, but appear morphologically indistinguishable from the primary ending (Landon, 1966 a). Both "plate" and "trail" (Barker & Ip, 1965) motor terminals have been identified in these spindles.

Much of the description that follows of the ultrastructure of many of the spindle components confirms the observations of Merrillees (1960), Landon (1966 a, b) and Ovalle (1971, 1972 a, b) of spindles in rat lumbrical muscle, and of a series of investigations in other species

(see Barker, 1973). For the sake of brevity, detailed acknowledgement has been omitted.

12.2 Capsule and axial sheath.

At its thickest part, the capsule consists of 6-8 layers of thin, flattened cells that are arranged in a concentric fashion (Figs. 12-14). Each of these "capsular sheet cells" (Merrillees, 1960) contains small mitochondria, scattered ribosomes and numerous small, round or oval micropinocytotic vesicles, in a fully-formed state or at various stages of assembly (Figs. 13 & 14), lining both sides of the cell membrane. Each cell closely interdigitates with its neighbour (Fig. 12), so that an almost continuous layer is formed. Cells of neighbouring layers may also form areas of close contact (Figs. 13 & 14), thus interrupting the channels between the cell layers.

Basement membrane is usually associated with both sides of the capsular sheet cell (Fig. 13). Occasionally, however, a pair of cells may have basement membrane on their outer surfaces only. The channel enclosed by the cells lacks basement membrane (Fig. 14) and the collagen and occasional elastic fibrils that are normally associated with the spaces between other cell layers. Banker & Girvin (1972) have described similar clear channels alternating with those filled with collagen as being a regular feature of canine muscle spindles.

Collagen fibrils in rat spindles are orientated longitudinally or, less frequently, encircle the capsule (Fig. 14). Elastic fibrils, when present, are always longitudinally orientated (Fig. 14) and often appear to

enclose an amorphous ground substance (see also Figs. 15 & 16).

Fibrocytes have been encountered in the outer layers of the spindle capsule (Fig. 12) as well as in the outer layers of the perineurium of the spindle nerve trunk. The axial sheath and endomysial cells (Merrillees, 1960) are composed of similar cells, the cytoplasm of which tends to be more electron dense than that of the capsular sheet cell (compare Fig. 15 to Figs. 13 & 14), which is presumably linked to the higher ribosome content of the endomysial cell. Like the capsular sheet cell, the endomysial cell is flattened and elongated, but the nuclei appear more compact, and the cytoplasm contains fewer micropinocytotic vesicles (Fig. 15). Basement membrane is always absent from both surfaces of endomysial cells (Shantha & Bourne, 1968). In the equatorial region of the spindle, the endomysial cells almost completely envelope each intrafusal fibre. The lateral edges of neighbouring cells are often closely opposed in this region (Fig. 15), to form the enclosure. In the more polar regions of the spindle several intrafusal fibres may be surrounded by each endomysial cell (Fig. 12). Both collagen and elastic fibrils are frequently associated with these cells and the intrafusal muscle fibres (Figs. 15 & 16) and are usually longitudinally orientated. Convolutions of branched and folded basement membrane (Adal, 1969) have also been observed between the endomysial cells and intrafusal fibres. Elastic fibrils are frequently associated with this aberrent basement membrane (Fig. 16).

Blood capillaries have frequently been observed between

the capsule lamellae (Fig. 12) but not in the periaxial space.

12.3 Intrafusal muscle fibres.

At the juxta-equatorial (Fig. 30) equatorial and extreme polar (Fig. 26) regions of the spindle, it is possible to distinguish 3 broad categories of muscle fibre of small, intermediate and large diameters, presumably corresponding to a similar classification of small (7-9μm), intermediate (10-12μm) and large (14-20μm) diameter intrafusal fibres in muscle spindles of the forelimb, hindlimb and trunk musculature of rat (James, 1971 a). These differences in diameter are frequently lost in the polar regions of the spindle (Figs. 27, 28, 29, 32 & 33). The small-diameter fibres are always of the nuclear-chain type, whereas those of intermediate and large diameter possess a nuclear bag. However James (1971 a) has recognized the intermediate-diameter fibre as most often possessing a chain of nuclei at the equator. On the basis of several ultrastructural and histochemical characteristics (Table 4), the large- and intermediate-diameter bag fibre have been distinguished as separate morphological types, namely the typical and intermediate bag fibres (Barker, 1973), respectively.

12.31 Nuclear-chain fibres. In the polar regions of nuclear-chain fibres, the myofibrils are packed as discrete units, with abundant interfibrillar sarcoplasm (Figs. 22 & 25). Glycogen is liberally scattered throughout the sarcoplasm, and may also be distributed within the myofibril, particularly at the I-band level (Fig. 22 and Ovalle, 1971). The mitochondria are large, unbranched and usually orientated

parallel to the longitudinal axis of the muscle fibre (Fig. 25). They appear most frequently at the I-band level, but may extend the length of one or two sarcomeres. They are therefore often encountered in transverse sections through the A-band region of the myofibrils (Fig. 25). Small, spheroidal, membrane-bound sarcoplasmic granules are frequently encountered within the interfibrillar sarcoplasm and in the central core sarcoplasm of the equatorial region. Each granule, as described by Ovalle (1971, 1972 a) consists of a dense, granular central core of material surrounded by a less dense zone (Fig. 22).

The SR is particularly well-developed at the I- and Z-band level of the myofibril. Transverse sections at these levels show the myofibrils almost completely encircled by a double layer of beaded tubules of SR (Fig. 25). Little SR, however, occurs in the A-band region. Junctional couplings of the SR and TT system, in the form of triads, are frequently encountered around the level of the I-band, and may be orientated longitudinally (Fig. 22) as well as transversely. Extensive dilations of the SR cisternal have also been observed, apposed to both the sarcolemma and mitochondria (Ovalle, 1971, 1972 a). Micropinocytotic vesicles, at various stages of assembly are commonly seen beneath the sarcolemma.

In longitudinal sections, a prominent M line can be seen in the centre of the pseudo H-zone.

The nuclei of chain fibres are peripheral in position in the polar regions but towards and at the equator, they become centrally placed to form a chain of elongated nuclei

(Fig. 19), separated by a core of sarcoplasm, rich in glycogen, mitochondria and ribosomes. Golgi complexes are usually juxta-nuclear in position. The myofibrils are reduced in this region to a thin cortical layer.

Most of the small nuclear-chain fibres exhibit appreciable ATPase, P'ase and SDH activity (Figs. 26-34), a profile similar to that of type C fibres extrafusally (Figs. 35-37; Yellin, 1969). The ATPase activity ranges from moderate (Fig. 32) to high (Figs. 26, 28 & 29), whereas extrafusal type C fibres always exhibit high activity. P'ase activity is always high in nuclear-chain fibres. Extrafusal type C fibres however, exhibit moderate P'ase activity (Fig. 36). SDH activity in the nuclear-chains ranges from moderate (Fig. 29) to high (Fig. 34), the diformazan granules having an even distribution over the whole fibre section in the polar regions of the spindle. In type C fibres the granules tend to accumulate beneath the sarcolemma (Fig. 37) while the core of the fibres shows few reaction products. Despite these differences, nuclear-chain and extrafusal type C fibres show similarities, in that each possesses the enzymes involved in both the glycolytic and oxidative pathways of respiration.

12.32 Nuclear-bag fibres. In their polar regions, both typical and intermediate bag fibres possess little inter-fibrillar sarcoplasm, containing few glycogen granules. The myofibrils are therefore poorly delineated, giving the appearance of a single myofibril bundle, particularly in transverse sections through the A-band region (Figs. 23 & 24). Mitochondria are smaller and fewer than those of nuclear-chain fibres. Those of the typical bag fibre are

small and confined to the I-band region (Figs. 20 & 23), seldom extending the length of the sarcomere. The intermediate bag fibre may possess larger mitochondria, compared to the typical bag fibre that are more numerous but still limited to the I-band region in their distribution (Fig. 24). Both types of mitochondria are unbranched and longitudinally-orientated, as in nuclear-chain fibres.

The SR network of both bag fibres is poorly developed at all levels of the myofibril. It consists of a single discontinuous layer around the myofibril in the I- and Z-band region, being totally absent from the A-band region. The network appears more abundant in intermediate bag fibres compared to typical bag fibres (Figs. 23, 24). Junctional couplings between the SR and TT systems are very sparse in typical bag fibres; only occasional triads have been located. Triads are, however, more numerous in intermediate forms of bag fibre (Fig. 21). Dilated cisternae of the SR have not been observed in either type of bag fibre, as previously noted in rat lumbrical muscle (Ovalle 1971, 1972 a). Micropinocytotic vesicles are present immediately beneath the sarcolemma in both forms of bag fibre.

In the two spindles successfully sectioned longitudinally for electron microscopy, the typical bag fibre was found to lack a distinct M line in the centre of an ill-defined pseudo H zone (Fig. 20). The presence of an M line, in the form of 2 faint parallel lines, as described by Ovalle (1971, 1972 a) in nuclear-bag fibres of rat lumbrical muscles, has not so far been observed in rat peroneal spindles. Intermediate bag fibres, however,

possess a more distinct M line, although the H zone may still be ill-defined (Fig. 21).

In the myotube regions of both typical and intermediate bag fibres, the nuclei lie centrally (Figs. 17 & 18) in a core of sarcoplasm, surrounded by a thin layer of myofibrils. The nuclei of the myotube and equatorial regions of both types of bag fibre are more compact than those of the nuclear-chain fibres (Fig. 19). The sarcoplasmic core is rich in mitochondria, ribosomes and Golgi complexes with scattered profiles of granular ER. Sarcoplasmic granules, of a similar structure to those described in nuclear-chain fibres have also been located in this region. At the level of the nuclear bag, the nuclei increase in number, filling the muscle fibre up to a maximum of 3 abreast. The nuclear bag of intermediate fibres may be smaller than that of the typical bag fibre, showing only 2 nuclei in cross-section. This is not however always the case; the nuclear bags of each type may be of equal size.

It is on the basis of their histochemical profiles that the difference between typical and intermediate bag fibres is most obvious. The larger typical bag fibre exhibits moderate to high ATPase, moderate P'ase and low SDH activity in the polar and juxta-equatorial regions of the spindle (Figs. 26-34). The large diameter type A fibres seen extrafusally in PL exhibit moderate ATPase, high P'ase and low SDH activity (Figs. 35-37). The largest diameter fibres both intrafusally and extrafusally are therefore similar in that they both have a predominance of those enzymes involved

in the glycolytic pathways of respiration. The intermediate bag fibre, however, exhibits low ATPase and P'ase activity. The SDH activity ranges from moderate (Fig. 29) to high (Fig. 34), but is always more positive than that of the typical bag fibre. Type B fibres extrafusally have similar low glycolytic enzyme profiles (Figs. 35 & 36) and show a correspondingly high SDH activity which may be equal to or exceed that of type C fibres (Fig. 37). These findings confirm the observations of Yellin (1969) and, in part, those of James (1971 b), of the histochemical profiles of intrafusal muscle fibres in lower hindlimb muscles of the rat. Yellin, however, describes a fourth histochemical type of intrafusal fibre, that exhibits low P'ase and SDH activity. This type has not yet been observed in rat PL muscle.

12.4 Sensory terminals.

The sensory terminals in muscle spindles of rat peroneal muscle have a similar ultrastructure to those described in rat lumbricals (Merrillees, 1960; Landon, 1966 a, b) and in other species (see Barker, 1973). It was not found possible to distinguish between the axon terminals of primary and secondary sensory nerve fibres in electron micrographs, even on the basis of the difference in shape suggested in cat (Corvaja, Marinozzi & Pompei ano, 1969) and in rat (Mayr, 1970). There is evidence that secondary sensory endings are located mainly on nuclear-chain fibres (Karlsen, 1965; Porayko & Smith, 1968; Mayr, 1969) in the jaw and lumbrical muscles of rat. If the same is true for spindles in PL, then secondary endings are best identified

Table 4.

Characteristics of intrafusal muscle fibres of adult rat peroneus longus muscle.

FEATURE	DIAMETER		
	LARGE	MEDIUM	SMALL
Equatorial nucleation	Bag	Bag	Chain
ATPase activity	High	Low	Medium
P'ase activity	Medium	Low	High
SDH activity	Low	Medium	High
Mitochondrial size	Small	Medium	Large
Mitochondrial density	Low	Low	High
M line	Absent	Present	Present

by their position on one or both sides of the primary (Gladden, 1969; tail muscles) on nuclear-chain fibres. Using these criteria, the extensive sensory terminal associated with one of the chain fibres in Figure 12 is therefore probably a secondary terminal.

Each sensory terminal contains numerous small mitochondria, many vesicles and occasional neurofilaments. It lies beneath the basement membrane of the intrafusal fibre (Merrillees, 1960; Landon, 1966 a, b). Schwann cell coverings of the terminal are absent (Fig. 12).

12.5 Motor terminals.

Motor terminals of both trail and plate types have been identified. Trail endings are normally juxta-equatorial in position and have been observed to overlap with secondary sensory endings (Fig. 12 & Barker, 1973). Such terminals have been located on typical (Fig. 38) and intermediate bag fibres and on nuclear chain fibres (Fig. 39). The ultrastructure of the terminal does not appear to vary according to the type of fibre innervated (Ovalle, 1972 b). The terminal is smaller than the plate ending and closely applied to the surface contour of the muscle fibre (Figs. 38 & 39). Junctional folds are usually absent (Fig. 40) although widely spaced shallow folds may occasionally be present (Figs. 41 & 42). Each terminal is packed with mitochondria and synaptic vesicles, with sparse scatterings of neurofilaments and neurotubules. Occasional dense-cored vesicles (Figs. 40 & 42) and flattened synaptic vesicles (Fig. 41) as described by Ovalle (1972 b) may be present. Each terminal has a Schwann cell covering (Figs. 38 & 39).

The sole-plate, when present, is confined to a thin subsarcolemmal aggregation of mitochondria-rich sarcoplasm (eg. Fig. 40).

A single motor terminal of plate morphology has been observed in the polar region of a nuclear-chain fibre. It shows an extensive area of myoneural contact (Fig. 43) with the terminals housed in indentations of the muscle fibre. The post-junctional folds are short and unbranched (Fig. 44) but not extensive in their distribution (Fig. 43). A well-developed sole-plate is present, in which the sub-neural sarcoplasm appears rich in mitochondria and glycogen. The terminals are packed with mitochondria and rounded synaptic vesicles. This single plate ending corresponds well with the "nuclear-chain plate" described by Ovalle (1972 b) in rat lumbrical muscle.

Chapter 13. The general morphology of developing rat spindles.

13.1 18.5 DF muscle.

Transverse sections through the mid-length region of shank muscles of 18.5 DF rats show the tissue to consist of groups of cells, at various stages of development, that are separated from similar groups by elongate fibroblasts, scanty collagen fibrils and a large intercellular space (Fig. 45), as previously described by Kelly & Zacks (1969 a) in 18-20 DF rat intercostal muscle. Each group is dominated by one or two large myotubes that possess central nuclei, a peripheral packing of slender myofibrils and extensive focal accumulations of glycogen granules. Several smaller immature muscle cells are closely applied to the walls of the larger "primary" (Kelly & Zacks, 1969 a) myotubes (eg. Figs. 45, 46, 50, 51) as well as occasional uninucleate myoblasts (Figs. 45, 52), that presumably correspond to the "Undifferentiated cells" reported by Kelly & Zacks (1969 a) in a similar location. A thin basement membrane ensheaths each group of cells and does not penetrate between the apposed cell membranes within each group (Figs. 46, 48, 49, 50, 51, 53; Kelly & Zacks, 1969 a; Landon, 1970, 1971).

The myofibril content of the immature cells varies from a thin peripheral scattering (Figs. 46-49) to a more extensive closely-packed arrangement as seen in the primary myotubes. Where the myofibril content is low (Landon, 1971) the apposed plasma membrane of the immature cell is thrown into folds or pseudopodial extensions (Figs. 47-49; Kelly & Zacks, 1969 a; Landon, 1970, 1971), that penetrate the

sarcoplasm of a more mature myotube. The pseudopodia vary in their morphology from broad, shallow invaginations (eg. Fig. 49) to elongate finger-like extensions that may penetrate deeply into the sarcoplasm of the parent myotube. In several micrographs a close association has been noted between the invaginated plasma membrane of the pseudopodium and the nuclear envelope of the apposed myotube (Fig. 47). The pseudopodia adopt both a transverse and longitudinal orientation in the sarcoplasm of the parent myotube, although no evidence has been found that they follow the path of expanded T tubules (Kelly & Zacks, 1969 a). Transverse sections reveal several rounded profiles of plasma membrane enclosing portions of membrane-bound sarcoplasm (Figs. 47, 49) that represent longitudinally-orientated pseudopodia. The sarcoplasm forming such pseudopodia contains glycogen granules and polyribosomes that are the distinguishing features of the immature cell. Myofibrils are not enclosed in the pseudopodia, although they are frequently located at the apposed surface of immature cells (Figs. 47, 49).

Pseudopodial extensions normally invade the sarcoplasm of a single parent myotube; only exceptionally are two myotubes apposed (Fig. 49). At their tips, plasma membrane separation may be indistinct (eg. Figs. 47, 49). Kelly & Zacks (1969 a) suggest this may be due to fusion or to differences in the plane of section. As the pseudopodia may adopt a longitudinal as well as a transverse course, it is more likely that the latter is true. Immature cells have never been seen to show extensive areas of continuity with the parent myotube, that Lipton & Konigsberg (1972) suggest

is an indication of fusion. It therefore appears improbable that such apposition indicates the process of fusion between uninucleate myocytes and myotubes (Kelly & Zacks, 1969 a).

Landon (1970, 1971) suggests that these apposing myocytes are multinucleate myotubes and that as myofibril formation proceeds and surpasses a critical density of 35% of the cytoplasmic cross-sectional area, the pseudopodial processes are lost. Myotubes of such an increased maturity have been observed in this study (Figs. 50, 51), which lack pseudopodia. Such apposition does not appear to occur between myoblasts and myotubes (Figs. 46, 52) or between nascent myotubes with a very low myofibril content and their more mature neighbours (Fig. 53). Specialized areas of membrane contact, consisting of localized thickenings of the apposed plasma membranes separated by a distinct intercellular space (Fig. 53), may occur between myogenic cells. Kelly & Zacks (1969 a) refer to such specializations as "close junctions".

Close inspection of the fine structure of the muscle groups failed to provide any evidence of muscle-spindle morphogenesis, although Landon (1972 a, b) found definite sensory terminals on myotubes of 18 DF rat gastrocnemius muscle. It is assumed therefore in this study, as suggested by Zelená (1957) that at the 18.5 DF stage of development the invading sensory axons are without terminals. The small intramuscular nerve trunks of 18.5 DF rat (Fig. 54) consist of groups of naked axons, of various diameters, that are enclosed by a single cell that possesses an outer covering of basement membrane. Because of its close relationship to

the axons, and on the basis of observations of developing nerve trunks in man (Cravioto, 1965; Gamble & Breathnach, 1965; Gamble, 1966; Ochoa, 1971) and rat (Peters & Muir, 1959; Allt, 1969) such a cell is regarded as a Schwann cell. A few cytoplasmic septa (Fig. 54) that are the processes of Schwann cells penetrate the axon bundle, subdividing it into smaller groups. No axons are completely encircled by Schwann cell processes; only part of the surface of a few axons is in contact with a Schwann cell, most axons being in contact with one another.

The Schwann cell cytoplasm contains a prominent Golgi network, abundant ribosomes in the form of "free" polysomes and those that are membrane-bound (granular ER) and a few mitochondria, microtubules and cytofilaments (Fig. 54). The cells forming the first layers of the perineurium differ from Schwann cells in their more flattened appearance, lack of basement membrane and more extensive profiles of granular ER. The distinction between perineural epithelial cells (Shantha, Golarz & Bourne, 1968) and fibroblasts cannot be made at this stage, as both types of cell during the early stages of development have a similar morphology (Shantha & Bourne, 1968). Developing axons of all diameters contain abundant neurotubules and neurofilaments. Mitochondria appear to be confined to the larger axons (Fig. 54; Ochoa, 1971).

13.2 <u>19.5 DF muscle spindles</u>.

The first evidence of muscle-spindle morphogenesis was found in the shank muscles of 19.5 DF rats (Figs. 6, 55, 56). Unmyelinated nerve axons that lack individual Schwann

cell coverings, extend from the nearby spindle nerve trunk to form small terminals on a single myotube (F1). No basement membrane intervenes between the adjacent axolemmal and sarcolemmal membranes. Schwann cell coverings of the terminals are always absent. It appears therefore that the first axon terminals of the muscle spindle are sensory in nature. Such terminals are frequently housed in depressions of the wall of F1 and never lie singly on the surface but occur as either groups of adjacent terminals, in which each ending is applied to the wall of the myotube (Figs. 56, 76, 78) or as groups of endings that overlap with one another, only one of the terminals associating with the myotube (Figs. 55, 77). Similar "overlapping" sensory terminals have been described by Landon (1972 b) in 18 DF rat spindles and by Uehara (1973) in both foetal and young rat spindles. Mayr (1970) describes a similar arrangement of aggregated secondary sensory terminals, some of which do not contact the nuclear-chain fibres that receive such terminals in adult rat spindles.

The flattened cells that ensheath the spindle nerve trunk spread out around the intrafusal fibre in the innervation zone, enclosing the myotube and its sensory terminals, to form the first layer of the capsule (Fig. 55; Landon, 1972 b).

The initial intrafusal fibre is always at the myotube stage of development, and differs from extrafusal myotubes of a comparable size only by the sensory terminals borne on its surface and, in 3 of the 5 spindles examined, by a small aggregation of myonuclei in the innervated region. The

number of nuclei in transverse section never exceeds 3 (Fig. 55). In two spindles, the innervated zone of F1 showed no nuclear bag (Fig. 56, spindle B); the equatorial myonuclei form a single column in the central-core sarcoplasm, a morphological profile that is identifcal to that of the primary extrafusal myotubes, the juxta-equatorial and polar regions of differentiated nuclear-bag fibres at this stage of development and to the first-innervated myotubes described by Landon (1972 b) in foetal rat spindles.

The polar regions of the early spindles could not be identified with any certainty, because of the absence of nerve terminals and capsule cells. By serial sectioning described elsewhere (11.21), transverse sections of the presumed polar regions of intrafusal myotubes were examined and found to exhibit a similar ultrastructure to extrafusal myotubes. Both contain extensive glycogen deposits, large central nuclei and peripheral myofibrils.

At 19.5 days gestation, the morphology of developing shank muscle is similar to that described in 18.5 DF rat. Myoblasts and myotubes, at various stages of differentiation, are clustered around the walls of primary myotubes. Myoblasts similarly associate with intrafusal myotubes (Figs. 55, 56, 113) in both the equatorial and more polar regions of the spindle. The myoblasts are orientated with their long axes parallel to the intrafusal myotube, beneath its basement membrane (Fig. 113). In only one spindle at this stage of development was a multinucleate myotube seen in association with F1. Its morphology was similar to those observed in 20 DF spindles.

13.3 20 DF muscle spindles.

The 20 DF stage of development is distinguished by the appearance of a small, nascent myotube that lies within the basement membrane of F1 (Figs. 57, 58). There is no intervening basement membrane at the apposed surfaces of the two intrafusal fibres. The two fibres lie together in close apposition, the deep surface of the smaller, nascent myotube (F2) often extending into the sarcoplasm of F1, to form pseudopodial inpushings (Figs. 58, 123), as described in extrafusal myotubes of 18.5 DF rat (Figs. 47-49) and by Landon (1972 a, b) in foetal rat spindles. The pseudopodia follow a transverse and longitudinal path within the sarcoplasm of F1. The apposed sarcolemmal membranes may show areas of specialization (eg. Fig. 58) that resemble the close junctions described by Kelly & Zacks (1969 a). Coated vesicles are also a common feature of the subsarcolemmal sarcoplasm (Fig. 58).

The sarcoplasm of fibres 1 and 2 shows distinct differences in maturity. The myofibrils of F1 are well-packed, although their distribution is limited to the periphery of the myotube. F2 by comparison has a morphology nearer to that of the myoblast. Myofibrils are usually sparse and peripherally positioned in such newly-formed myotubes (Fig. 58). The sarcoplasm is packed with polyribosomes, granular ER cisternae and various cytofilaments.

Sensory terminals are still small at this stage of development, of the overlapping (Landon, 1972 b) or bundled (Uehara, 1973) type (Fig. 57) and are invariably confined to the outer surface of the axial bundle. A distinct nuclear

bag occupies the central region of F1, whereas its smaller partner, F2 is myotubular throughout its length.

The capsule is now at least bilamellate as reported by Landon (1972 b) in spindles of a similar age. Two morphologically distinct types of cell appear to be present. Those flattened cells that tightly enclose the axial bundle and spindle nerve trunk possess numerous ribosomes, some glycogen granules and occasional small mitochondria. Micropinocytotic vesicles may be observed at various stages of formation (Fig. 102) at their cytoplasmic borders. Their appearance is therefore similar to capsular sheet cells (Merrillees, 1960) and perineural epithelial cells (Shantha, Golarz & Bourne, 1968). Basement membrane and lateral overlappings, that are distinctive features of perineural epithelial cells, have not been observed in such capsule cells. In contrast, several flattened cells with electron dense cytoplasm (Figs. 57, 102) are frequently located close to the axial bundle. An abundance of granular ER cisternae is the distinctive feature of this cell type and the fibroblasts that form the endomysial rudiments extrafusally. Such cells may therefore represent the developing fibrocytic elements of the capsule and axial sheath.

Transverse sections of the juxta-equatorial and polar regions of 20 DF spindles failed to provide any evidence of a developing fusimotor innervation.

13.4 20.5 DF muscle spindles.

The muscle spindles of rats at the 20.5 day of gestation may have a similar structure to those of 20 DF

rats. Fibre 2 may still exhibit myofibrillar immaturity compared to F1 (Figs. 59, 120, 124) or, more frequently, show an increased maturity compared to F2 in 20 DF spindles (Fig. 60). Pseudopodial apposition between the two developing fibres is still a common feature, even where F2 exhibits an increased myofibril content (Fig. 60; Landon, 1972 b), a condition that does not prevail extrafusally in developing rat muscle (Figs. 50, 51; Landon, 1971). The pseudopodial interlocking is interrupted in some areas by myoblasts that invariably adopt a position between the developing intrafusal fibres and within their common basement membrane (Fig. 60).

Transverse sections of the equatorial regions of all 9 spindles examined at this developmental stage showed F1 to possess a nuclear bag of a maximum of 3 nuclei abreast. In 5 spindles, F2 also exhibited a small aggregation of myonuclei. This fibre in the remaining 4 spindles was always at the nascent myotube stage, as illustrated in figure 59, with a chain of central nuclei throughout its length. The polar regions of both intrafusal fibres are myotubular and distinguishable from similar extrafusal configurations by a sirgle covering of capsule cells.

Sensory terminals may still be of the overlapping type (Fig. 60) or more commonly adopt an individual spiral course around the outer surface of the axial bundle.

13.5 Newborn muscle spindles.

It was found that the development of spindles in FHL of postnatal rats (see 6.1) followed a similar course to those in a selection of unidentified shank muscles. The

muscle source of the spindles is therefore not referred to in the following account.

By the time of birth, the capsule has increased to several layers at the equator (Fig. 61). Capsular sheet cells completely encircle the axial bundle and spindle nerve trunk. Fibroblast-like cells still border the outer surfaces of the intrafusal fibres (Figs. 61, 103). A single layer of capsular sheet cells demarcates the polar regions of the intrafusal fibres (Fig. 62).

The distinctive feature of newborn rat spindles is the appearance of a third intrafusal fibre (F3), that is always of a smaller diameter than F1 and F2. This nascent myotube possesses few myofibrils in an electron-dense sarcoplasm (eg. Fig. 61) that is packed with polyribosomes and occasional granular ER cisternae. It always adopts a position between the more mature intrafusal myotubes (Figs. 61, 62), into which it may protrude pseudopodial extensions (Figs. 62, 121, 125) of a similar structure to those described in foetal spindles. Fibre 3 is not, however, a multinucleate myotube throughout its length, but in the polar regions of the spindle, may be replaced by a string of myoblasts (Figs. 128, 129). Myoblasts may also separate the apposed surfaces of fibres 1 and 2, so that the original pseudopodial interlocking of these intrafusal myotubes may be lost by this stage of development (Fig. 62). Equatorial nuclear bags are invariably present in F1 and F2 (Fig. 62), as confirmed by Landon (1972 b), although Marchand & Eldred (1969) suggest that the intrafusal-fibre complement of neonatal rat spindles is two, the equatorial nucleation

being a nuclear bag and a nuclear chain.

All of the intrafusal fibres are myotubular in the polar region (Fig. 62). In addition, F3 possesses a central chain of nuclei at the equator (Fig. 61).

Muscle fibres are a common feature of extrafusal shank muscle at birth (Figs. 63, 64, 66). Myofibrils, that are separated by interfibrillar sarcoplasm, occupy the peripheral and central core regions of the fibre. Nuclei are typically peripheral in position (Fig. 63). Immature myotubes may still be encountered in pseudopodial apposition to their more mature neighbours (Figs. 64, 65), even in those nascent myotubes that apparently lack myofibril assemblies (Figs. 66, 67).

The equatorial sensory terminals of newborn rat spindles appear greater in number and size than those of foetal spindles although they are still confined to the outer surface of the axial bundle, with only occasional terminals extending between the intrafusal fibres (Fig. 61). In such cases, the invading terminal is shared between two fibres, a situation also recognized in mature spindles (see review by Barker, 1973). Overlapping sensory terminals may still be encountered (Fig. 61). The axons of the spindle nerve trunk lack myelin and individual Schwann cells, although the number of axons sharing a single Schwann cell is less than in foetal rats. The outer surface of the axial bundle and the sensory terminals remains invested by a common basement membrane.

The first evidence of the arrival of the fusimotor innervation was observed in spindles of newborn rats

(eg. Fig. 62), as reported by Landon (1972 a, b) in rat spindles 12 hr after birth. Both polar (Figs. 62, 93, 96) and juxta-equatorial (Fig. 95) motor terminals have been located.

13.6 1-2 DPN muscle spindles.

In rat muscle spindles one day after birth, F3 invariably exhibits an increased myofibril content compared to that at birth, although the myotubular morphology of the polar regions and the equatorial nuclear chain are retained (Fig. 68). The pseudopodial apposition between the intrafusal fibres is frequently lost in the equatorial region (Fig. 68), although Landon (1972 b) suggests that this does not occur until the latter part of the first postnatal week. However, pseudopodial interlockings are still a common feature of the polar regions of the axial bundle at this stage of development, except in those areas occupied by myoblasts.

By the second postnatal day, a fourth intrafusal fibre (F4) has started to differentiate within the axial bundle. F4 shows a distinct immaturity compared to the other fibres and a diameter approaching that of F3 (Fig. 69). It invariably extends pseudopodial inpushings into one or more of the other neighbouring fibres (Figs. 69, 70, 122, 126, 127). A common basement membrane still ensheaths the outer surfaces of the developing fibres (Figs. 69, 70), although some evidence has been found of individual basement membrane formation (Fig. 132).

Sensory terminals are still largely confined to the outer surfaces of the intrafusal fibres (Figs. 69, 70).

Collagen fibrils are associated with the capsule cells, as well as longitudinally orientated elastic fibrils (Fig. 108), as reported by Landon (1972 b) in spindles of 2.5 DPN rats.

13.7 4 DPN muscle spindles.

The muscle spindle of 4 DPN rats contains the full complement of 4 intrafusal fibres, although F4, the second nuclear-chain fibre may still exhibit a relative immaturity (Fig. 71) and lie in apposition to one or more of the other fibres. Myoblasts may still be encountered within the axial bundle (Fig. 72). The most distinctive development of the spindle at this stage is in the polar morphology of the intrafusal fibres. The differences in the diameter of these fibres, observed during their development, are maintained in 4 DPN spindles (eg. Fig. 71) even in the polar regions where, in the adult spindle, such differences are often lost. Myonuclei are frequently peripheral in this region, particularly in F1 (Figs. 71, 72).

All of the intrafusal fibres have a similar arrangement of abundant interfibrillar sarcoplasm ensheathing each myofibril, that contains small mitochondria. Longitudinal sections of intrafusal fibres at this stage of development, and of those present in neonatal rat spindles (Figs. 133, 134) show a distinct M line in the centre of the pseudo H zone of all fibres, as confirmed by Landon (1972 b).

Each intrafusal fibre may now possess an individual basement membrane. The axial bundle is often invaded by cells that form the inner layers of the capsule (Fig. 71). Pseudopodial interlockings are not often encountered at this developmental stage.

Motor terminals are now more abundant. Those located were always intracapsular in position, with smooth post-junctional membranes (Figs. 71, 97).

13.8 12-16 DPN muscle spindles.

On the twelfth postnatal day of development, the intrafusal fibres are separated in the equatorial region by cells forming the first vestiges of the endomysial enclosures and axial sheath (Fig. 73). A developing periaxial space separates the axial bundle from the multi-lamellate capsule which now lacks an extensive lining of fibroblast-like cells. In some regions, where the periaxial space is narrow (Fig. 73), such cells can be observed extending from the inner layer of the capsule to form one or more of the endomysial envelopes, a situation that also prevails in some adult spindles (see Barker, 1973).

The periaxial space is wider in 16 DPN spindles and may contain large vacuolated cells (Figs. 75, 112) of a similar morphology to those reported in the spindles of adult cat (Goglia, 1970).

Equatorial sensory terminals by the 16 DPN stage adopt a semi-lunar profile (Figs. 75, 86), typical of the adult primary annulo-spiral ending. Only at this morphogenetic stage could secondary sensory terminals be identified with any certainty, on the basis of their juxta-equatorial location on nuclear-chain fibres (Fig. 74). Motor terminals now approach adult frequency and dimensions (Fig. 99).

In 12-16 DPN spindles the diameter differences noted during development are maintained in the equatorial and juxta-equatorial regions (Figs. 73-75) but are lost in the

polar zones as in adult rats. Separation of the intrafusal fibres is not always complete. In some areas pairs of fibres may lie in close apposition within a common basement membrane (Fig. 75). Similar associations have been reported between nuclear-chain fibres of newborn and adult cats (Scalzi & Price, 1971). Uninucleate cells are often encountered beneath the basement membrane of the intrafusal fibres (Figs. 75, 119), adopting a similar position to the myoblasts of developing spindles and the satellite cells of adult forms (Landon, 1966 b).

Ultrastructural variations may now exist between the intrafusal fibres, although Landon (1972 b) suggests that no significant differences are present in 14 day-old rats. Nuclear-chain fibres now possess larger mitochondria in the abundant interfibrillar sarcoplasm of the I-band region. A well-defined M line is still present (Fig. 138). The large diameter nuclear-bag fibre (F1) lacks a distinct M line and possesses a few, small mitochondria (Fig. 135) as noted in the typical bag fibre of the adult spindle (Table 4; Figs. 20, 23). In one fibre a double M line (Fig. 136) occupied the centre of the sarcomeres. The smaller nuclear-bag fibre (F2) exhibits a more distinct single M line in the centre of the pseudo H zone (Fig. 137) and a mitochondrial size and distribution approaching that of F1.

Chapter 14. The fine structure of developing rat spindles.

14.1 Sensory terminals.

The overlapping sensory terminals of 19.5 DF rat spindles contain a few, small clear vesicles and several of the dense-cored type (see review by Smith, 1971; Figs. 76-78), in a finely flocculent cytoplasm as described by Landon (1972 b). The vesicles are usually distributed randomly in the cytoplasm of the terminal although occasional aggregations of clear vesicles may be observed close to the nerve-terminal membrane (Fig. 76). Mitochondria, when present, are small and tend to accumulate at the core of the terminal (Figs. 76, 78). Some terminals that lack mitochondria, possess more neurotubules and neurofilaments (Fig. 77) than the normal type. Such terminals are often not associated with intrafusal fibres but are enclosed by neighbouring terminals.

The grouped sensory terminals, from their earliest observation, have a similar ultrastructure to those of the adult spindle (12.4), except for the large number of dense-cored vesicles, and their topography in relation to the single intrafusal myotube present at this stage. Their orientation, although irregular to the axis of Fl, tends to be longitudinal rather than the typical circular alignment seen in the adult and more mature spindle, as reported by Uehara (1973). Basement membrane, in its early form, covers the outer surface of the sensory terminals. At several points, the apposed nerve terminal and muscle membranes show areas of specialization, consisting of paired areas of increased density (Figs. 76, 78; Landon, 1972 b), described

in adult rat spindles as "desmosome-like" (Mayr, 1970). Such specializations also occur between adjacent nerve terminals in foetal rats (Fig. 79).

Micropinocytosis is a common activity of the synaptic sarcolemma, almost invariably resulting in the formation of coated vesicles (eg. Fig. 80). The presence of coated vesicles in the axoplasm of foetal sensory terminals (eg. Fig 79) suggests that this activity is also a feature of the axolemma.

At the later foetal stages of development, the ultrastructure of the sensory terminal differs little from that of 19.5 DF rats. However, multiaxonal terminals frequently adopt a circular course, enwrapping the external surface of the intrafusal fibres (Fig. 79). By the early neonatal period (Figs. 81-84) coiled endings are the common feature of the equatorial region and are now single axonal. Such terminals may be associated with more than one developing intrafusal fibre (eg. Fig. 82). The cytoplasm of the sensory terminal contains a greater number of mitochondria, neurotubules, neurofilaments and clear vesicles but fewer dense-cored vesicles than in foetal spindles. Desmosome-like specializations of the synaptic membrane are still encountered (Fig. 84), as well as coated vesicles in the sub-terminal sarcoplasm (Fig. 83) and the cytoplasm of the terminal (Fig. 84).

Smaller terminals, of the "bundled" type, described by Uehara (1973) in developing rat spindles, have been observed in the juxta-equatorial region of newborn rat spindles. They differ from the equatorial sensory terminals by their lack

of mitochondria in a cytoplasm that is packed with neurotubules, neurofilaments and vesicles of both the lucent and dense-cored types. They therefore resemble the enclosed terminals (Fig. 77) described in foetal spindles. Figure 80 illustrates a "bundled" type of terminal that is single-axonal, although its identification as a sensory terminal is obscured by a basement membrane that appears to pass both over the terminal and its Schwann cell covering (typical of such terminals) and possibly between it and the intrafusal fibre.

By the second to third postnatal week, the equatorial sensory innervation is similar to that of the adult. Each intrafusal fibre is enwrapped by a coiled terminal that adopts a spiral course (Fig. 85). In transverse section, these terminals, as described by Landon (1972 b), consist of a core of cytoplasm filled with neurotubules and mitochondria, surrounded by a peripheral zone of cytoplasm containing many small vesicles, only a very small proportion of which possess dense cores (Figs. 86, 90, 91). Membrane specializations may be encountered at the sensory synapse (Fig. 87) and coated-vesicle formation is still an activity of the sarcolemma (Figs. 88, 89). It was only at the 12 and 16 DPN stages that possible evidence of exocytosis was found (see review by Smith, 1971). On two occasions, invaginating coated areas of the sarcolemma appeared to contain an electron-dense core of material (Figs. 91, 92), similar to that contained in dense-cored vesicles. The core appears to be continuous with the plasma membrane and cytoplasm of the sensory terminal over-

lying it, although this continuity is not always clear (Fig. 92). It could be that the protruding core of material merely represents an entrapped portion of axoplasm. The relative electron density of the core compared to the terminal cytoplasm suggests this is improbable. The delineation of the plasma membrane of the terminal in the supposed area of exocytosis is indistinct (Fig. 92); it does however, appear to be of a double nature (arrows, Fig. 92), as would result from a discharging vesicle.

14.2 Motor terminals.

The first evidence of developing fusimotor terminals was observed in muscle spindles of rats 12 hr after birth, in both a juxta-equatorial (eg. Fig. 95) and polar (eg. Fig. 93) location. The terminals adopt the form of single or multiple small end-bulbs (see also Landon, 1972 b), containing a varying number of clear vesicles, only a small proportion of which possess dense cores, that have been reported in immature extrafusal motor terminals both *in vivo* (Terävänen, 1968 b; Kelly & Zacks, 1969 b) and *in vitro* (Pappas *et al*, 1971).

Polar terminals of newborn rat spindles lack a primary synaptic cleft (Fig. 93), or any folding of the postjunctional sarcolemma that suggests secondary-cleft formation. Each terminal is covered, at least in part, by Schwann cell cytoplasm. Extrafusal motor terminals at this stage of development have a similar morphology, with the exception of their greater accumulations of synaptic vesicles in somewhat larger terminals that may be housed in depressions of the wall of the innervated muscle fibres,

representing rudimentary primary clefts (Teräväinen, 1968 b; Kelly & Zacks, 1969 b). In all intrafusal and extrafusal motor terminals of newborn rats a distinct basement membrane is interposed between the nerve terminal and the innervated fibre (Figs. 93-96).

In both of the polar terminals examined, the post-synaptic region of the intrafusal fibre has shown some degree of specialization, consisting of a thin aggregation of sarcoplasm containing many mitochondria and ribosomes, occasional profiles of granular ER and small accumulations of glycogen (Fig. 93). It is interesting to note that the innervated muscle fibre illustrated in figure 62 is bordered by a myoblast close to the innervation zone. The fusion of myoblasts with such innervated fibres has been suggested as a possible method, by which such accumulations of sarcoplasm, that will eventually form the sole-plate, may form (Kelly & Zacks, 1969 b).

Micropinocytosis appears to be an activity of the post-synaptic membrane. Coated vesicles are frequently encountered in the sarcoplasm of this area (Fig. 93). Extrafusal motor end-plates possess a more distinctive sole-plate at birth (Fig. 94) than the polar fusimotor terminals. The post-synaptic sarcolemma also shows areas of increased density (Fig. 94; Teräväinen, 1968 b; Lentz, 1969 a) that have not been observed at fusimotor junctions until the later postnatal stages (eg. Fig. 98).

Juxta-equatorial and mid-polar motor terminals in neonatal rat spindles are usually single structures that do not always possess a Schwann cell covering (eg. Fig. 95).

The clear cytoplasm contains numerous synaptic vesicles and occasional structures of the dense-cored type (Fig. 95). Mitochondria are usually absent (eg. Fig. 96). The post-synaptic membrane is always smooth and lacks any invaginations suggestive of primary or secondary cleft formation. Extensive subsarcolemmal aggregations of sarcoplasm are usually absent (eg. Fig. 95).

None of the fusimotor terminals observed in newborn rat spindles innervated immature myotubes but were limited to the more mature nuclear-bag fibres (F1 and F2).

During the first and second postnatal weeks, motor terminals are encountered progressively more frequently in the juxta-equatorial and mid-polar regions of the spindle. Such terminals have a similar content of synaptic and dense-cored vesicles as those described at birth, the post-synaptic region of the intrafusal fibres still lacking primary and secondary synaptic clefts and any evidence of sole-plate formation (Figs. 97, 98). As post-junctional folds and sole-plate are fully developed in extrafusal motor end-plates by the second postnatal week (Kelly & Zacks, 1969 b and personal observation), and some fusimotor terminals in the polar regions of 12 DPN rat spindles appear to be of a plate morphology (Fig. 99), it seems reasonable to assume that the small juxta-equatorial and mid-polar terminals (Figs. 97, 98) are of the adult trail type (Figs. 38-42). Such trail terminals have been observed on both nuclear-bag (F1 and F2) and nuclear-chain (F3 and F4) fibres of postnatal rat spindles.

A single motor terminal of possible plate morphology

has been observed in the polar regions of a nuclear-bag (F1) fibre of 12 DPN rat (Fig. 99). The clear terminal cytoplasm contains numerous synaptic vesicles that tend to accumulate near the junctional membrane, occasional dense-cored vesicles, neurotubules, neurofilaments and mitochondria. Basement membrane passes between the terminal and intrafusal fibre. Post-junctional folds, where present, are wide and shallow, protruding into a thinly spread sole-plate of mitochondria and ribosome-rich sarcoplasm. The terminal possesses a Schwann cell cover. The morphology of this terminal corresponds well to the nuclear-bag plates described by Ovalle (1972 b) in rat lumbrical muscle.

14.3 Capsule and periaxial space.

The capsule of muscle spindles at the 19.5 DF stage of development consists of an aggregation of flattened cells, some of which partially surround the nearby supplying nerve trunk (Figs. 55, 101). These cells are easily identified by extensive profiles of granular ER cisternae in a cytoplasm rich in free polyribosomes and glycogen. They invariably lack basement membrane and so can be distinguished from Schwann cells that surround the bundles of axons constituting the spindle nerve trunk (Fig. 101; Gamble & Breathnach, 1965; Gamble, 1966). The cells that form the first layer of the capsule have no regular orientation, although most of them present an elongate flattened profile suggesting a transverse orientation around the single intrafusal fibre (Figs. 55, 56). Their morphology is similar to that of the extrafusal fibroblast

(Fig. 100). It seems probable that the first layer of the spindle capsule consists of a massed group of fibroblasts, some of which surround the spindle nerve trunk. It has been suggested that developing perineural cells are fibroblastic in nature (Gamble & Breathnach, 1965; Gamble, 1966), lacking several of the distinguishing features of the adult form such as basement membrane and lateral overlaps. However Shantha & Bourne (1968) suggest that perineural epithelial cells are not derived from fibroblasts, but that they may show some of the features of a highly active developing cell that are subsequently lost during maturation. It may be therefore that those fibroblast-like cells that surround the spindle nerve trunk and intrafusal fibre (Fig. 101) are immature perineural epithelial cells. Landon (1972 b) also suggests that such ensheathing cells cannot be distinguished as perineural cells.

At the later stages of foetal development, two distinct types of cell enclose the axial bundle. Those immediately adjacent to the intrafusal fibres are arranged en masse. At their points of overlap (Fig. 104), the apposed plasma membranes may show areas of specialization, similar to the close junctions noted between myogenic cells in developing rat muscle (Kelly & Zacks, 1969 a) and described as the "adhaerens" type by Landon (1972 b) in similarly-aged spindles. These cells contain numerous granular ER cisternae and polyribosomes, that are features still held in common to extrafusal fibroblasts (see particularly Fig. 105). The cytoplasm is frequently electron dense (Fig. 102), which has been attributed, in developing Schwann cells, to

the accumulation of ribosomes (Ochoa, 1971). Basement membrane is always absent.

Bordering the outer surface of the fibroblast-like cells, a second cellular component is encountered that also surrounds the spindle nerve trunk (Figs. 57, 59), its distinctive feature being the thin flattened appearance of the cytoplasmic projections that extend from the cell body (Figs. 102, 104). Micropinocytotic vesicles at various stages of formation, similar to those of the capsular sheet cells in adult spindles (Figs. 13, 14), may be present within the cytoplasm of these cells (Fig. 102), as well as numerous ribosomes and a few granular ER cisternae. Only occasionally do such cells completely envelope the spindle nerve trunk and axial bundle. More often two or three cells form one layer of the capsule, overlapping at their tips. The areas of overlap show similar membrane specializations (Fig. 104) to those reported in the inner capsule cells. The perinuclear cytoplasm of the outer capsule cells, however, exhibits many of the morphological characteristics of the inner cells and extrafusal fibroblasts, including an abundance of granular E^R cisternae and the absence of basement membrane (Fig. 105).

By the time of birth, the inner capsule layer of compact fibroblast-like cells can still be distinguished from those forming the outer layers, on the basis of shape and cytoplasmic electron density (Figs. 61, 103). The inner cells never completely encircle the axial bundle, whereas those of the outer lamellae, on occasions, may. The elongate cytoplasmic extensions of the outer cells again possess a high proportion of micropinocytotic vesicles, many of which are of the coated type (Fig. 106). In the early

neonatal period, many of these vesicles and the ER cisternae, have distended lumens (Figs. 68, 107). Collagen and occasional elastic fibrils are now frequently encountered between the closely interdigitating cells of the outer lamellae (Figs. 107, 108; Landon, 1972 b) and in association with the more compact cells of the inner layer (Fig. 109). The first indication of collagen formation was recognized in spindles at birth (Fig. 103). Basement membrane is usually absent in both cell types, although, in some areas, broken profiles bordering the plasma membrane of the outer capsule cells may be observed (Fig. 108).

In the cell-body area of the outer capsule cells fewer profiles of granular ER cisternae are observed in the electron-lucent cytoplasm. In similar areas of the inner capsule cells, numerous ER cisternae are encountered in the dense cytoplasm (Fig. 69). Occasionally the inner capsule cells may possess a cilium that arises from one of the juxta-nuclear centromeres (Figs. 70, 109) and is enclosed in a deep invagination of the cell membrane. In transverse section, the cilium may be observed to lack central fibres (inset, Fig. 109), possessing the 9+0 formula noted in cilia of cultured rat fibroblasts (Wheatley, 1969).

It is not until the second postnatal week of development that the two cellular components of the adult capsule, capsular sheet cells and endomysial cells, become distinct in immature spindles. At the time of the appearance of the periaxial space and the separation of the intrafusal fibres, the axial bundle and its individual fibres are surrounded by the processes of several cells that have a morphology

similar to those of the inner capsule layers of immature spindles (compare Figs. 111 & 69). Both types of cell possess numerous granular ER cisternae and free polyribosomes in an electron-dense cytoplasm, bounded by plasma membrane that lacks an outer covering of basement membrane. The cytoplasmic processes of the endomysial cells are often closely apposed to one another to form the endomysial enclosures and may, on occasions, be associated with similar cells that form the depleted inner capsulelayer (Fig. 73). Collagen and elastic fibrils are associated with the axial sheath cells and the intrafusal fibres, as in the adult spindle.

The multi-lamellate capsule, by the 16 DPN stage, is composed of concentric layers of thin flattened cells, the cytoplasm of which contains a few small mitochondria, scattered ribosomes and numerous oval or rounded micropinocytotic vesicles (Fig. 110). Basement membrane borders the outer surface of such cells, although its continuity is not always distinct. Collagen fibrils occupy the spaces between the cell layers, adopting both a circular and longitudinal orientation (Fig. 110). Atypicial cilia, of a similar construction of those described in the inner capsule cells of immature spindles, occasionally arise from a juxta-nuclear centriole of a capsular sheet cell (Fig. 75).

The developing periaxial space may contain vacuolated cells, similar to those described by Goglia (1970) in adult cat spindles. The distinguishing feature of these cells is their highly vacuolated appearance (Fig. 112), which led Goglia (1970) to suggest a secretory function for these

cells. The vacuoles are frequently located close to the plasma membrane (Fig. 112), suggesting that they may arise by pinocytotic activity of the membrane, as occurs in capillary epithelial cells (Fawcett, 1966). It is interesting to note that in an illustration of a newborn cat spindle (Fig. 11, Scalzi & Price, 1971) one of the endomysial cells shows similar peripheral vacuoles. Large, dense, granular inclusions are also a feature of these cells in developing rat spindles (Fig. 112). There is no bordering basement membrane.

14.4 Developing intrafusal fibres.

14.41 The first-innervated myotube. In fine structure, F1 of the earliest detected spindles is indistinguishable from extrafusal myotubes (Fig. 56; Landon, 1972 b) but varies only in some cases in its equatorial nuclear arrangement and innervation. Both intrafusal and extrafusal myotubes have a typically rounded outline in transverse section and contain central nuclei or aggregations of nuclei that are separated from the enveloping sarcolemma by layers of peripheral sarcoplasm containing myofibrils at various stages of formation and assembly. At this stage of development, F1 contains many vesicles of distended granular ER that are continuous with narrow, tubular smooth-surfaced vesicles (Figs. 76, 113), that form the first vestiges of the SR, as previously reported in cultured chick myotubes (Ezerman & Ishikawa, 1967) and in developing rat extrafusal muscle (Edge, 1970). These beaded smooth-surfaced tubules may partially encircle individual myofibrils, particularly at the Z-band level (Fig. 114). A similar observation by

Walker & Edge (1970, 1971) in foetal rat myotubes led them to propose that SR tubules may be involved in Z band formation and thin filament aggregation. In transverse section, SR tubules have a flattened outline and diffuse dense contents similar to that of the granular ER cisternae (Fig. 114; Ezerman & Ishikawa, 1967). Such tubules are frequently apposed to the sarcolemma (eg. Fig. 58), particularly in those areas of invagination of the muscle membrane (inset, Fig. 114), that indicate T tubule formation (Ezerman & Ishikawa, 1967; Edge, 1970; Kelly, 1971). Similar subsarcolemmal couplings have been reported in adult rat intrafusal fibres (Ovalle, 1971).

It has been suggested that the beaded T tubules of immature muscle arise by an incomplete micropinocytotic activity of the sarcolemma (Edge, 1970). Its subsequent development includes the extension of the tubules in both a longitudinal and transverse direction within the myotube (also Ezerman & Ishikawa, 1967; Kelly, 1971). T tubules in the first-innervated intrafusal myotube of rat spindles, identified by their smooth, sharp limiting membranes and clear lumen (Ezerman & Ishikawa, 1967) appear to adopt a predominantly longitudinal course (Figs. 113, 114), as do the junctional complexes between the SR and TT systems (Fig. 114). Similar longitudinally-orientated triads have been described in extrafusal myotubes of foetal and newborn rats (Edge, 1970; Schiaffino & Settembrini, 1970; Kelly, 1971; Luff & Atwood, 1971). These junctional complexes have no direct association with the developing myofibrils but are always located in the peripheral subsarcolemmal

sarcoplasm (Fig. 114), which is probably linked, as in extrafusal myotubes, to the initial peripheral coupling of SR tubules to the sarcolemma (Kelly, 1971).

The most common type of junctional complex in all developing intrafusal myotubes, is the triad (eg. Fig. 114), that consists of a central T tubule apposed on two opposite sides by terminal SR tubules. As reported in extrafusal myotubes of rat (Edge, 1970), junctional complexes consisting of 3 SR tubules apposed to a single longitudinally orientated T tubule, have been observed (Fig. 114); such complexes have not been reported in adult rat intrafusal fibres. The various multiple junctional complexes described by Ovalle (1971) in rat lumbrical intrafusal fibres have not been observed in immature spindles.

The subsarcolemmal, interfibrillar and central-core sarcoplasm of F1, at the early stages of its development, contains numerous ribosomes, most of which are not attached to a membrane system but lie either singly, or in the form of polysomes (Fig. 113) in the sarcoplasm. Paler staining glycogen granules may be located in a similar position or, more frequently, be aggregated into large "lakes", where they intermingle with ribosomes, in a juxta-or inter-nuclear position (Fig. 56), as described in extrafusal myotubes (eg. Kelly & Zacks, 1969 a; Schiaffino & Hanzlíková, 1972 a). Many small mitochondria aggregate predominantly in the central-core sarcoplasm and are usually orientated parallel to the longitudinal axis of the myotube, presenting a rounded outline in transverse section (Fig. 56). A modest Golgi complex of flattened and distended vesicles is

usually located in a juxta-nuclear position (Fig, 114). The myonuclei are polymorphic and elongate, with dispersed nuclear chromatin and only slight peripheral clumping (Figs. 55, 56, 114). A prominent nucleolus may be present (Fig. 55) as in the nuclei of extrafusal myotubes.

A thin basement membrane (Figs. 113, 114) borders the outer surface of the sarcolemma, except at the sites occupied by sensory nerve terminals, myoblasts or nascent myotubes, that are always enclosed within the basement membrane of the innervated, parent myotube.

14.42 Intrafusal myoblasts. Myoblasts have been observed in association with the developing axial bundle at most of the morphogenetic stages studied. These uninucleate cells are always situated beneath the basement membrane that ensheaths the intrafusal fibres (Figs. 113, 115-118) or may be totally enclosed by the fibres or their processes (eg. Fig. 117). The dense chromatin-packed nucleus is large and occupies most of the cytoplasm (Figs. 115, 117). Nucleoli do not appear to be a prominent feature of myoblast nuclei, although they have been reported in extrafusal myoblasts (eg. Bergman, 1962; Przybylski & Blumberg, 1966; Fischman, 1970; Fig. 1). However, Landon (1970) suggests that nucleoli are not found in myoblast nuclei of rat gastrocnemus muscle.

The electron-dense cytoplasm of the myoblast is packed with numerous free ribosomes (Figs. 115-118). Mitochondria are usually small and few in number, although they may, in the anucleate regions of the cytoplasm, occupy a large portion of the cross-sectional area (Fig. 116).

Vesicles of granular ER are encountered infrequently in the cytoplasm of myoblasts (Fig. 116, 118) and may, on occasions, show distended lumens (eg. Fig. 118). No connections have been observed between strands of granular ER and the nuclear envelope, as reported by Landon (1972 b) in intrafusal myoblasts, nor have peripheral couplings between smooth-surfaced vesicular precursors of the SR and plasma membrane (Kelly, 1971) been seen.

Glycogen granules may be present in some myoblasts (Fig. 117; Bergman, 1962; Fischman, 1970). Their identification is frequently obscured by the large number of membrane-free ribosomes. Focal accumulations of glycogen, that are a common feature of immature myotubes (see 14.41; 14.43), are not found in myoblasts.

The juxta-nuclear area of the myoblast cytoplasm may house a typical Golgi complex. Centrioles are also a common feature of this area in both myoblasts (Fig. 118) and nascent myotubes (Figs. 123, 129), although they have not yet been encountered in adult muscle fibres (Muir, 1970). Paired centrioles have, however been reported in the satellite cells of bat web muscle (Muir et al, 1965; Church, 1969), one of which may give rise to an atypical cilium as observed in this study, in the capsule cells of developing rat spindles (Figs. 75, 109). Atypical cilia have not been encountered in the myoblasts or myotubes of immature rat spindles, although such structures have been reported in cultured chick skeletal and cardiac muscle (Przybylski, 1971). Transverse sections of the centrioles of intrafusal myoblasts and myotubes show them to have a

typical hollow, cylindrical profile, with the wall composed of 9, evenly-spaced, triplet hollow tubules (inset Fig. 180), embedded in an amorphous matrix.

The intrafusal myoblasts are orientated parallel to the longitudinal axis of the myotubes that they border (Fig. 129), lying in strings beneath the basement membrane. This orientation and grouping has also been noted in extrafusal myoblasts (Landon, 1970).

Uninucleate myogenic cells are still encountered in muscle spindles of 12 and 16 DPN rats, when the intrafusal-fibre complement is complete (Landon, 1972 b). Such cells (eg. Fig. 119) exhibit the same basic ultrastructure as the myoblasts of immature spindles and are located beneath the basement membrane of the parent intrafusal fibre. Their position and morphology also links them to the satellite cells of adult rat intrafusal fibres (Landon, 1966 b) which Landon (1972 b) assumes they form.

14.43 Intrafusal myotubes. In both the late foetal and early postnatal stages of development, multinucleate myotubes, at various stages of development are present in the axial bundle, adopting a similar sub-basement membrane position to the intrafusal myoblasts. As described previously (Chapter 13), the intrafusal fibres develop and mature in a definite chronological sequence in a similar manner as the secondary and tertiary generations of myotubes in extrafusal muscle (eg. Wirsen & Larsson, 1964; Kelly & Zacks, 1969 a; Landon, 1970). The nascent myotube of 20 DF rat spindles (F2 in Figs. 57, 58, 120), that occupies a similar position to the myoblasts of 19.5 DF

spindles, exhibits an increased myofibrillar maturity and an equatorial aggregation of nuclei in spindles at birth, but remains of a smaller diameter than the first-formed (F1) nuclear-bag fibre (Figs. 61, 62). A still smaller myotube, F3, appears between F1 and F2 at birth (Figs. 61, 62, 121) in a position often occupied by myoblasts in foetal spindles (eg. Fig. 60). By the second postnatal day, F3 is more mature (Figs. 69, 70) but retains its equatorial nuclear chain. A third nascent myotube, F4, is often present a this stage (Figs. 69, 70, 122) but, by the fourth postnatal day (Figs. 71, 72) is frequently as mature as the first formed nuclear-chain fibre.

Although the nascent myotubes, that may be observed in foetal and early postnatal rat spindles, do not have the same morphological fate, they all appear to develop in the same way and pass through the same series of ultrastructural stages before maturity is attained. From their very earliest stages of formation, the intrafusal myotubes lie in close association with those myotubes constituting the remainder of the axial bundle, within a thin basement membrane that ensheaths the outer surface only of all intrafusal myogenic cells (eg. Figs. 120-122). This association also includes an interlocking of the superficial sarcoplasm of the developing and apposed myotubes. Nascent myotubes possess long pseudopodial projections of their sarcoplasm that penetrate the apposed surface of the adjacent, more mature structures (Figs. 58, 120-127), as described by Landon in both extrafusal (1970, 1971) and intrafusal (1972 a, b) muscle fibres. Such pseudopodial extensions may be

relatively short and unbranched (eg. Figs. 120, 126), long and unbranched (eg. Figs. 124, 125) or elaborate branched structures that only penetrate the superficial sarcoplasm of the parent myotube (eg. Fig. 127). On several occasions, as reported by Landon (1972 b), an invaginating pseudopodium may extend as deeply as the central-core sarcoplasm of the apposed myotube, so that a close association may be observed between the invaginated sarcolemma and the nuclear envelope of the myonucleus (eg. Figs. 123, 124). The sarcoplasmic extensions usually follow a transverse pathway (eg. Figs. 124, 125). Longitudinal sections of postnatal rat spindles have shown that such pseudopodia may also adopt a longitudinal orientation. Transverse sections of developing fibres therefore frequently contain rounded profiles of sarcoplasmic pseudopodia, surrounded by the invaginated sarcolemma, that are totally enclosed within the sarcoplasm of the apposed or apposing fibre (Figs. 121, 123, 125). The possibility cannot be excluded that some pseudopodia may be engulfed into the sarcoplasm of the invaginated fibre by some form of phagocytic activity of the sarcolemma.

It is of interest to note that the pseudopodia invade only the interfibrillar and central-core sarcoplasm of the parent myotube (eg. Figs. 124, 125, 126) and never the developing myofibrils. This may lend some support to the suggestion of Kelly & Zacks (1969 a) that the invaginated areas of the more mature myotubes in foetal rat intercostalis represent expanded T tubules. However, the pseudopodial extensions are not always in register with the I-band region of the myofibril, which is the main evidence

for their hypothesis.

The membrane separation of a nascent myotube and its neighbour may occasionally be indistinct (eg. Fig. 123). This is probably due to variations in the plane of section (Lipton & Konigsberg, 1972) rather than a possible indication of fusion, which Kelly & Zacks (1969 a) suggest is the fate of such apposed muscle cells. Lipton & Konigsberg (1972) suggest that the fusion of myoblasts and myotubes, in cultures of Japanese quail muscle, is indicated by inter-communicating pores contained within a pair of cul-de-sacs formed by the fused membranes of both cells. Such configurations have not been observed between the intrafusal myotubes of rat muscle spindles, nor any evidence of an increase in the number of fibres by longitudinal splitting (Cuajunco, 1940; Marchand & Eldred, 1969) or by the incorporation of extrafusal myotubes (Cuajunco, 1927, 1940). It appears therefore that the suggestion made by Landon (1970, 1971) that pseudopodial apposition is but a transient feature in the development of all extrafusal myotubes also prevails intrafusally (also Landon, 1972 a, b).

Pseudopodial extensions are a feature not only of newly-formed myotubes, whose ultrastructure is similar to that of the myoblast (eg. compare Figs. 122, 117) but also of myotubes with a high myofibril content (Figs. 60, 125; Landon, 1972 b), a situation that does not exist in extrafusal muscle (Figs. 50, 51; Landon, 1971). The time at which this close interlocking is lost varies considerably. The extensive apposition between F2 and F1 is usually lost by birth (Figs. 61, 62) when the nuclear-bag fibres are

separated by a nascent myotube and numerous myoblasts. Fibres 3 and 4 may remain closely apposed to one or more of the other fibres until the end of the first postnatal week, when the axial bundle is separated into its individual components by the invading inner capsule cells, nerve fibres and growing sensory terminals (Figs. 71-74). This separation coincides with the peripheral migration of the intrafusal myonuclei in the polar regions of the spindle (Fig. 72) and the acquisition of individual basement membrane. The appearance of basement membrane on the inner surface of the developing intrafusal fibres may begin as early as the second postnatal day in F1 and F2 (Fig. 132), when only the outer surfaces of the nuclear-chain fibres (F3 & F4) are coated (Figs. 130, 131). The newly-formed inner basement membrane appears to stem from the original outer coat (Fig. 132).

At any single morphological stage, up to the fourth postnatal day, only a single nascent myotube is present in the axial bundle (eg. Figs. 57, 62, 69). Maturation of the myotubes, which involves the assembly of myofibrils in the peripheral sarcoplasm of the equatorial and juxta-equatorial regions and throughout the whole core in the polar region, appears to follow the same sequence as their initial formation, as also reported by Landon (1972 b). At birth, therefore, F2 has a peripheral packing of myofibrils, when myofilament assembly is in its early stages in F3 (Figs. 128, 129). By the second postnatal day, this assembly may be approaching completion in F3, when F4 is only at the nascent myotube stage (eg. Figs. 126, 127). The construction of myofibrils, from the many free cytofilaments that are present

in nascent myotubes (Figs. 123, 124, 126) appears to proceed at the expense of the extensive accumulations of ribosomes that contribute to the electron density of nascent myotubes (Fig. 122, Konigsberg, 1965). Focal accumulations of glycogen, that are a conspicuous feature of both intrafusal (eg. Figs. 55-58) and extrafusal (Figs. 45, 48, 50, 51) foetal myotubes are also depleted in the early postnatal stages of development, as described by Schiaffino & Hanzlíková (1972 a) in rat skeletal muscle. Sparse glycogen deposits are confined to the interfibrillar and central-core sarcoplasm of more mature intrafusal fibres (eg. Figs. 128, 132).

Even at the 4 DPN stage, when myotube maturation is often complete in the polar regions of the spindle (Fig. 72), no significant differences could be found between the ultrastructure of the intrafusal fibres, as confirmed by Landon (1972 b). Many small mitochondria are distributed throughout a relatively abundant interfibrillar sarcoplasm in both nuclear-bag and nuclear-chain fibres (Fig. 72). The myofibrils of all intrafusal fibres possess a well-defined M line at birth (Figs. 129, 133, 134) and at the 4 and 8 DPN stages. A distinct M line was frequently encountered in newly-formed myofibrils before any other banding pattern, apart from the Z line could be discerned (Figs. 129, 134), an observation in agreement with that of Allen & Grisnik (1971) in cultured chick myotubes.

It was not until the end of the second postnatal week of development that ultrastructural variations between the intrafusal fibres were observed, although Landon (1972 b)

failed to find any differences in 14 DPN rat spindles. The large-diameter nuclear-bag fibre (F1) was then found on occasions to possess little interfibrillar sarcoplasm, a poorly-developed sarcotubular system and very few small mitochondria in the I-band region (Fig. 135). The centre of the pseudo-H zone is normally devoid of a distinct M line although in a single spindle from PL of a 16 DPN rat a double M line was visible (Fig. 136), consisting of two parallel thin densities similar to that described by Ovalle (1971, 1972 a). The smaller-diameter nuclear-bag fibre (F2) possesses more abundant interfibrillar sarcoplasm that contains a variable number of small mitochondria and a more elaborate network of SR tubules. A single M line bisects the pseudo-H zone (Fig. 137). The nuclear-chain fibres (F3 & F4) now possess many elongate mitochondria that may extend the full length of the sarcomere, in an abundant interfibrillar sarcoplasm that is packed with ribosomes and some glycogen granules. A distinct M line is retained (Fig. 138). A more elaborate network of SR tubules encircles the myofibrils at the I- and Z-band level. Junctional coupling of the SR and TT systems, of the diad (Fig. 119) or triad types may still however be longitudinally orientated, although such structures are a feature of adult rat intrafusal fibres (Ovalle, 1971; Fig. 22).

Chapter 15. The histochemistry of developing rat muscle fibres.

15.1 Extrafusal fibres.

In agreement with the findings of previous investigations into the histochemistry of developing rat muscle fibres (Zamieniecka & Ostenda, 1969; Kelly & Schotland, 1972), no definite evidence of histochemical variations between the myotubes of foetal rat EDL muscle has been found in this study (Figs. 139-141). Several of the larger primary myotubes at the 20.5 DF stage may exhibit a pale although still positive ATPase reaction in the peripheral sarcoplasm (Fig. 139). The same myotubes are positive in their staining reaction for both P'ase and SDH (Figs. 140, 141). All other primary and successive generations of myotubes exhibit a high activity for all three enzymes, although variations in the intensity of staining for P'ase may be observed between the myogenic cells (Fig. 140). No evidence however has been found of the successive development of histochemical fibre types in a manner described by Wirsen & Larsson (1964) in the thoracic musculature of mouse.

Even at birth, when several of the larger myotubes have reached the muscle-fibre stage of development, histochemical differentiation of EDL is not at an advanced stage, a finding in agreement with that of previous investigations (Dubowitz, 1965; Engel & Karpati, 1968; Zamieniecka & Ostenda, 1969; Kelly & Schotland, 1972; Brooke et al, 1971; Shafiq et al, 1972). Some of the large myotubes or muscle fibres may still however, exhibit

a reduced ATPase activity compared to their neighbours (Fig. 142) although a weak, negative reaction, as found in the B fibre of the adult (Fig. 35), is never found. The position, size and reaction of the pale fibres of neonatal rat muscle, suggests they are comparable to the Type I fibres described by Kelly & Schotland (1972) in 2-4 DPN rat intercostalis, and by Brooke et al (1971) in 2 DPN rat soleus, gastrocnemius and tibialis anterior muscle. As both of these investigations involved the ATPase reaction only, the oxidative enzyme reaction of the pale fibres is not reported. In this study, such fibres like their more positive neighbouring myotubes, were seen to possess a high SDH activity (Fig. 144). The P'ase content of the pale ATPase fibres appears reduced (Fig. 143) when compared to that of the larger fibres that exhibit a very positive reaction for ATPase. It may be, therefore, that part of the B fibre population that in the adult exhibits a high oxidative but low glycolytic enzyme reaction (Figs. 35-37), is beginning to differentiate at birth although Dubowitz (1965) suggests that newborn rat skeletal muscle is histochemically undifferentiated. The progressive development of muscle fibres with weak ATPase reactivity is known, however, to be a common feature in the histochemistry of muscle development (see Davies, 1972 b).

Fenichel (1963) showed that the large 'b' fibres of Wohlfart (1937) are low in myosin ATPase. It seems probable that this fibre type is the ATPase-low fibre seen in neonatal rat muscle (Fig. 142). The large myotubes of 20.5 DF rat muscle, that have a pale ATPase reactivity, may

represent the early stages in the differentiation of the B fibre population, as it is known that the histochemical differentiation of muscle fibres may proceed more rapidly for one enzyme, compared to others (Dubowitz, 1965).

The majority of myotubes that constitute foetal and neonatal rat muscle exhibit a strong, uniform activity for all enzymes (Figs. 142-144) and therefore approach the adult type C fibre.

By the beginning of the second postnatal week however, the first evidence was found of the development of the anaerobic type A fibre (see Table 1). These fibres, that are of the largest diameter in the muscle fasciculi, exhibit a moderate ATPase (Fig. 145), high P'ase (Fig. 146) and a low SDH activity that is characterized by a fine scattering of small diformazan granules over the whole sectional area of the fibre (Fig. 147). The majority of the smaller-diameter fibres exhibit a positive reaction for all 3 enzymes and therefore constitute the type C fibre population. The B fibre population is represented by occasional intermediate-diameter fibres that now exhibit a low ATPase activity (Fig. 145), a P'ase activity that is generally weaker than that of the type A fibres and a high SDH activity that is comparable to that of the C fibres. Variations of such profiles are commonly found, suggesting that the differentiation is by no means complete at this postnatal stage of development. Such variations are not a common feature of EDL muscle of 12-17 DPN rats, where the checker-board of histochemical fibre types, typical of adult muscle, may be observed (see also Dubowitz, 1965;

Zamieniecka & Ostenda, 1969).

15.2 Intrafusal fibres.

Both of the previous investigations into the histochemistry of developing intrafusal fibres of mouse and rat depended almost exclusively on the P'ase reaction. Wirsen & Larsson (1964) suggest that during the foetal development of the mouse spindle, successive generations of intrafusal fibres decrease both in diameter and P'ase activity. Ostenda & Strugalska (1971) also propose, in rat, that the large-diameter nuclear-bag fibres progressively acquire a high P'ase and low SDH activity whereas the nuclear-chain fibres have an exactly opposite profile, by the 20 DPN stage. However, neither of these studies included an investigation of the histochemical profiles of the intrafusal fibres of the adult.

In this investigation, the intrafusal muscle fibres of 5 DPN rat spindles exhibit similar variations in P'ase activity as those described by Wirsen & Larsson (1964) in early foetal mouse spindles. The largest-diameter nuclear-bag fibre exhibits the highest activity followed in intensity by the smaller diameter nuclear-bag and nuclear-chain fibres, ~~that exhibit an activity that varies from~~ intermediate to low (Fig. 149). However, both ATPase (Fig. 148) and SDH (Fig. 150) activity is equal and positive in all of the fibres, irrespective of size or type.

It was not until the 17 DPN stage that evidence of histochemical variations was found. The P'ase reaction was found unreliable for both extrafusal and intrafusal fibres at this stage and is therefore omitted. The ATPase reaction

however consistently presented 3 types of intrafusal fibre, based on their enzyme activity and diameter, as reported in adult rat spindles (Table 4; Figs. 28, 30). The large-diameter nuclear-bag fibre exhibits a high ATPase (Figs. 151-153) and low SDH (Figs. 155, 156) activity although occasionally the SDH activity may appear high (Fig. 154). It seems therefore that oxidative-enzyme differentiation is not necessarily complete by this postnatal stage.

The small diameter nuclear-bag fibre exhibits a very low ATPase activity (Figs. 151-153) of a weakness comparable to that of the extrafusal B fibres, and the intermediate bag fibre of the adult spindle (Figs. 28, 30, 32). Its SDH activity is always high (Figs. 154-156). The ATPase activity of the small nuclear-chain fibres varies from intermediate (Fig. 152) to high (Fig. 151), but is always positive. The chain fibres are always positive for SDH (Figs. 154-156).

Chapter 16. The fine structure of newborn mouse and guinea-pig muscle spindles.

16.1 Newborn mouse.

The axial bundle of neonatal mouse spindles consists of a group of tightly-packed myotubes and myoblasts at various stages of differentiation, enclosed by a common basement membrane (Figs. 157, 161), that does not penetrate the inner surfaces of the developing fibres. Each of the 5 spindles examined contained 4 intrafusal myotubes, of which 3 were at an advanced stage of differentiation and 1 was consistently at the nascent myotube stage, a finding in agreement with that of Wirsen & Larsson (1964) who suggest that the intrafusal-fibre complement of newborn mouse spindles varies between 3 and 5. However the general appearance of the axial bundle as a tightly-packed group of fibres is of a comparable structure to the illustrated 20 DF mouse spindle in the same study, suggesting some degree of delay in the developmental sequence in spindles of FHL compared to those of the trunk musculature.

Of the 3 more mature myotubes present at birth, one is of a larger diameter in both the equatorial (Fig. 157) and polar (Fig. 161) regions of the spindle, and always possesses a nuclear bag. The polar myonuclei are frequently peripheral in position in these fibres (Fig. 161), whereas the other intrafusal fibres retain their myotubular structure in this region.

In two spindles, one of the remaining myotubes has been observed to possess a small equatorial aggregation of myonuclei. In all other myotubes in all spindles, a nuclear

chain occupies the equatorial region. The centre of the axial bundle houses an immature myotube which, like those identified in immature rat spindles, possesses many pseudopodial extensions of the superficial sarcoplasm that invaginate the neighbouring myotubes, the invaginated sarcolemma frequently showing a close association with the nuclear envelope of the apposed myotube (Fig. 158). The peripheral sarcoplasm of such nascent myotubes contains aggregations of free filaments and occasional incompletely-assembled myofibrils (Fig. 160). Numerous free ribosomes and glycogen granules are distributed throughout the peripheral and central-core sarcoplasm of both nascent (Figs. 158, 160) and more mature myotubes (Fig. 157). The first rudiments of the sarcotubular system may be observed in those myotubes with few myofibrils. Tubules of SR appear to bud from vesicles of granular ER (Fig. 160; Ezerman & Ishikawa, 1967), while the spherical T tubules are often encountered in a subsarcolemmal position (Fig. 160).

Intrafusal myoblasts adopt a similar position to the nascent myotubes, in the centre of the axial bundle. They possess large central nuclei (Fig. 161) and a cytoplasm packed with ribosomes and occasional glycogen granules (Fig. 162). Mitochondria are small and intermingle with occasional dilated vesicles of granular ER. The juxta-nuclear cytoplasm may be occupied by a typical Golgi complex of tubules and vesicles and paired centrioles (Fig. 162) that have also been identified in rat intrafusal myoblasts (Fig. 118) and myotubes (Fig. 123).

The axial bundle is enclosed by a capsule that is

multi-layered in the equatorial region (Fig. 157) but only bi-lamellate in the polar zones (Fig. 161). There is no periaxial space. As reported in neonatal rat spindles (Figs. 61, 103) the capsule cells that border the axial bundle may differ from those of the outer layers that also enclose the spindle nerve trunk (Fig. 157). The inner capsule cells possess more compact nuclei and more profuse profiles of granular ER than the outer cells. Paired centrioles have been observed in the abundant cytoplasm, one of which may form an atypical cilium (Fig. 157) of a similar structure to those observed in similar cells of newborn rat spindles (Fig. 109). The lateral cytoplasmic extensions of the outer capsule cells are more extensive and thinner than those of the inner cells. Micropinocytotic vesicles may be encountered in the cytoplasm. There is no evidence of basement membrane formation in these cells that would confirm their identification as capsular sheet cells (Merrillees, 1960) or perineural epithelial cells (Shantha, Golarz & Bourne, 1968). Collagen fibrils are associated with all cells of the capsule.

Large primary sensory terminals occupy shallow depressions of the outer wall of the intrafusal muscle fibres in the equatorial zone (Fig. 157). Occasional terminals of the overlapping type (14.1; Landon, 1972 b) have been observed (Fig. 157). The majority however are single-axonal and adopt a spiral course around the axial bundle. The terminal cytoplasm contains mitochondria, occasional neurotubules and neurofilaments, and several smooth-surfaced vesicles, a small proportion of which

possess dense cores. Basement membrane never intervenes between the terminal and intrafusal fibre.

In the polar and juxta-equatorial regions motor terminals have been identified (Fig. 159) innervating the large-diameter nuclear-bag fibre. Such terminals have a similar morphology to those observed in the polar regions of neonatal rat spindles (Fig. 93), adopting the form of multiple, small end-bulbs that contain a varying number of clear vesicles, some of which have a flattened appearance (Fig. 159; Ovalle, 1972 b). Occasional dense-cored vesicles may be present. Basement membrane occupies the post-junctional space, but a distinct sole-plate is not present. The post-junctional membrane may adopt a "crenate" appearance that could represent the early stages of cleft formation. A Schwann cell covering wraps the outer surface of the motor terminals. The axons within the spindle nerve trunks that presumably supply the sensory and motor terminals are unmyelinated at this stage of development (Figs. 157, 161), without individual Schwann cells.

16.2 Newborn guinea pig.

In contrast to the immature structure of neonatal rat and mouse spindles, the muscle spindle of the newborn guinea pig shows several of the characterstics that in rat spindles have indicated a fully-differentiated structure.

A multi-lamellate capsule, consisting of flattened, elongate sheets of cells that overlap in a concentric, tubular fashion, is separated, in the equatorial region of the spindle, from the axial bundle by a wide periaxial space (Fig. 163). Cells that lack an outer coat of basement

membrane and possess extensive cisternae of granular ER in the elongate cytoplasm, surround individual or pairs of intrafusal fibres forming the endomysial enclosures (Fig. 163). Both collagen and elastic fibrils are associated with the intrafusal fibres, the endomysial cells and the capsular sheet cells. Convolutions of branched and folded basement membrane (Fig. 163; Adal, 1969), similar to that described in adult rat spindles (Fig. 16) may be located close to the intrafusal fibres, myelinated nerve fibres or the endomysial cells. Elastic fibrils commonly associate with such aberrant membrane formations.

In neither of the spindles examined did the intrafusal fibres show any degree of myofibrillar immaturity like that of the nascent myotube nor any form of pseudopodial apposition. The closest association observed consisted of the ensheathment of two nuclear-chain fibres by a common basement membrane and endomysial cell (Figs. 163, 164). However some basement membrane interposed the adjacent plasma membranes. The two nuclear-chain fibres also shared a single sensory terminal (Fig. 164). Such close apposition and common sensory innervation are also features of newborn and adult cat spindles (Scalzi &Price, 1971) and postnatal rat spindles (eg. Fig. 75).

The intrafusal fibres may be separated into nuclear-bags or nuclear chains on the basis of their equatorial morphology. Both the large and small-diameter nuclear-bag fibres (Fig. 163) possess few mitochondria and myofibrils with a double M line in the centre of the pseudo H zone (Fig. 165). The 3-4 nuclear-chain fibres may possess more

mitochondria and a single solid M line. However, one of the intrafusal fibres, in one spindle (Fig. 163), although its equatorial nucleation and diameter distinguished it as a nuclear-chain fibre, possessed a double M line in common with the nuclear-bag fibres. Such fibres are known to be a feature of spindles in the lumbrical muscle of guinea pig (Banks & James, 1973).

The large, equatorial, primary, sensory terminals adopt a spiral course and are housed in shallow depressions of the intrafusal fibres, within the basement membrane. The terminal cytoplasm is packed with mitochondria, clear vesicles and occasional neurotubules and neurofilaments. Few dense-cored vesicles have been located. The junctional membranes of the terminals and intrafusal fibres exhibit numerous areas of specialization that are "desmosome-like" (Mayr, 1970).

Although few fusimotor terminals have been identified, their structure may also be used as an indication of the maturity of guinea-pig spindles at birth. Both terminals were of the plate type and innervated nuclear-bag fibres. The elongate terminals are packed with mitochondria and synaptic vesicles, some of which appear flattened in outline (Fig. 166). Many dense-cored vesicles are scattered throughout the cytoplasm of the terminal, which is covered by a layer of Schwann cell. The post-junctional sarcolemma is thrown into a series of folds that extend into the sarcoplasm and are lined with basement membrane that interposes the terminal and intrafusal fibre. The subsarcolemmal sarcoplasm is packed with mitochondria, ribosomes and glycogen

granules, although no sole-plate nuclei have as yet been identified.

The differentiation of the capsule, periaxial space and endomysial sheath and the separation and maturity of the intrafusal fibres suggest that in newborn guinea pig, the development of the spindle is approaching completion, whereas newborn rat and mouse spindles are only in the early stages of their morphogenesis.

Chapter 17. The operated developing rat spindles.

17.1 De-efferented spindles.

As described previously (see 5.3, 8.2) the neonatal peripheral de-efferentation of muscle spindles in 12 litters of rats was attempted by sectioning the FHL nerve. The operated muscles were screened at sacrifice for operative success by estimating the damage to the affected nerve with a dissecting microscope. The number of successful operations was subsequently reduced to 15 (Table 3). Transverse sections of FHL of unoperated 12 DPN rats revealed that at the level of entry of the FHL nerve (Fig. 167), the interosseous nerve may be observed in an extramuscular superficial position. In the more distal regions of the muscle, the interosseous nerve penetrates the muscle for a short distance before adopting its course between the perimysium of the muscle and the tibial periosteum and interosseous membrane, terminating in Pacinian corpuscles (Fig. 2).

By the examination of transverse sections of operated muscles at the level of entry of the FHL nerve, a secondary, more precise method of evaluating the success of the nerve section was devised. A successfully de-efferented muscle exhibits extensive damage to the FHL nerve. Myelinated axons, that are a normal feature of the control, unoperated nerve (Fig. 167) are absent in operated nerves (Fig. 168). However the interosseous nerve has a similar content of myelinated axons in both the control and successfully operated animals. Healthy Pacinian corpuscles (Fig. 3) are also a diagnostic feature of an unaffected interosseous nerve.

Using this method of inspection at the cellular level the number of successful operations was further reduced to 2 (see Table 5, Lt. 10, N6; Lt. 12, N3). Of the remaining operated animals, no evidence was obtained, in several cases, of the condition of the interosseous nerve because of its loss during the removal of the muscle (Table 5, eg. Lt. 1, N4; Lt. 1, N5; Lt. 6, N3; Lt. 12, N6). These muscles were still serially examined with the light microscope. In other cases, the neonatal sectioning of the FHL nerve was either incomplete, or regeneration of the axons had occurred to such an extent that it was discernible with the light microscope (Lt. 6, N2; Lt. 6, N3; Lt. 9, N2; Lt. 9, N3; Lt. 9, N5; Lt. 10, N5; Lt. 10, N7; Lt. 11, N4; Lt. 12, N5). In one case (Lt. 5, N2) the operative procedure on FHL nerve had been totally unsuccessful, indicated by an apparently normal nerve trunk and the presence of several normal muscle spindles and extrafusal motor end-plates.

The examination of the 2 successfully operated muscles (Lt. 10, N6; Lt. 12, N3), as described elsewhere (11.22) yielded no evidence of muscle-spindle formations (eg. Fig. 168), whereas transverse sections at a similar level of the normal FHL muscle may present profiles of as many as 12 spindles (Fig. 167).

Flexor hallucis longus of 12 DPN rats contains only occasional myotubes but consists of muscle fibres with only small differences in diameter, aggregated into well-defined fascicles, particularly in the peripheral areas of the muscle (Fig. 167). The denervated muscles contain more myotubes that are randomly distributed among muscle fibres of a more variable diameter (Fig. 168) than in control muscles.

The persistence of myotubes in neonatally-denervated rat muscle has been reported previously (eg. Zelená, 1959, 1962; Engel & Karpati, 1968). No definite conclusions could be made in this study, however, on the effects of denervation on the maturation of myotubes into muscle fibres, as FHL of newborn rats was found to be predominantly at the muscle-fibre stage of development and some re-innervation had occurred in each of the operated animals.

In some areas of the denervated muscles adjacent to the pathway adopted by the FHL nerve, several fascicles of muscle fibres appeared to be of a normal structure differing from surrounding fascicles by the increased size of many of the constituent fibres (Fig. 168). Such islands of hypertrophied fibres appear to indicate areas of re-innervation (Yellin, 1967 b) in denervated muscle. In each of the successfully operated muscles occasional intramuscular nerve trunks were also observed to contain small, unmyelinated axons. A distinct motor end-plate was also identified in the operated muscle Lt. 12, N3, on the sixteenth postoperative day. Re-innervation therefore appears to occur within this postoperative period, as noted by Zelená & Hník (eg. 1960 a, b) on the 10th day after neonatal nerve crush.

The normal interosseous nerve (Fig. 168) supplied several Pacinian corpuscles in both operated muscles. These sense organs, at this stage of development (12-16 DPN), consist of a prominent central sensory terminal containing many mitochondria, neurotubules, neurofilaments and occasional profiles of smooth-surfaced reticulum (Fig. 172),

that is surrounded by concentric layers of flattened cells that vary in their morphology. Those layers of cells immediately surrounding the central terminal (Fig. 169) are closely applied to each other by cytoplasmic interdigitations and "desmosome-like" thickenings of the plasma membrane (Fig. 172). The lamellae are arranged bi-laterally with two intervening clefts (arrows, Fig. 169) that establish an open connection with the innermost interlamellar space of the outer cells. The cytoplasm contains ribosomes and numerous micropinocytotic vesicles, a small portion of which are of the coated type. Only occasionally has basement membrane been observed associated with these cells. The more peripheral lamellae are composed of compact cells that are separated by numerous fibrils, a large proportion of which are collagen. The fibrils extensively intermesh (Fig. 170) to form a thick coating on the outer surfaces of the cell layers. The compacted cytoplasm of these cells is distinguished by its many profiles of granular ER cisternae, the lumens of which may be dilated (Figs. 169, 170), and numerous mitochondria, ribosomes and glycogen granules. Basement membrane is absent.

The cells composing the outer lamellae and capsule are flattened elongate structures that possess numerous micropinocytotic vesicles and ribosomes and only occasional strands of granular ER and peripheral cytofilaments (Fig. 171). The lamellae cells are separated by randomly orientated collagen fibrils and a subcapsular space that varies in thickness according to the level of sectioning (Fig. 169). Basement membrane is not a distinct feature of these cells.

The normality of these Pacinian corpuscles (Pease & Quilliam, 1957; Rhodin, 1963) in the denervated muscles suggests that the interosseous nerve has remained undamaged at the time of operation, as it seems reasonable to assume that such sense organs would depend, to some extent, on their innervation during development (see review by Zelená, 1964).

The absence of differentiated muscle spindles in the operated muscles suggests that, in rat, the interosseous nerve does not supply certain spindles, as in cat (see 5.3), with a sensory nerve component only, as such de-efferented spindles would not have degenerated by the twelfth postoperative day (Zelená, 1965).

17.2 Atypical spindles.

An encapsulated structure with muscle-spindle characteristics was located in one operated animal (Table 5, Lt. 1, N5), in which the FHL nerve showed some signs of regeneration. By electron-microscopic examination, it was found to contain 2 small muscle fibres that were myotubular at their mid-length, enclosed by a capsule of flattened cells. The remnants of a nerve trunk (Fig. 173), consisting of a few unmyelinated axons (Fig. 179) contained by a single Schwann cell adopts an inter-lamellar position. In the myotube region, both intrafusal fibres are, at some point, enwrapped by a spiral sensory terminal (Figs. 174, 176) that contains many vesicles, some of which possess dense cores (Fig. 176). Both the large- and small-diameter intrafusal fibre (Fig. 173) lacks a nuclear bag. In the more polar regions, both fibres possess an individual basement

membrane, peripheral myonuclei and a distinct M line in the centre of the pseudo H zone. Interfibrillar sarcoplasm, SR tubules and transversely-orientated triads (Figs. 177, 178) appear abundant at all levels of the myofibril. No evidence was found, however of myofibrillar degeneration, motor terminal formation or of intrafusal myoblasts.

In the innervated region, the capsule consists of 2 cellular components (Fig. 173), of a similar structure to those described in developing rat spindles (see 14.3). A narrow periaxial space separates the outer and inner capsule cells. A single vacuolated cell was located in the periaxial space. It did not differ in structure from those described in immature rat spindles, (Fig. 112; 14.3) and adult cat spindles (Goglia, 1970).

The morphology of this encapsulated structure is identical to that of the atypical spindle observed in re-innervated rat muscle 5 months after neonatal nerve crush (eg. Zelená & Hník, 1960 a; Hník & Zelená, 1961), which, they suggest, originates from re-innervated spindle remnants that survive denervation. The presence of regenerating nerve axons in the spindle capsule and of sensory nerve terminals in the atypical spindle located in this study, confirms this suggestion.

17.3 Denervated spindles.

From the results of a previous investigation (Zelená, 1957, 1959, 1962, 1964), it was proposed that those spindles denervated by the sectioning of the FHL nerve at birth, would degenerate by the twelfth postnatal day (Fig. 3). This degeneration was monitored in the operated animals of Lt. 13.

17.31 First postoperative day.

By the first postdenervation day, axons are absent from both the intramuscular and spindle (Figs. 180, 181) nerve trunks, although Schwann cells are well preserved with an intact basement membrane. A common feature of both perineural epithelial and spindle capsule cells is the presence of many, large, multivesicular bodies (Fig. 182) that protrude from the peripheral cytoplasm into the intercellular space. Extrafusal muscle fibres differ little from those of the contralateral unoperated muscle, although T tubules may occasionally appear dilated.

Nerve terminals were absent from all 3 spindles examined, even in the equatorial region (Fig. 181) where sensory terminals are invariably encountered in normal spindles at the same morphogenetic stage (Fig. 68). Two well-formed intrafusal myotubes are contained in the axial bundle of the denervated spindles, although a distinct nuclear bag was located in the large-diameter myotube of only one spindle (Fig. 181); all other intrafusal fibres appeared myotubular throughout their length. In one spindle, a third nascent myotube contributed to the axial bundle, as in normal spindles at this morphogenetic stage.

The axial bundle of all denervated spindles contains few myoblasts and is enwrapped by basement membrane. No signs of myofibrillar atrophy were found in such denervated intrafusal fibres (eg. Fig. 180). The sarcotubular system appears no more developed than in the intrafusal fibres of control spindles. Paired centrioles (inset, Fig. 180), of a similar structure to those described in normal developing

intrafusal myoblasts and myotubes (14.42; Figs. 118, 123) have been observed, on one occasion, in the smaller-diameter myotube. Lipid droplets are a common feature of denervated extrafusal and intrafusal (Figs. 180, 181) fibres.

17.32 Third postoperative day. Some axonal regeneration had occurred in the FHL nerve by the third post-denervation day (Fig. 187), although no re-innervated spindles were located. The small, regenerating axons lie adjacent to or within the cytoplasmic extensions of Schwann cells, within the preserved basement membrane (Fig. 187). The axoplasm contains many neurotubules and neurofilaments and occasional mitochondria and dense-cored vesicles. No motor terminals were observed in the denervated muscles, although a few large-diameter muscle fibres with obvious sole-plate nuclei, sarcoplasm and shallow post-junctional folds are present (Fig. 185). Such fibres exhibit some degree of denervation atrophy in the end-plate region that includes a disorganization of the myofibril orientation and the presence of numerous dense bodies that are presumably degenerating Z bands. However, the majority of extrafusal fibres exhibit a normal myofibril architecture (Fig. 186).

None of the 3 denervated spindles examined contained any nerve terminals. Only 2 intrafusal fibres are present in each spindle (eg. Fig 183) and only on one occasion did the large-diameter fibre possess a nuclear bag. Myoblasts may occasionally be located in the axial bundle. The outer basement membrane of the intrafusal fibres often appears collapsed (Fig. 184), particularly at the junction of the neighbouring fibres. Similar configurations of basement

membrane have been described in degenerating extrafusal fibres of the fruit-bat (Church, 1970 a) and rat and rabbit (Vracko & Benditt, 1972).

The intrafusal myotubes, although they differ markedly in number, equatorial morphology and innervation from those of normal (Figs. 69-72) and control spindles, show no signs of denervation atrophy.

17.33 Fifth postoperative day.

It was at the fifth postoperative day that myofibrillar atrophy was observed in the intrafusal fibres of denervated spindles. Three of the 4 spindles examined contained only 2 intrafusal fibres, neither of which possessed nuclear bags or nerve terminals. In the polar region, central nuclei and sarcoplasm are absent. The fourth spindle contained an additional small-diameter fibre, enclosed within a basement membrane that ensheaths the outer surface of the axial bundle (Fig. 188). None of the intrafusal fibres possessed nuclear bags, although Zelená (eg. 1957, 1962) suggests that discrete equatorial zones are present in muscle spindles of 5 DPN rats, denervated at birth.

In some areas, the intrafusal fibres may exhibit extensive atrophy, consisting of disarranged myofibrils that apparently lack a distinct banding pattern (Fig. 189). T tubules are abundant in the subsarcolemmal regions of the fibre (Figs. 189, 190). Myelin figures (Fig. 189) are common in all fibres. Such atrophy is not found in all regions of the intrafusal fibres; in some areas the myofibril architecture may be normal (Fig. 188). No fragmentation of the fibres was found. Control spindles

at this stage of development contain two well-developed nuclear-bag and nuclear-chain fibres.

17.34 Seventh to twelfth postoperative day. All the animals sacrificed at the seventh and ninth postdenervation days showed signs of extensive axonal regeneration in both the intramuscular (Fig. 194) and spindle (Figs. 191, 193) nerve trunks. Regenerating axons vary in diameter (Fig. 194) and are enclosed as groups by the cytoplasmic extensions of Schwann cells that are bordered by a distinct basement membrane (Fig. 194). Myelinated axons were never encountered.

The re-innervated spindle examined contained 3 intrafusal fibres enclosed by a multi-lamellate capsule. Occasional nerve terminals of a similar structure to those described in atypical spindles (see 17.2) were located in the myotube region of the fibres. None of the intrafusal fibres appear to possess a nuclear bag. In their more polar regions (Fig. 191) each fibre is packed with normal myofibrils that are separated by abundant interfibrillar sarcoplasm and SR tubules, particularly at the I- and Z-band levels (Fig. 192), differing little from extrafusal fibres (Fig. 191). Both intrafusal and extrafusal fibres possess dilated T tubules that adopt a predominantly longitudinal course. The axial bundle is separated by invading Schwann cells and occasional endomysial cells (Fig. 191). Each fibre possesses an individual basement membrane. No motor terminals were encountered in the spindle.

In one of the muscles examined for de-efferented spindles (Lt. 6, N2), the re-innervated fragments of a

denervated spindle were observed. The lamellate capsule of flattened cells encloses numerous unmyelinated axons and their associated Schwann cells (Figs. 199, 201), fibroblast-like cells (Fig. 195) and occasional anucleate fragments of atrophic muscle fibres (Fig. 195), that possess numerous disorganized, disintegrating myofibrils (Figs. 196, 197). Only occasionally can a normal hexagonel lattice of actin and myosin filaments be observed (Fig. 196). Numerous dense-bodied lysosomes, that are a characteristic of neonatally-denervated extrafusal muscle fibres (Schiaffino & Hanzlíková, 1972 b), are scattered throughout the sarcoplasm.

In some areas of the degenerating spindle, the capsule encloses only regenerating axons (Figs. 199, 201) and occasional red blood corpuscles (Fig. 198). No phagocytic cells (Zelená 1957) or myclinated axons, that are a feature of the regenerating FHL nerve (Figs. 200, 202), have been encountered.

Table 5.

A summary chart detailing the results obtained from the animals of litters 1-12, in which the FHL nerve was sectioned at birth.

LITTER	ANIMAL	CONDITION OF FHL NERVE	CONDITION OF INTEROSSEOUS NERVE	RESULTS
Lt. 1	N4	complete section	no evidence	no spindles
Lt. 1	N5	regenerated	no evidence	Atypical spindle
Lt. 5	N2	normal	no evidence	normal spindles and motor end-plates
Lt. 6	N2	some regeneration	no evidence	Degenerated spindle
Lt. 6	N3	some regeneration	no evidence	no spindles
Lt. 9	N2	incomplete section	no evidence	no spindles
Lt. 9	N3	incomplete section or regeneration	no evidence	motor end-plates
Lt. 9	N5	incomplete section	normal	normal spindles and motor end-plates
Lt. 10	N5	some regeneration	no evidence	no spindles
Lt. 10	N6	complete section	normal	normal Pacinian corpuscles no spindles
Lt. 10	N7	some regeneration	normal	no spindles
Lt. 11	N4	some regeneration	no evidence	no spindles
Lt. 12	N3	complete section	normal	normal Pacinian corpuscles no spindles
Lt. 12	N5	some regeneration	normal	no spindles
Lt. 12	N6	complete section	no evidence	no spindles

PART IV - DISCUSSION.

Chapter 18. The mode of increase in the number of intrafusal fibres.

Figure 203 illustrates the morphological characteristics of the method of increment of intrafusal fibres in foetal and neonatal rat spindles. It has been shown (see Chapter 13) that this increase is a rapid, sequential process that is completed within 5.5 days. The morphogenetic fate of the successive generations of intrafusal fibres is also a precisely determined sequence, in that the development of the two nuclear-bag fibres outspaces and precedes in time that of the nuclear-chain fibres. The nuclear-bag fibres are essentially a foetal acquisition, whereas the nuclear chains develop in the first postnatal days. However, the developmental and adult structure of the nuclear-bag fibres also differs. The first-innervated myotube (F1) develops an aggregation of myonuclei that is larger than its next-formed companion, F2. The second nuclear-bag fibre is also consistently of smaller diameter than the primary bag fibre. This heterogeneity was also recognized by Landon (1972 b) in foetal rat spindles as "one large myotube in close association with another smaller and more recently formed "secondary" myotube" (p. 192). Nuclear-chain fibres are the smallest-diameter intrafusal fibres from their time of inception.

There are three distinct generations of intrafusal fibres, differing in their diameter and equatorial nucleation that develop separately and sequentially during muscle-spindle morphogenesis. A similar sequence was observed by Cuajunco in spindles of pig (1927) and man (1940), where the large-

diameter fibres were recognized as the most advanced and included in the initial formation of the spindle. In pig he suggests that the medium-sized fibres form from myotubes grouped with the first fibres, while the small fibres form last of all. On the other hand, Marchand & Eldred (1969) suggest that neonatal rat spindles contain a nuclear-bag and nuclear-chain fibre, that subsequently split longitudinally to produce the adult number.

Although this developmental sequence of the different morphological types of intrafusal fibre may be easily observed in electron micrographs, (see also Landon, 1972 a, b) their mode of increase as a continuing active process requires some degree of interpretation and hypothesis. It has been shown (see 13.2) that at its earliest morphogenetic stage, the muscle spindle contains a single intrafusal myotube. Its formation presumably follows the same course as all extrafusal myotubes at the same and earlier stages of development, by the fusion of myoblasts or nascent myotubes (Kelly & Zacks, 1969 a). None of the myotubes at the "pre-spindle" foetal stage of development exhibit any ultra-structural characteristics to suggest their specialization, prior to innervation, as potential intrafusal fibres, an observation confirmed by Landon (1972 a, b) and supported by an identical proposal made by the light microscopists (Tello, 1917, 1922; Cuajunco, 1940; Zelená, 1957; Bowden, 1963). The fact that regenerating sensory nerve fibres may induce the formation of spindles de novo from normal and presumably unspecialized extrafusal myotubes and myoblasts (Zelená & Hník, 1963 a; Zelená, 1964) also supports the suggestion

that the myotubes of developing spindles are not formed from myoblasts that are "seeded" and specialized in the muscle primordia.

It has been shown (see 14.43), that the development of successive generations of intrafusal myotubes is characterized by a series of morphological events also recognised by Landon (1972 a, b). Newly-formed myotubes develop at a site previously occupied by intrafusal myoblasts (eg. Figs. 128, 129), that are known in extrafusal muscle to proliferate by mitotic divisions and subsequently to fuse to form myotubes (see 3.12). The pseudopodial extensions of nascent intrafusal myotubes have been recognized in extrafusal muscle as a transient feature of their development (Landon, 1970, 1971), that are lost with maturation, although Kelly & Zacks (1969 a) suggest they are a prerequisite for fusion. However, no positive indications of fusion between nascent myotubes and their more mature myotubes were found in this study, of a type described in the elaborate study of Lipton & Konigsberg (1972). These investigations used a tilting-stage analysis to avoid differences in the plane of section being interpreted as fusion.

The function of such pseudopodia is not clear. It is known that an interface is a suitable site for pseudopodial growth (Weiss, 1941). However, the absence of such structures in myoblasts that adopt a similar sub-basement membrane position to nascent myotubes suggests that they are not simply a reflex formation of cells in this position. Nascent myotubes differ from those cells identified as myoblasts in this study (Fig. 1) by their multinucleation and myofibril

formations, both characteristics being held in common with their more mature neighbouring myotubes, that differ only in diameter and stage of maturation.

Moscona (1962) suggests that the ability to adhere is peculiar to cell membranes during the early stages of differentiation, and is lost as the cells progress towards their specialized mature form. It may be therefore that pseudopodia are an extreme example of membrane adherence between homotypical cells that are lost, certainly in extrafusal myotubes (Landon, 1971), with increasing maturity. Such pseudopodia may simply follow the course of invaginating T tubules, as proposed by Kelly & Zacks (1969 a) and supported in part in this study (see 14.43). Their variable orientation may reflect the tortuous course adopted by T tubules during their formation (see eg. Edge, 1970; Schiaffino & Settembrini, 1970; Kelly, 1971; Luff & Atwood, 1971; 14.41).

The separation of closely-apposed myotubes in extrafusal muscle is assisted by the insinuation of undifferentiated cells and fibroblast-like cells (Ishikawa, 1966; Kelly & Zacks, 1969 a). In the developing spindle, the only cells separating the intrafusal myotubes at the time of their increment are myoblasts, their later separation being carried out by nerve fibres and their terminals and the endomysial cells of the axial sheath. This difference in separating agents, and subsequently the time of separation, may account for the retention of pseudopodia in intrafusal myotubes that exhibit a high density of myofibrils (14.43; Landon, 1972 b), while in extrafusal muscle pseudopodia are

a feature of immature myotubes only (Landon, 1971).

Although the ability to adhere may explain the presence of pseudopodial extensions, it throws little light on their function. It may be that such an intimate association between a newly-formed and a more mature myotube is a means of rapidly gaining information on the tension and subsequent orientation of the whole muscle from myotubes that have already obtained and used this information during their development. In this way, as suggested by Kelly & Zacks (1969 a) in extrafusal muscle, successive generations of intrafusal fibres composing the mature spindle, will be aligned parallel to each other. The close association of pseudopodia with the nuclear envelope of its mature neighbour (Fig. 142; Landon, 1972 b), may be a gross morphological representation of this information exchange. The formation and uptake of coated vesicles at the interface (eg. Fig. 58) may also be a means of exchanging such information.

It seems therefore that intrafusal fibres are formed by the continual proliferation of myoblasts (see Fig. 203) that subsequently fuse to form myotubes which mature in close association with one another, in a manner suggested by Couteaux (1941), and confirmed by Landon (1972 a, b). Although only occasional mitotic figures have been observed in myoblast nuclei in this study, Landon (1972 b) reports myoblast mitoses at all stages of muscle-spindle formation. Marchand & Eldred (1969) report a complete absence of mitotic figures in the myogenic cells of early postnatal rat spindles, although their illustrations of a 1 DPN (Fig. 2, B) and 2 DPN (Fig, 2, F) spindle show distinct mitotic figures

in the nuclei of cells enclosed within the axial bundle, which, they suggest, are axial-sheath cells. In the light of this and Landon's (1972 b) study, it is obvious that such cells are myoblasts, as axial-sheath cells do not invade and surround the axial bundle until the latter part of the first postnatal week.

In the same study, Marchand & Eldred monitored the uptake of tritiated thymidine, administered to rats from 0 to 12 days old, sacrificed 8 hr later. They found labelled nuclei in the cells of the capsule but none in the fibres of the axial bundle, although cells that they identify as axial-sheath cells do possess labelled nuclei. Again, in the light of this study, such cells in neonatal rat spindles are most certainly myoblasts or nascent myotubes, so that their illustration of an injected 2 DPN rat spindle (Fig. 3, E) more probably provides evidence of a labelled and therefore mitotically-dividing myoblast nucleus that lies singly, or has been incorporated into a nascent myotube.

Landon (1972 b) suggests that the radioautographic experiment of Marchand & Eldred is inconclusive as the incorporation time (8 hr) is inadequate to permit the inclusion of a labelled nucleus within a forming myotube. Labelled nuclei may however appear in cultured chick myotubes within 8-10 hr after thymidine administration (Bischoff, 1970). An incorporation time of 8 hr is certainly sufficient to label similarly dividing satellite cells in skeletal muscle (eg. Moss & Leblond, 1970, 1971). More probably, the invalidity of these experiments lies in the resolution of the light microscope. Labelled myoblast nuclei were present

in the axial bundle of neonatal rat spindles, but their identification as such was not made by Marchand & Eldred because of their similarity to axial-sheath cells. The morphogenetic stages selected by these investigators were also probably unsuitable for the maximum detection of dividing myoblasts. It has been shown in this study (eg. Fig. 203) that myotube formation is generally completed by the second postnatal day; the subsequent development of the axial bundle involves the maturation and separation of the intrafusal fibres. It may be that most of the intrafusal myoblasts, that subsequently fuse to form the fourth intrafusal fibre, present at birth (the earliest developmental stage tested in Marchand & Eldred's study), are of the postmitotic type (Bischoff, 1970). Only the occasional proliferative divisions of stem-satellite cells (Church, 1970 b) would produce labelled nuclei at later developmental stages. Under these conditions, few labelled nuclei would be observed in animals treated at birth. A more productive exercise would be to carry out radioautographic experiments in gravid females at the 19.5 day of pregnancy, when the mitotic divisions of stem-myoblasts in foetal spindles would presumably result in the labelling of myoblast nuclei and, over a longer incorporation time, of nascent intrafusal myotubes.

Similar criticism may be levelled at the pursuant experiments of Bravo-Rey et al (1969) who studied the effects of a single dose of ionizing irradiation, that normally arrests nuclear mitotic activity, administered to the hindlimbs of 0-5 DPN rats and to the whole body of

females at various stages of pregnancy, the pups of which were examined 4 weeks later. The effect of colchicine, which arrests mitosis at the metaphase stage, was also examined in 8 DPN rats, treated at the 3 DPN and various foetal stages of development. Neither x-radiation nor colchicine produced any significant changes in the development of the spindle, supporting the proposal that the mitotic activity of myoblast nuclei is not a stage in the increment of intrafusal fibres.

By their own suggestion however, the single radiation dose administered to foetal rats was so low that insufficient damage of the mitotic process may have resulted. In the ensuing weeks recovery may have occurred, mitotic activity resumed and the fusion of myoblasts to form intrafusal myotubes completed to form a normal spindle. The absence of any effect on the dimensions and components of the spindle capsule, which is known to include cells that divide mitotically at the postnatal stages of development (Marchand & Eldred, 1969), is also attributed to such a compensatory recovery of mitotic activity in both experimental sets of animals. They suggest that a similar recovery could not occur in dividing myoblasts of spindles, as the occurrence of the first replicatory phase of the intrafusal fibres is confined to the first and second postnatal days. Irradiation damage at this time might therefore be expected to severely upset such a developmental schedule. However, in the light of this study, as previously suggested, mitotic stem-myoblasts do not necessarily need to be present at birth or the later postnatal stages; the full

complement of intrafusal myoblasts may instead be present by the time of birth. Severe damage would not be expected to result from x-radiation or colchicine administration to spindles at the 2-3 DPN stage, when myoblast proliferation and fusion is complete (Fig. 203).

The presence of paired centrioles in intrafusal myoblasts of foetal and postnatal spindles (eg. Fig. 118) may offer some evidence of the mitotic division of such cells. Przybylski (1971) suggests that centrioles are a feature of mitotic and fusing myoblasts in embryomic chick skeletal muscle, but do not commonly occur in myotubes or muscle fibres. However, differentiated myocardial cells retain centrioles. He correlates the absence of centrioles in skeletal myotubes and muscle fibres with the loss of "their mitotic and DNA synthetic machinery" (p. 219) from their nuclei, while their presence in myocardial cells may be related to their continuing ability to divide. If this is so, then the centrioles of intrafusal myoblasts and capsule cells may indicate mitotic activity. Their presence in nascent myotubes (eg. Fig. 123) probably represents a pre-degeneration phase, as such structures have not been encountered in older myotubes or intrafusal fibres.

The previously proposed methods of intrafusal-fibre increase during spindle development (see 1.22) are readily understandable with the knowledge of the prolonged tight apposition and pseudopodial interlocking of intrafusal myotubes. The space between adjacent apposed myotubes is well beyond the resolution of the light microscope and indeed not always distinct in low-power electron micrographs

(eg. Fig. 60). The "budding fibres" described by Marchand & Eldred (1969, Fig. 2, K), as representing the configurations adopted by single fibres just prior to splitting, are probably closely-apposed pairs of fibres as described in this and Landon's (1972 b) study. Their proposed time of longitudinal splitting, within the first postnatal week of development, corresponds to the period of time when pseudopodial interlockings are lost and the fibres separate.

A similar misinterpretation may have been made by Cuajunco (1940), who suggested that the intrafusal fibres of human foetal spindles increase in number by longitudinal splitting. Cuajunco (1927, 1940) also suggested that the source of the small (presumably nuclear-chain) fibres may lie in extrafusal fibres that are incorporated into the spindle after capsule formation. While it is possible that individual "free" myoblasts may enter the axial bundle from the surrounding tissue in the early stages of spindle morphogenesis, it seems unlikely that multinucleate myotubes can be incorporated into a closely-bound group of myotubes, surrounded by basement membrane and axon terminals.

This investigation into the fine structure of developing intrafusal fibres has revealed that the origin of their increment must lie in prolifering myoblasts, that fuse to form myotubes in a manner first suggested by Couteaux (1941) and Wirsen & Larsson (1964). The polar regions of the intrafusal myotubes subsequently adopt the profile of muscle fibres. The implications of the morphology of this mode of increase are twofold relating to the development and structure of extrafusal muscle fibres and to the fine structure of adult

intrafusal fibres.

The histogenesis of extrafusal muscle involves the formation of a few primary generations of myotubes and the subsequent progressive development of secondary orders around their walls (eg. Wirsen & Larsson, 1964; Kelly & Zacks, 1969 a). This ordered pattern of myogenesis has been confirmed in the development of intrafusal fibres. A diagnostic feature of the formation and maturation of intrafusal myotubes is the development of pseudopodial extensions of the sarcoplasm. It appears that similar forms of pseudopodia, that have been recognized in extrafusal muscle cells (Kelly & Zacks, 1969 a; Aloisi, 1970) represent but a transient feature in the formation of myotubes, thus confirming a similar proposal made by Landon (1970, 1971).

In only a single investigation (Ishikawa, 1966, 1970) has it been suggested that satellite cells may be identified as a separate and distinct entity in developing muscle. Their position and structure, however, link such cells to the myoblasts identified in this study. It has been suggested that some of the satellite cells in developing muscle may contain myofibrils (Muir et al, 1965); may differentiate into definite myotubes (Ishikawa, 1970), and that their numbers decrease at the later stages of myogenesis (Muir et al, 1965; Church, 1969). The confusion that has obviously arisen in the identification of satellite cells in immature muscle must offer the first evidence of a morphological and functional link between the myoblasts of developing muscle and the satellite cells of the adult. The results of investigations into regenerating muscle have

implicated satellite cells in a myoblastic role, in that they provide the nuclei, by mitotic division, for muscle-fibre reformation (eg. Church et al, 1966; Shafiq et al, 1967; Church, 1970 a; Shafiq, 1970). The suggestion is, therefore, that satellite cells are dormant myoblasts that are retained in the adult as a potential source of nuclei, a proposal made by Mauro (1961) at their first identification. Church (1970 b) labels the satellite cell as the stem pre-myoblast of adult muscle, that is capable of mitotic division to provide more satellite cells and sub-populations of myoblasts that have the ability to fuse.

Muscle spindles of 8-16 DPN rats, that have the full adult complement of intrafusal fibres frequently contain uninucleate cells in association with an intrafusal fibre, beneath its basement membrane. Such cells have a similar morphology and position to the myoblasts of developing spindles (see 14.42) and to the satellite cells of the adult (Landon, 1966 b). Their presence in the axial bundle when muscle-fibre formation is complete, identifies them as satellite cells, which may provide a source of myonuclei, during postnatal growth as in extrafusal muscle (Shafiq et al, 1968; Moss & Leblond, 1970, 1971). Their origin must lie in the myoblasts of immature spindles because of the similarity in structure and position. It is difficult to envisage another cell form invading the basement membrane of the muscle fibre to adopt such a typical position. The possibility that some myonuclei and peripheral sarcoplasm may be "pinched off" from the intrafusal fibre, in a manner described by Hess & Rosner (1970) and Reznik (1970) in

regenerating muscle, to form satellite cells cannot be excluded, although no such configurations have been identified.

Bearing in mind that intrafusal fibres are undoubtedly influenced by specialized forces during their development (see Chapter 2), the axial bundle may still offer a suitable model for the morphological examination of myogenesis, as the evidence offered here suggests that this process is similar in extrafusal and intrafusal fibres. Such a closed system of 4 muscle fibres is ideal for studying the temporal sequence of formation of myotubes and muscle fibres and the subsequent fate of satellite cells.

A common feature of the encapsulated regions of nuclear-chain fibres in adult spindles is their enclosure by a common basement membrane (Corvaja, Marinozzi & Pompeiano, 1967; Adal, 1969; Scalzi & Price, 1971). In the light of this investigation, it seems probable that such areas represent the incomplete separation of the intrafusal fibres during postnatal development. It has been shown that the formation of individual basement membrane coincides with the peripheral migration of myonuclei in the polar regions of the intrafusal fibres (see 14.42). The absence of basement membrane at the apposed surfaces of nuclear-chain fibres in the myotube region only (Scalzi & Price, 1971) may offer further support to the linking of these two morphological events during development.

Chapter 19. The morphogenetic influence of the sensory innervation.

19.1 Proliferation of nuclei in developing nuclear-bag fibres.

The mechanism by which the adult complement of nuclei is attained in developing nuclear-bag fibres has been investigated recently in neonatal rats (Marchand & Eldred, 1969; Bravo-Rey et al, 1969). Earlier studies of the foetal development of muscle spindles had indicated that the myonuclei of potential nuclear-bag fibres divide repeatedly (Cuajunco, 1927, 1940; Zelená, 1957), possibly by amitosis (Tello, 1917, 1922; Mavrinskaya, 1967). The more recent investigations present evidence that the total complement of nuclei is present in the two "parent" fibres of rat spindles at birth. At the time of supposed longitudinal splitting the nuclei are said to be merely re-distributed to the "daughter" fibres without further duplication. No evidence is presented, however, of the prenatal mode of nuclear replication.

Marchand & Eldred found the number of nuclei in the single nuclear-bag fibre of newborn rat spindles to be greater than that of the adult bag fibre by a factor of approximately two, suggesting that at the time of the supposed longitudinal splitting of this fibre, the nuclei are simply shared between the daughter bag fibres. With the knowledge of the fine structure of neonatal rat spindles, it is obvious that the nuclei counted by these investigators included those belonging to the interposed immature nuclear-chain fibre (eg. their figure 2A illustrates such a

configuration). The smaller nuclear-bag fibre identified in this study of spindles at birth (eg. F2, Fig. 61), possesses a much smaller nuclear bag that is only rarely aligned parallel to that of the larger bag fibre. This staggering of the nuclear bags (eg. Figs. 69, 70) may have led Marchand & Eldred to define the smaller fibre of neonatal rat spindles as a nuclear chain, and thus to its exclusion from nuclear counts. The reduction in nuclear number observed by these investigators in the bag fibres of 6 DPN rats compared to that of newborn rats, corresponds to the period of time reported in this and Landon's (1972 b) study, when the separation of intrafusal fibres occurs.

The fact that a concomitant halving of equatorial cross-sectional area does not occur at the supposed longitudinal splitting of the nuclear-bag fibre in postnatal rat spindles, offers further evidence that Marchand & Eldred's observations were of the separation of a small-diameter nuclear-chain fibre from the larger nuclear-bag fibre. Their suggestion that a subsequent development of the peripheral cytoplasm of the "daughter" bag fibres increases the cross-sectional areas to adult values is not supported in this study. A critical analysis of the measurements, given in their Table 2 of the equatorial cross-sectional areas of adult intrafusal fibres, indicates that nuclear-bag fibres belong to one of two groups. The mean cross-sectional area for b1 is $110\mu^2$, while that of b2 is $72\mu^2$. This would mean that following postnatal splitting, one of the daughter fibres must increase in area, on average from approx. $46\mu^2$ (from their Table 4) to $110\mu^2$, without any increase in nuclear number. Observations of

adult spindles (see 12.32) show that the nuclear bag is surrounded by only a thin shell of myofilaments and sarcoplasm, whereas according to the suggestion of Marchand and Eldred, a similar peripheral area should be as large as that occupied by the myonuclei.

Their failure to find mitotic figures in the nuclei of intrafusal fibres, or labelled nuclei following the administration of tritiated thymidine at birth, is not surprising with the present knowledge that at the time of administration and observation both nuclear-bag fibres are fully developed. Likewise, the ionizing irradiation and colchicine experiments of Bravo-Rey et al (1969) were also carried out at an inappropriate time.

The possibility of myonuclear mitotic or amitotic division remains open. In this study, no evidence was found of mitotic figures in the myonuclei of developing intrafusal myotubes. Investigations into the development of cultured and regenerating (Holtzer, 1959) extrafusal myotubes have shown that neither mitotic nor amitotic nuclear division occurs in muscle cells containing myofibrils. The evidence supporting amitotsis comes mostly from the configurations adopted by some nuclei. Cooper & Konigsberg (1961) have shown, by time-lapse photography, that the dumb-bell shape is reversible and therefore does not indicate amitotic nuclear division. It seems thus unlikely that nuclear proliferation in developing bag fibres is by division, although it may be that the developing sensory terminals exert an influence on the underlying nuclei that enables them to retain their myoblastic ability to divide.

Equatorial nuclei may increase in number by the fusion of myoblasts with the developing myotube in this region. If this is so, we might expect to find evidence of large numbers of myoblasts in a partial state of fusion with the intrafusal myotubes, in the innervated region of muscle spindles at the time of nuclear proliferation. No such evidence has been found.

19.2 Hypothetical model for the differentiation of nuclear-bag and nuclear-chain fibres.

It could be that the nuclear bag is a morphological representation of an attraction between the intrafusal myonuclei and sensory nerve terminals. It is possible that the fluid core of sarcoplasm, that is the central feature of the entire length of developing intrafusal fibres and of the myotube and equatorial regions of adult fibres, may permit nuclear movement. It may be that developing sensory terminals exert some form of attractive force on the nuclei of developing myotubes that causes their movement towards the innervated area and their aggregation there. The aggregated nuclei may be replenished by the appositional recruitment of myoblasts. However, such a proposition requires some amendment, because of the absence of a nuclear bag in nuclear-chain fibres.

Figures 204 and 205 are schematic representations of one possible method by which a heteromorphic population of intrafusal fibres might develop. The model is built around six established facts:-

1. The intrafusal fibres develop in a set morphological sequence (see Chapter 18).

2. The intrafusal fibres develop mainly under the influence of sensory nerve terminals (Zelená, 1957, 1964), as de-efferentation of developing spindles (Zelená, 1965) does not affect this morphogenetic sequence.
3. Primary sensory terminals are the exclusive type present during the formation of nuclear-bag fibres (Zelená, 1964) and presumably during the morphogenesis of nuclear-chain fibres, as secondary sensory terminals could not be identified in early postnatal rat spindles (Zelená, 1957; 14.1).
4. Intrafusal fibres are totally dependent on the morphogenetic influence of sensory nerve terminals, during their formation (eg. Zelená, 1957).
5. The morphogenetic effect of sensory nerve fibres can be exerted only when muscle cells are capable of differentiation (see 2.3), which, during the normal development of the spindle, is called the "morphogenetic period".
6. Following de-afferentation of adult cat spindles, the equatorial nuclei of the intrafusal fibres are replaced by myofibrils, while the polar regions are relatively unaffected (Tower, 1932; Boyd, 1962).

   It is proposed that:-

   a. the morphogenetic influence of primary sensory terminals is of a varying strength that decreases with the advancement of the morphogenetic period (Figs. 204, 205).
   b. the strength of the influence also decreases per unit distance from the first point of contact of the primary axon with the intrafusal muscle

fibre (Fig. 204). This may be related to the decrease in the area of synaptic contact towards the extremities of the primary ending.

c. the influence is confined to the immediately-innervated area.

d. the morphogenetic influence can only be exerted during the formation of the intrafusal muscle fibres. Once myogenesis is complete, the influence adopts a maintaining role.

An overall maximum strength of influence is therefore exerted at the first contact with a myotube and diminishes as growth advances towards the poles. The first-innervated myotube of the developing spindle (F1, Figs. 55, 204, 205a) is thus subjected to a maximum influence and consequently develops a large aggregation of nuclei at the first point of nerve contact. The aggregation diminishes in proportion to the strength of influence, so that at the extremities of the primary sensory innervation, the influence is sufficient only to maintain the myotubal nature of the intrafusal fibre (Fig. 204). At the time of formation of the second intrafusal myotube (F2, Figs. 57, 204, 205a) the morphogenetic influence has decreased in its overall strength, because of its time-dependency. However, that of the mid-region is still sufficiently strong to attract some nuclei, but the aggregation is proportionately small (Figs. 204, 205a). The peripheral influence merely retains the central nuclei of the myotube. The second bag fibre may therefore possess fewer equatorial nuclei than its older neighbour.

Towards the end of the morphogenetic period, the sensory

influence is reduced to an overall minimal value, similar to that required, in the adult spindle, for the maintenance of the equatorial nucleation. The intrafusal myotubes that develop at this time are therefore subjected to an influence that is only capable of retaining the central nuclei of the myotube, and so adopt a nuclear-chain morphology (Figs. 204, 205a).

Such a varying influence allows for some plasticity during development, so that various morphological forms may result. If the initial sensory terminal contact is with two myotubes (see Landon, 1972 b), F2 will be subjected to the same strength of influence as F1. The equatorial nucleation will then be equally large in both nuclear-bag fibres (Fig. 205b). Similarly, if F3 is somewhat premature in its development, it may be influenced to a similar extent as F2, or at some level between F2 and F3 (Fig. 205b) so that a double row of nuclei may occupy the equatorial region (eg. F3, Fig. 73).

In spindles of a variety of mammals, nuclear-chain fibres outnumber nuclear-bag fibres. It may be that the strength of influence that is necessary for the formation of nuclear bags is restricted to a small, initial fraction of the morphogenetic period in each species, while the minimal influence for nuclear-chain formation is present for the remainder of this period, the length of which may vary but will normally be in excess of that of the initial period (Fig. 205). The number of nuclear-chain fibres may therefore be governed by the length of this latter period and by the number of myoblasts available for myotube formation, but

should lead to an excess of chain fibres.

An overall weak morphogenetic influence, that at its maximum is only as strong as that affecting F2 (Figs. 204, 205b) in rat hindlimb spindles, may result in only small differences in nucleation between nuclear-bag and nuclear-chain fibres, as is apparently found in hindpaw lumbrical muscles of rat (Ovalle, 1972 a, Fig. 1) and guinea pig (Banks & James, 1973). The relative strength of the sensory influence may decrease with distance from the spinal cord, so that the spindles of peripheral muscles (such as hindpaw lumbricals) might be expected to contain fibres that differ little in their equatorial nucleation.

Such a model may offer some explanation of the results obtained following nerve section or crush in immature or adult animals. The quality or strength of influence that is necessary for the retention of a central nucleus during the formation of intrafusal muscle fibres may differ in some way from that maintaining the nucleation in the adult spindle (Fig. 204). The results of its withdrawal would therefore differ in the immature animal compared to the adult. It is also necessary to postulate that once the formation of an intrafusal muscle fibre is complete, even a large nuclear-bag fibre requires only a minimal influence for its long-term maintenance. Such a postulation agrees with that of Zelená (1964), who suggests that the balance of influences may be quite different during development than in maturity.

If the influence of sensory terminals is removed at the onset of spindle differentiation in rat, or at birth when the nuclear-bag fibres at least are present, rapid

disintegration of the muscle component of the spindle follows (eg. Zelená, 1957, 1962, 1964; 17.3). J. K. Werner (personal communication) has attempted to determine the period during which the spindle must be innervated in order to develop all components to a point where they will not degenerate rapidly following denervation. Zelená (eg. 1957) found such a relative independency in 20 DPN rats. Werner examined the spindles in medial gastrocnemius muscle 2 weeks to 2 months after nerve crush at the 4, 6, 8 and 10 DPN stages. The spindles in those animals, in which the nerve was crushed at the 8 and 10 DPN stages appeared normal in every respect when viewed with the light microscope. At the other experimental stages, muscle spindles showed a deficiency in components as well as number. The majority of spindles in the 4 and 6 DPN animals contained 3 or less intrafusal fibres, with several containing 2 nuclear-bag fibres only. These results suggest that the morphogenetic influence of primary sensory terminals is essential for the maintenance of structure during the formation and maturation of individual intrafusal fibres. The nuclear-chain fibres, that are the only immature fibres of the spindle at the 4-6 DPN stage of development, are thus the only fibres to degenerate in the denervation period. Once the morphogenesis of individual fibres is complete (eg. at the 4-6 DPN stage for nuclear-bag fibres and 8-10 DPN stage for nuclear-chain fibres), the removal of the primary influence does not cause short-term degeneration, as in adult spindles where equatorial atrophy after de-afferentation follows a long time course (Tower, 1932; Boyd, 1962).

The proposal made here, that the morphogenetic influence

of primary sensory terminals is confined to the immediately-innervated area, suggests that the polar regions of intrafusal myotubes develop independently of this influence. We might expect the polar areas to exhibit some resistance to denervation carried out during their formation. This has been shown in part (see 17.3; Zelená, 1957), where the nuclear bags of spindles denervated at birth appear to degenerate before myofibrillar atrophy of the polar regions occurs. The degeneration of the polar regions of such intrafusal fibres may be caused in part by the prolonged absence of motor terminals, or by the disintergration of the fibres in their central regions by equatorial degeneration.

Such a time-limited morphogenetic influence may be attributed to regenerating axons of nerves crushed at birth. Those axons that re-innervate the muscle soon after denervation (eg. Zelená & Hník, 1963 a; Zelená, 1964) may therefore have the power to induce a normal sequence of intrafusal fibre formation. Beyond this time the influence may decrease in strength so that at later re-innervation periods, the morphogenetic effect is only sufficient to maintain the myotubal nature of the re-innervated spindle remnants (Fig. 205b), as in atypical spindles (Hník & Zelená, 1961; 17.2). It has been shown that the two intrafusal fibres that may survive neonatal denervation are the remnants of the two nuclear-bag fibres normally present at birth. Atypical spindles are formed by the re-innervation of these fibres. Few intrafusal myoblasts or satellite cells are located in degenerating spindles (see 17.3). Re-innervating axons may therefore be unable to induce the formation of further generations of intrafusal fibres, because of the

paucity of myogenic cells, or their inappropriate stage of differentiation (cf. Zelená, 1964; Zelená & Sabotková, 1971).

However, such a model ignores the possible function of the secondary sensory innervation in spindle morphogenesis. If secondary sensory terminals are located almost exclusively on nuclear-chain fibres (Boyd, 1959) then their arrival must occur postnatally, as suggested by Zelená (1957; 1964). It may be therefore that primary sensory terminals during the morphogenetic period always have the effect of producing an equatorial nuclear bag, but the arrival and establishment of secondary terminals overrides this effect in immature fibres and merely maintains their myotubal character. However, not all spindles possess a secondary sensory innervation (eg. Porayko & Smith, 1968), while nuclear-chain fibres are the normal feature of almost all spindles. It may be that the morphogenetic influence of the primary nerve terminals is so low at the time of nuclear-chain formation that the secondary terminals are needed to supplement their effect on the immature fibres of the spindle. Such a teleological explanation must involve some degree of attraction between the immature terminals of secondary sensory fibres and nascent myotubes of the spindle or of resistance to secondary-terminal establishment in the more mature nuclear-bag fibres.

The probability of the postnatal arrival of secondary sensory terminals is somewhat reduced by the fact that basement membrane is well developed on the outer surface of the axial bundle at birth (eg. Fig. 80). Zelená & Sabotková

(1971) suggest that the persistance of old basement membrane on the premature formation of new basement membrane in muscle regenerates may prevent the contact of sensory nerve terminals with myotubes and thus account for the absence of spindles in such tissue. It may be that secondary sensory nerve fibres invade the basement membrane of the axial bundle prenatally with the primary fibre, and grow away from the equatorial region before forming terminals postnatally. The bundled endings observed in newborn rat spindles (Fig. 80), that appear to lie between two layers of basement membrane, may represent such growing pre-terminal axons of the secondary sensory innervation. Likewise, some of the non-terminal structures in the overlapping terminals of immature spindles (Fig. 77) may represent the growing tips of primary nerve axons. The separation and maturation of the pre-terminal axons probably coincides with the acquisition of individual Schwann-cell coverings and subsequent myclination of the nerve fibres, that occurs postnatally in rat (eg. Peters & Muir, 1959). On the other hand, the growing tips of secondary sensory axons may have the ability to penetrate the basement membrane of the axial bundle, which may then re-form over the terminals. Muscle fibres are known to have the ability to produce new basement membrane during regeneration (Vracko & Benditt, 1972). Such an ability may also be a feature of developing intrafusal fibres.

The model also ignores the fact that during their formation, the intrafusal fibres do not possess individual primary sensory terminals but are innervated communally on

their outer surfaces only (14.1). Cross-terminals that make contact with two intrafusal muscle fibres, are also common (eg. Fig. 61). We might therefore expect this influence to have an accumulative effect, reaching its peak at the end of the morphogenetic period. This would manifest itself in a progressive development of myonuclei in the nuclear-bag fibres. However, no evidence was found of such a progressive formation. If the morphogenetic influence acts on all fibres throughout the morphogenetic period, then the removal of the influence at any time within this period should result in the degeneration of all fibres. As this has been shown not to be the case (cf. p. 160), it seems probable, as proposed in this model, that the morphogenetic effect can only be exerted at the time of intrafusal fibre formation. Once maturity is attained, the influence has only a maintaining effect. It appears therefore that the morphogenetic influence of primary sensory terminals during muscle-spindle formation is limited by time, distance from the first point of contact of the primary axon with the axial bundle, distance from the spinal cord and by the maturity of the myogenic cells innervated.

19.3 The nature of the morphogenetic influence.

As long ago as 1920, Olmsted postulated that the trophic effect of gustatory nerves on taste buds is due to some substances "of the nature of hormones" (p. 392) that are continually released by the sensory nerve terminals. This hypothesis was elaborated by May (1925) who suggested that the hormone-like substance stemmed from the cell body of the neuron. Parker (1932) discovered that the latent period

between cutting the nerve and the onset of atrophic changes in denervated sense organs and muscles is proportional to the length of the distal stump of nerve. After cutting the lateral line of the catfish, degeneration of the lateral-line organs spread proximo-distally from the cut at a rate of about 2 cm per day (Parker, 1932; Parker & Paine, 1934). This suggests that the latent period might be due to the gradual depletion of a resevoir of trophic substance in the peripheral stump of the nerve, which is released at the nerve terminals. There is now an impressive body of evidence to show that various materials flow proximo-distally in both sensory and motor nerve axons (see reviews by Jacobson, 1970; Smith, 1971).

During their development, sensory terminals of the spindle differ little in structure or content from those of the adult (14.1; Landon, 1972 b), except for a greater concentration of both empty and dense-cored vesicles, both of which progressively decrease in concentration with the advancement of the morphogenetic period. Dense-cored vesicles are presumed to subserve a neurotrophic function (Smith, 1971). Similarly, dense-cored granules in the neurosecretory axons of invertebrates (Bunt, 1969; Normann, 1969) are known to release their contents at the axolemma by exocytosis. Exocytosed vesicles may then be recycled as coated vesicles or small "synaptic" vesicles (Bunt, 1969; Normann, 1969; Smith, 1971). A similar cycle has been postulated for regenerating peripheral nerves (Pellegrino de Iraldi & de Robertis, 1968; Lentz, 1969 b), where the similarity of large granule-containing vesicles to those in other neurones

producing hormonal substances was noted. It was proposed that the granule-containing vesicles may contain neurotrophic factors in a protein-bound form, for release by exocytosis. These investigations also suggest that the small clear vesicles, which are also a feature of sensory terminal cytoplasm (see 12.4, 14.1; Adal, 1969), represent an aspect of a recycling process for redundant membrane materials.

Zacks & Saito (1969) have proved that the coated vesicles of mouse motor nerve terminals and the underlying sarcoplasm are involved in the uptake of material from the synaptic cleft. It may be therefore that the coated vesicles of sensory terminals (eg. Fig. 79) do not necessarily have their origin exclusively in exocytosed dense-cored vesicles, but may form, in addition, spontaneously at the axolemma. However, their presence in the subsarcolemmal sarcoplasm of sensory neuromuscular junctions, reported in this (14.1) and Landon's (1972 b) study may implicate them in the endocytosis of protein cores, in a manner described by Smith (1971) and Zelená (1972).

It has been suggested (see pp.156-157) that the morphogenetic influence of sensory terminals decreases with the advancement of the morphogenetic period; if this is so, then this decreasing effect may be correlated with the decreasing numbers of dense-cored vesicles in the sensory terminal axoplasm, during the development of the spindle. The only possible evidence of exocytosis of dense-cored vesicles was observed in two spindles towards the end of the morphogenetic period. It may be that their discharge is rapid and synchronized in the early stages of development, so that the

chance of their fixation and observation in a state of discharge, at any one time, is very limited. At the later stages of development, exocytosis may be occasional and asynchronized, in harmony with a weak, maintaining morphogenetic influence, so that the chance of observing a single discharging vesicle is increased.

Both of the configurations that may represent discharging dense-cored vesicles consist of a flask-shaped core of electron-dense material that protrudes from the axolemma into a coated vesicle of the junctional sarcolemma (cf. pp.94-95; Figs. 91, 92). The core of material is of a similar density and size to neighbouring dense-cored vesicles of the terminal. Several empty vesicles also border the junctional axolemma and have a similar collapsed appearance to the presumed exocytosed vesicle (Fig. 92). It may be that the core of material is discharged intact into coated vesicles (see Fig. 206) that are subsequently "pinched off" from the sarcolemma. In neither case did the vacated vacuole have a coated appearance. However, this does not exclude the possibility that the coated or clear vesicles of the terminal axoplasm develop from such discharged vesicles by micropincytotic activity of the axolemma.

It may be, as suggested by Katz (1969), that exocytosis only occurs when two specific sites meet, one on each membrane, and perhaps only at that region of the axolemma directly apposed to an invaginating coated vesicle (see Fig. 206). Coated-vesicle formation is a feature of the apposed sarcolemmal membranes and capsule cells in immature spindles. It is thus more probable that the formation of coated vesicles at a compatible area of the sarcolemma induces the exocytosis

of dense-cored vesicles rather than the reverse.

No evidence was found of the subsequent fate of the endocytosed cores of protein. Holtzman (1971) has shown that the proteins taken up by coated vesicles in the central nervous system are ultimately digested by lysosomal enzymes. It may be that the neurotrophic effect of exocytosed dense-cored vesicles is released in a similar way in developing intrafusal fibres (Fig. 206).

If the number and rate of discharge of dense-cored vesicles is related to the strength of the morphogenetic effect, regenerating sensory nerve terminals (eg. in atypical spindles) should contain fewer vesicles of this type compared to normally growing terminals. The cursory examination of regenerating spindles (see 17.2) did reveal a paucity of dense-cored vesicles in regenerating sensory nerve terminals.

Chapter 20. The role of fusimotor innervation in the postnatal development of the spindle.

The main postnatal development of the spindle involves the growth and the histochemical and ultrastructural maturation of individual intrafusal fibres (see 15.2, 14.43). These three developmental processes coincide with the arrival and establishment of the fusimotor terminals. The fusimotor innervation would thus appear to be implicated in the development of these characteristics.

20.1 Postnatal growth of intrafusal fibres.

It was found in this, and Landon's (1972 b) study, that the successive generations of intrafusal muscle fibres can be separated on the basis of diameter during their development. A similar difference in diameter has been noted between successive generations of extrafusal muscle fibres (Joubert, 1955; Wirsen & Larsson, 1964; Kelly & Zacks, 1969 a; Bridge & Allbrook, 1970; Kelly & Schotland, 1972). It is suggested that the foetal differences in size are lost by a differential growth rate, the growth of the small fibres being greater than that of the large first generation fibres.

A similar differential growth pattern appears to prevail in developing spindles, where the developmental differences in diameter between the intrafusal myotubes are lost in the late postnatal stages (13.8). This is due, presumably, to the accelerated increase in diameter of nuclear-chain fibres compared to typical and intermediate bag fibres. Such growth is apparently confined to the polar regions of the intrafusal fibres, as their encapsulated regions retain the foetal differences in size. This differential growth may occur

independently of nerve influence, and may be due simply to the differences in the time of formation of these fibres. The increase in size of extrafusal first generation fibres may be approaching completion at the time of the formation of the later generations of fibres (eg. at the late foetal stages of development in rat), so that postnatal growth involves mostly an increase in size of the later-formed muscle fibres. As the method of increment of extrafusal and intrafusal muscle fibres are similar and adopt the same time course in both, we might expect an additional similarity in the pattern of postnatal growth, if such growth depends simply on intrinsic differences in the time of formation of muscle fibres.

However, the postnatal growth of intrafusal fibres does not proceed, overall, at the same rate as in extrafusal fibres. The equatorial and juxta-equatorial regions of intrafusal fibres show no differential growth and their polar regions never attain the dimensions of adult extrafusal fibres (eg. Fig. 11). Growth of the encapsulated regions of intrafusal fibres may be inhibited by the development of the capsule and periaxial space. It may be as Tello (1922) puts it : "The Weismann fascicle is thus nothing else but one of the primitive myotube bundles that form the muscle at the moment when the development of the spindle is initiated, as is maintained in a slightly modified embryonic form even in adult man ....." (translation, p. 407).

There is evidence (see 4.2) that it is muscle tension that primarily influences the growth of extrafusal muscle, although the growth increment of denervated developing muscle "is negligible compared with normal muscle" (Zelená,

1959, p. 83). It appears therefore that the motor innervation of skeletal muscle fibres also plays a role in determining the rate of growth. It may be that the tension on developing limb muscles caused by skeletal elongation provides the influence for the differential growth of extrafusal and intrafusal fibres, beyond which it is the motoneuron that mainly influences the growth of individual fibres. In this way, the differences in the pattern of innervation of intrafusal and extrafusal muscle fibres may account for the differences in size between these fibres. In support of this Zelená (1963) found that neonatal tenotomy of muscle spindles leads to a decrease in the longitudinal growth of intrafusal fibres, although not in equatorial diameter (polar diameters are not given), and that neonatal de-efferentation (Zelená, 1965) leads to a 25% reduction in the diameter of the polar regions. It seems therefore that both tension and motor innervation are involved in the growth of intrafusal fibres.

20.2 Histochemical maturation of intrafusal fibres.

In both this (15.1) and previous investigations (see 3.32) evidence has been provided that histochemical differences between extrafusal muscle fibres differentiate during development and are not, as suggested by Wirsen & Larsson (1964), present from the moment of muscle-fibre formation. The "undifferentiated" fibre appears histochemically to approach the adult type C, although their profiles are not identical (see also Ashmore & Doerre, 1971; Brooke et al, 1971). The most striking feature of histochemical differentiation is the progressive development of fibres with weak ATPase activity

that eventually form the B fibre population (15.1; Kelly & Schotland, 1972; Davies, 1972 a, b). The development of the predominantly anaerobic type A fibre may occur at the same time (Davies, 1972 b) or, in rat, after the establishment of the B population.

A progressive development of histochemical differences between intrafusal fibres was also reported in this study (see 15.2). It was found that at a time when ATPase and SDH activity is uniformly high in all intrafusal fibres (ie. at 5 DPN stage), P'ase activity varies in intensity according to the diameter of the fibre. Thus, in agreement with Wirsen & Larsson (1964) and Ostenda & Strugalska (1971) the large diameter typical bag fibre, at this time, exhibits the highest P'ase activity, while the intermediate bag fibre and nuclear-chain fibres have an activity that varies from intermediate to low (eg. Fig. 149). As neither of the previous investigations included profiles of adult intrafusal fibres, it is not surprising that the general conclusion was that such variations in P'ase activity indicate the mature state. However, it was found in this study that the P'ase activity of adult intrafusal fibres does not have a reciprical relationship to diameter (Table 4; Figs. 27, 33). According to the suggestion of Wirsen & Larsson (1964), the large first generation fibres of the spindle exhibit the highest P'ase activity from their time of inception, followed by the secondary (intermediate) and tertiary (nuclear-chain) fibres. However if such a correlation between the intensity of P'ase activity and the sequence of development is applied to adult rat intrafusal fibres, their developmental sequence should

be in the order, nuclear chains, typical bag fibre, intermediate bag fibre. This study of the fine structure of developing rat spindles has shown that this is not so. It is thus obvious that the decreasing P'ase activity of successive generations of extrafusal and intrafusal fibres, observed by Wirsen & Larsson and in this study, is, as Dubowitz (1970) suggests, merely variations in the intensity of staining and not a manifestation of inherent differences in their P'ase content. The fact that even at its greatest intensity the P'ase staining of immature muscle is in no way comparable with the blue-black colour of high activity in adult muscle (see 9.3), supports this argument.

It seems therefore that the differentiation of P'ase activity as well as that of ATPase and SDH, occurs progressively during postnatal development. Histochemical maturity is attained by the third postnatal week.

There is evidence that the histochemical properties of extrafusal muscle fibres are determined, at least in part, by their motor innervation (see review by Guth, 1968). It is thus probable that the histochemical differentiation of extrafusal fibres is controlled by developing motoneurons. If this is so, the removal of such a neuronal influence at the outset of the maturation process might be expected to prevent its completion. That the postnatal histochemical differentiation of muscle fibres is impeded by neonatal denervation in rat has been shown (Engel & Karpati, 1968; Shafiq et al, 1972), although it appears that some muscle fibres in rat soleus successfully mature and acquire weak reactivity for ATPase despite denervation (Engel & Karpati,

1968). Cultures of aneural chick muscle also fail to differentiate histochemically (Askanas et al, 1972).

Kelly & Schotland (1972) have suggested that the checker-board pattern of histochemically-distinct fibre types may develop by the progressive arrival of different nerve motor axons that innervate and thus regulate the biochemistry of only those muscle fibres that have matured sufficiently to receive innervation. The first myotubes to receive motor terminals from "pioneering" axons would thus be those of the primary generation, which, following the pattern of ATPase differentiation, would subsequently develop into type B fibres. The large myotubes of foetal rat EDL, that exhibit a weak ATPase activity (Fig. 139) presumably correspond to these first-innervated primary-generation myotubes, as do the large "b" fibres of foetal human muscles (Wohlfart, 1937). Terminal axon sprouts of the pioneering axon may innervate nearby maturing myotubes, that would subsequently, similarly, differentiate into type B fibres. Other secondary generations of myotubes are innervated by later-differentiating axons, that supply and determine the differentiation of type A or C fibres.

It may be that the sequence of the arrival of the motoneurons in the muscle primordium is related to the diameter of the motoneurons in the adult. The first-innervated myotubes may therefore differentiate into type A fibres, that are supplied by the largest motoneurons (Henneman & Olson, 1965). The remaining primary and secondary generation myotubes may be innervated by the smaller motoneurons that supply type B and C fibres in

maturity. The histochemical differentiation of type A fibres may adopt a longer time course than that of type B and C fibres. Although the differentiation process may thus begin earlier in type A than in type B and C fibres, its completion may occur later in development. By thus correlating the pattern of neural innervation with that of muscle assembly, a checker-board intermingling of histochemical fibre types would result.

A similar correlation may be applied to the developing spindle, as the histochemical differentiation of intrafusal fibres occurs at the same time as the arrival and establishment of the fusimotor innervation (see Fig. 207). It is now accepted that during the morphogenesis of the spindle, primary sensory nerve fibres arrive before the fusimotor innervation (Tello, 1917, 1922; Zelená, 1957; Bowden, 1963). The progressive arrival of the spindle nerve fibres may therefore be linked to axon calibre, so that the large primary axons are the first to arrive at the site of spindle formation, while those to arrive last of all belong to the smallest diameter group of fusimotor axons. On the same basis, it is not unreasonable to assume that the various types of fusimotor fibres might also arrive at differing times. In support of this, Skoglund (1960 b, c) suggests that "mixed" extrafusal-intrafusal innervation develops before the γ. Using the nomenclature and degeneration times proposed by Barker, Stacey & Adal (1970), the sequence of motor-terminal formation might be P1, P2 and trail.

The earliest detectable spindles were found in this study of lower hindlimb shank muscles, in the 19.5 day

foetus. However, Landon (1972 a, b) found simple contacts between primary sensory terminals and single myotubes at the 18 DF stage although none were found at a similar developmental stage in this study. The difference may be accounted for, in part, by the differences in estimating foetal age and variations in individual animals (Cuajunco, 1927). It is possible as Landon (1972 b) suggests, that the earliest spindles described in his study, that lack any aggregation of myonuclei in the innervated myotube, are at an earlier stage of development than the specialized myotubes described in this study. It may be that the first primary axons contact unspecialized myotubes at about the 18-19 DF stage of development when α motoneurons are also innervating extrafusal myotubes (Zelená, 1959, 1962; Landon, 1972 b).

Fusimotor terminals were located in the juxta-equatorial and polar regions of spindles at birth (see 13.5), innervating nuclear-bag fibres only. Polar (plate) terminals may make contact prenatally (Fig. 207) at a time when only potential nuclear-bag fibres are available for innervation. It could be that at the time of the arrival of β nerve fibres, only F1 has reached a sufficiently mature stage of differentiation (Kelly & Zacks, 1969 b; Lentz, 1970) to accept the nerve terminals, a suggestion that is substantiated in adult rabbit spindles, where 52% of P1 terminals supply the typical bag fibre (Barker & Stacey, 1970). There is evidence in rat spindles, that β collateral end-plates are distributed to nuclear-bag fibres (Karlsen, 1965; Porayko & Smith, 1968). The collateral innervation of extrafusal and intrafusal fibres may develop in a similar way to the motor units of

extrafusal muscle, where neighbouring myotubes are mutually innervated by axon sprouts from the same neuron (Kelly & Zacks, 1969 a; Kelly & Schotland, 1972). In adult cat spindles, P1 and P2 plates are located in the polar regions of intrafusal fibres, in both their extracapsular and intracapsular portions (Barker, Stacey & Adal, 1970). At the proposed time of arrival of P1 terminals in rat, the capsule of the spindle is confined largely to the zone of sensory innervation, so that in their polar regions the intrafusal myotubes closely abut extrafusal types. Collateral sprouting is therefore simplified by the absence of a capsule, which may overgrow the terminals during its postnatal development. Kelly & Zacks (1969 b) propose that adjacent axon sprouts are separated by the myelination of the nerve fibre and the formation of primary synaptic clefts. Similar agents, as well as the extending capsule, may also separate the β terminals of extrafusal and intrafusal myotubes.

The fusimotor neurons supplying P2 terminals may similarly arrive at a stage in the morphogenesis of the axial bundle when only F1 or both F1 and F2 are sufficiently mature to receive the terminals (Fig. 207). The premature formation of nuclear-chain fibres or the late arrival of P1 or P2 terminals may account for the distribution of plate terminals on nuclear-chain fibres in rat lumbricals (Hennig, 1969; May, 1969; Ovalle, 1972 b).

If the nervous system totally regulates the metabolic and histochemical properties of intrafusal fibres, then their histochemical heterogeneity in rat (Table 4) and

other species (Barker, 1973) may be a reflection of the selective distribution of plate terminals between nuclear-bag fibres. However, in cat spindles bag fibres may be supplied with one, two or all three types of motor ending, that may themselves vary in degree of density whereas most chain fibres receive trail endings only (Barker, Stacey & Adal, 1970). Once a neuromuscular connection is established, further neurotization of nuclear-bag fibres is thus not necessarily inhibited. In this way, γ trail fibres may form terminals on both nuclear-chain and nuclear-bag fibres in the early postnatal stages of muscle-spindle development in the rat.

In extrafusal muscle, both acetylcholine release and acetylicholine sensitivity are correlated with the resistance to hyperneurotization (see review by Guth, 1968). It may be that such a correlation does not prevail in the axial bundle of developing spindles, or that acetylcholine release from the newly-established fusimotor terminals is insufficient to build up this resistance and so individual fibres may receive a multiple and mixed motor innervation.

The nature of the trophic influences exercised by motoneurons on bag fibres is therefore likely to be very variable. The trophic effects of the different types of terminal may differ in their dominating power according to their relative density. In rat, therefore the three types of fusimotor terminal may ultimately determine the three histochemical profiles of intrafusal fibres that have been reported in this study, by their relative predominance on each fibre. Such a proposal allows for the many variations

in histochemical properties of intrafusal fibres between different species (see Barker, 1973) and within the same species (eg. James, 1971 a, b).

If the nervous system is solely responsible for the evolution of enzyme variation, then denervation should totally halt this process. However Engel & Karpati (1968) found that type B fibres could still differentiate in rat soleus following neonatal neurectomy. It may be, as Kelly & Schotland (1972) suggest, that this differentiation process is modified by intrinsic differences between the generations of myotubes. On the other hand, the denervation of hindlimb muscles at birth in rat, does not prevent the arrival of motoneurons, as the first contacts are made in the foetus. It may be, as suggested in this study (15.1) that the histochemical differentiation of type B fibres has started by the time of birth, the process of differentiation having a slow but autonomous time-course, as suggested by Lewis (1973) in his investigation into the development of muscle contractile properties in cat. Neonatal denervation may thus have only a minimal effect on the subsequent development of type B fibres. Type A and C fibres may be innervated by later arriving motoneurons, as suggested by Kelly & Schotland (1972). Their denervation at birth would therefore halt the differentiation process in its early stages and severely impair their subsequent differentiation. On the other hand, the onset of differentiation of type A and C fibres may precede that of type B fibres (cf. pp.174-175) but the process may take longer to complete. The immature type A and C fibres may therefore still be dependent on

their innervation at the time of birth for the completion of histochemical differentiation. The withdrawal of the nerve influence at this time would thus severely impair their postnatal differentiation.

20.3 Ultrastructural differentiation of intrafusal fibres.

It was shown in this study (see 14.43) that in the early stages of the postnatal development of the spindle, the polar regions of all intrafusal fibres exhibit a similar ultrastructural morphology. The variations in ultrastructure typical of mature intrafusal fibres appear to develop within the same time as the establishment of fusimotor terminals.

A similar progressive differentiation of ultrastructure characteristics has been described during the early postnatal development of extrafusal fibres in rat (Bunting, 1969; Shafiq et al, 1972). Neonatal denervation severely impairs this differentiation process (Shafiq et al, 1972). Cultures of aneural chick muscle also fail to develop ultrastructural variations (Askanas et al, 1972). The implication of the nervous system in the myofibrillar maturation of muscle fibres is also supported by the fact that during the postnatal development of the diaphragm in rat, neuromuscular junctions exhibit differences in structure (Fitts, 1969 quoted from Gauthier, 1970), as in adulthood (Padykula & Gauthier, 1970) before the distinct types of muscle fibre appear (Bunting, 1969).

It may be that such a dependent relationship of muscle fibre differentiation on motor innervation also pertains to the developing spindle. The obervation of neonatally-

de-efferented spindles of the rat, by Zelená (1965), led her to suggest that fusimotor nerve fibres only exert a trophic influence on the polar regions of intrafusal fibres. In the muscle spindle of guinea pig (see 16.2), mature ultrastructural variations between intrafusal fibres are exhibited within 1 hr of birth. The fusimotor terminals are also of an adult structure (Banks & James, 1973). It is therefore improbable, as Landon (1972 b) suggests, that the morphogenetic effects of the nerve terminals of the spindle are linked to the abrupt changes in metabolic activity associated with birth, as the muscle spindles of guinea pig are morphologically mature at this time.

It appears that the myotube that forms the anlage of the spindle (F1), completes part of its development in line with all other extrafual myotubes, before the arrival of any spindle nerve terminals. We might therefore expect that in its early stages of differentiation, such an immature typical bag fibre would not differ in ultrastructure from neighbouring extrafusal myotubes. The remaining fibres of the axial bundle develop under the influence of primary sensory nerve terminals (see 19.2), that is apparently confined to the immediately-innervated area. It has also been shown that the developmental distribution of the sarcotubular and mitochondrial systems is similar in all fibres. A physical function has been attributed to the M line both during development (Allen & Grisnik, 1971) and in maturity (Knappeis & Carlsen, 1968). It was reported in this study, in agreement with Allen & Grisnik's observations of developing myotubes of chick, that the M line in nuclear-

chain fibres appears after Z line formation, but before any other banding pattern becomes visible. It is difficult to imagine the development of myofibrils without an M line to maintain the spatial relationship between the myosin filaments of the A band in the typical bag fibre. It may be that the contractile properties of intrafusal fibres develop under the influence of motoneurones, as appears to occur in extrafusal muscle of rat (Close, 1964, 1965). The loss of the M line in typical bag fibres or, presumably, its reduction to a thin double-lined structure (Ovalle, 1971, 1972 a) may be a manifestation of the maturation of such contractile properties. Fusimotor neurones may induce the progressive development of the various patterns of SR and T tubules and similarly govern the size and distribution of mitochondria in individual intrafusal fibres.

If the pattern of motor innervation ultimately determines the ultrastructure of intrafusal fibres, in a similar manner to the histochemical properties (pp. 176-179; Fig. 207), then the variations in ultrastructure reported between intrafusal fibres of the same spindle (eg. Table 4), of spindles in different muscles of the same animal (eg. compare 12.3 and Ovalle, 1971, 1972 a) and between such fibres in different species (see Barker, 1973), may reflect the variable motor innervation of individual intrafusal fibres.

20.4 The nature of the trophic influence of fusimotor terminals.

The nature of the influence of motoneurons, that acts on developing and adult extrafusal muscle fibres, determining

their histochemical and contractile properties (see review by Guth, 1968) and, possibly, their related ultrastructure (Gauthier, 1970; Schiaffino, Hanzlíková & Pierobon, 1970) is unknown, but is probably due to either the pattern of nerve impulses or a chemical influence carried from the cell body, in the axon to the muscle fibre, or due to the interaction of both. There are reasons why the trophic effect at neuromuscular junctions cannot be due solely to the neurotransmitter (Gutmann, 1970), and there is some evidence that the trophic substance involved in the nerve-dependent regeneration of the newt's limb may be a protein (Lebowitz & Singer,1970). Lentz (1971, 1972) has also shown that the degeneration of motor end-plates, the development of which is known to be controlled by the motoneuron (Zelená, 1959, 1962; Teräväinen, 1968 c; Lentz, 1969 a), can be retarded by explants of sensory ganglia. This suggests that the trophic effect may be due to the liberation of substances unrelated to neurotransmission.

During development, fusimotor and sensory nerve terminals contain many dense-cored vesicles, that have been implicated in neurotrophism (see review by Smith, 1971), by the exocytosis of their contents. The morphogenetic effect of fusimotor nerve fibres may thus be exerted by the uptake of the exocytosed cores of dense-cored vesicles by the innervated fibres, in a manner suggested for sensory nerve terminals (see pp.166-167; Fig. 206). The marcromolecular content of the dense-cored vesicles of the different types of fusimotor terminals may vary in their quality so that

the histochemical and ultrastructural properties of the intrafusal fibres may be determined by the relative predominance of one particular type of transmitted protein, or by the interaction of the three possible types in nuclear-bag fibres.

Chapter 21. General implications of the pattern of development.

21.1 Intermediate forms of intrafusal fibre.

It has been shown that the morphogenesis of the spindle involves three major events:-

(a) an increase in the number of intrafusal fibres that differ in their equatorial nucleation.

(b) the histochemical and ultrastructural maturation of the intrafusal fibres.

(c) the development of the capsule and periaxial space.

It has been postulated that (a) and (b) occur under the morphogenetic influence of the spindle nerve terminals. Primary sensory axons induce the differential development nuclear-bags and nuclear-chains (a), whereas fusimotor axons influence their histochemical and ultrastructural differentiation (b).

Although the completion times of (a) and (b) are separated chronologically, the two developmental processes must overlap to some extent, as both depend upon a correlation between the arrival of the innervation (whether sensory or motor) and the pattern of muscle-fibre assembly (see 19.2, 20.2, 20.3 & Figs. 204, 205, 207). Equatorial morphology, ultrastructure and histochemistry can generally be linked together for any single intrafusal fibre of the adult (see Table 4) although the development of these features depends on separate trophic agents. This also suggests that the agents must act at some common time in development when only certain muscle fibres are available, or sufficiently mature to receive the influence. The only

other explanation is that the histochemistry and ultrastructure of individual intrafusal fibres are ontogenetically fixed and that specialized fusimotor axons wander towards the type of muscle fibre with which they are destined to associate, a proposal that receives little support with respect to extrafusal muscle fibres (Mommaerts, 1970).

If sensory and fusimotor nerve axons influence developing intrafusal fibres at approximately the same time (cf. Mavrinskaya, 1967), why is it that the histochemical and ultrastructural characteristics of the mature form are not present from the time of muscle-fibre formation, whereas the differences in equatorial nucleation are? The reason for the difference in maturation time may lie in the nature of the trophic influences of sensory and fusimotor terminals. It has been suggested that the morphogenetic influence of the primary axon is of a constant single quality with an immediate but decreasing effect with the advancement of the morphogenetic period (see 19.2). On the other hand, the trophic influence of fusimotor terminals may be an accumulative mixture of three individual influences that are exerted separately and sequentially with the advancement of muscle-spindle morphogenesis. Their overall influence, as with that of α motoneurons (see 3.32), may not be immediate but may adopt a long time-course that is determined by the time taken for the development and establishment of the motor terminals. It is not surprising therefore that at a time when individual intrafusal fibres have only just received their full complement of fusimotor terminals (ie. at 4 DPN stage), there are no histochemical or ultrastructural

variations between the fibres of the axial bundle.

Barker (1973) has recently reviewed the evidence for the existence of different types of bag fibre, which he designates as "typical" and "intermediate". He suggests that intermediate forms of bag fibre may differ in histochemistry, length, diameter, size of the bag and, in ultrastructure in one or more respectsfrom typical bag fibres. This investigation of mature spindles in adult rat peroneal muscle has also presented evidence of the heterogeneity of nuclear-bag fibres (see Table 4). In the light of the proposals made here of the nature, time and method of action of the trophic influences of sensory and motor nerve terminals, the presence of intermediate forms of intrafusal fibre in the adult spindle is conceivable and even predictable.

Only a small asynchrony in the pattern of intrafusal-fibre assembly with respect to the arrival of the primary axon, may lead to variations in equatorial nucleation (cf. pp. 158-159). Similarly, variations in the time of arrival of fusimotor nerve fibres or in the development of the axial bundle, may lead to the innervation of the smaller nuclear-bag (F2) by a predominantly "nuclear-chain selection" of motor terminals. An intrafusal fibre of nuclear-bag equatorial morphology, but with nuclear-chain ultrastructure may result (medium-diameter bag fibre, Table 4). The premature formation of nuclear-chain fibres, or the late arrival of β or γ plate nerve fibres, may lead to the innervation of these fibres by a "nuclear-bag selection" of fusimotor terminals. The intermediate fibre in this case would exhibit

a nuclear-chain equatorial morphology, but a nuclear-bag ultrastructure. Such fibres have been reported in spindles of guinea-pig lumbrical muscle (Banks & James, 1973), and in this study of newborn guinea-pig spindles (see 16.2).

This proposal, that intermediate forms of intrafusal fibre are a result of neuromuscular asynchrony, suggests that these fibres are "developmental accidents" that have no morphological or functional significance in the adult. It appears, however, that in spindles of some muscles (see Barker, 1973) the intermediate bag fibre consistently constitutes part of the axial bundle. In such cases, the presence of an intermediate fibre may be the norm, whereas its absence may represent a developmental asynchrony. Its presence or absence in different muscles of the same animal may be a manifestation of the varying demands and functions of different muscles.

21.2 Physiological immaturity of the muscle spindle.

Landon (1972 b) has suggested that Skoglund's (1960 b, c) finding, that in neonatal kitten only the phasic component of the normal spindle afferent response to stretch can be obtained from the gastrocnermius muscle, may be due to the relative maturity of nuclear-bag fibres compared to nuclear-chain fibres, in spindles of the kitten at birth. He proposes that the micrographs of Scalzi & Price (1971) provide evidence that the developmental state of the neonatal kitten spindle is comparable to that of the newborn rat. A closer inspection of their illustrations (eg. Fig. 11) shows that such a comparison is not valid. The axial bundle of neonatal kitten spindles is invaded by

sensory axons that form terminals on all surfaces of all intrafusal fibres, some of which are enclosed, at least in part, by endomysial cells of the inner capsule (see particularly Fig. 11). In the same spindle, Scalzi & Price identify at least 5 nuclear-chain fibres. A periaxial space is present, although it is not apparently of adult dimensions. These characteristics suggest that the developmental state of newborn kitten spindles approaches more closely that of the rat in the second postnatal week of development (see 13.8), when both bag and chain fibres are distinguishable. Such a developmental state of the newborn kitten spindle may be predicted from the gestation length of 65 days, as compared to 21-22 days in rat. Its immaturity compared to that of the newborn guinea-pig (see 16.2), where gestation is of a similar duration (68 days) is due to the difference in the placenta and consequent maturity of the young at birth between the two species.

The morphological explanation of Skoglund's (1960 b, c) findings probably lies therefore, as he first suggested, in the immaturity of the newly myelinated afferent nerve fibres of newborn cat.

21.3 The nature of the capsule and periaxial space.

The capsule of 19.5 DF rat spindles consists of a single layer of flattened cells, some of which partially surround the spindle nerve trunk. Their morphology is very similar to that of the extrafusal fibroblast (see 14.3). In the later stages of development, two types of cell constitute the capsule. The cells of the inner layer are similar to those that form the first layer of the capsule in the spindle

anlage, while those of the outer layers are identical to and continuous with the perineural epithelial cells of the spindle nerve trunk (eg. Fig. 102). The latter observation confirms the proposal of Shantha, Golarz & Bourne (1968), that the capsular sheet cells of the adult spindle stem from the perineural epithelial cells of the intramuscular nerve trunk. At a time when the inner capsule cells are depleted, endomysial cells can be seen enclosing the axial bundle and its constituent fibres. The endomysial enclosures are often connected to the few cells that line the inner portion of the capsule (eg. Fig. 73). At a time when the periaxial space begins to develop, the outer capsule cells, that form the capsular sheet cells of the mature spindle, may contain several profiles of granular ER and micropinocytotic vesicles with distended lumens (eg. Figs. 68, 107).

A similarity in structure of fibroblasts and immature perineural epithelial cells has been reported (Gamble & Breathnach, 1965; Gamble, 1966; Shantha & Bourne, 1968). Hence the cells of the first layer of the capsule may be immature perineural epithelial cells that subsequently form the endomysial enclosures of the adult spindle, retaining their immature morphology in the adult. On the other hand, their origin may be mesodermal, in the endomysial fibroblasts that surround all myotubes and nerve trunks at the early stages of development. Those cells surrounding the spindle nerve trunk in the 19.5 day foetus may form the fibrocytic elements of the endoneurium, while those surrounding the axial bundle may mature into the endomysial cells of the adult spindle (Fig. 15), the structure of which does not

differ significantly from the endomysial fibrocytes of extrafusal muscle, that form by the maturation of fibroblasts.

The separation of the outer and inner capsule cell layers coincides with the development of the periaxial space. Brzezinski (1961 a, b) has shown that the periaxial fluid of guinea-pig spindles does not have the same histochemistry as lymph, which according to Sherrington (1894), constitutes the fluid of the periaxial space. Brzezinski suggests that this fluid is formed by the spindle sheath. It may be that the distended ER cisternae and micropinocytotic vesicles of postnatal rat spindles represent the formation and accumulation of such fluid prior to its discharge, presumably by some form of exocytosis, into the space between the inner and outer capsule cells. It is unlikely that the vacuolated cells observed in the periaxial space of 16 DPN rat spindles (Fig. 112), first reported by Goglia (1970) are implicated in the production of periaxial fluid because of their absence at the time of its formation. The similar vacuolated appearance of endomysial cells (Scalzi & Price, 1971, Fig. 11; cf. pp. 102-103) and of the pinocytotic cells of capillary epithelium (Fawcett, 1966) suggests that such cells may be endomysial cells that engage in pinocytosis when freed into the periaxial fluid.

21.4 Concluding remarks.

Although this study into the fine structure of developing muscle spindles, the need for which was stressed by Bowden (1963), has elucidated some of the morphological characterstics of adult spindles and solved some of the

events in muscle-spindle formation, the nature and origin of the controlling trophic influences remains vague and speculative. In particular, the role of the fusimotor innervation in the histochemical and ultrastructural maturation of intrafusal fibres requires experimental analysis, by the observation of the effects of neonatal de-efferentation on these differentiation processes. In relation to the proposed morphogenetic influence of motoneurones, it would be interesting to monitor the formation of myofibrils in the equatorial region of nuclear-bag fibres, in the de-efferented adult spindle (Tower, 1932), and in nascent myotubes of vertebrate non-twitch skeletal muscle. It may be that the newly-formed myofibrils of both de-afferented nuclear-bag fibres and pre-innervated non-twitch myotubes possess an M line.

In a similar vein, the ultrastructure of the "nuclear-bag spindles", experimentally produced by J. K. Werner (cf. pp. 159-160) needs investigation, as it may be that the histochemical and ultrastructural maturation of the re-innervated nuclear-bag fibres is retarded by temporary denervation. The finding of Andrew, Part & Wait (1971), that the spindles in some lateral segmental muscles of rat's tail lack a γ component in their fusimotor innervation, could provide evidence of the morphogenetic influence of fusimotor neurons, if the intrafusal fibres of such spindles exhibit a different ultrastructure and histochemistry from those that possess a γ component.

The uptake of tritiated thymidine by intrafusal myoblasts following administration at the late (18-20 days) foetal

stages of development would confirm the method of intrafusal-fibre formation proposed here. Similarly the effects of foetal x-radiation and colchicine treatment, as attempted by Bravo-Rey et al (1969), on the formation of nuclear bags would throw some light on their origin, and either confirm or negate the development of intrafusal fibres as a mitotic process. It may be that a cessation of mitosis in the cells of the capsule, using similar pathogens, would lead to a reduction in the dimensions of the periaxial space if, as suggested here, the two structures are developmentally linked.

The experimental analysis of the proposals made here regarding the morphogenetic influences that induce muscle-spindle formation has already started with the investigations of J. K. Werner (1973, in press). The importance of this developmental process to the understanding of the adult structure and neuromuscular development in general has thus been recognized. "As wonders these things have grown stale through familiarity" commented Sherrington (1940) with reference to the development of the nervous system. The advent of the electron microscope has obviously aroused fresh interest in neuromuscular development, so that Sherrington's speculation does not hold true now.

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