AN ANATOMICAL STUDY OF ADAPTIVE PROCESSES

IN MUSCLE

A thesis presented in partial fulfilment
of the requirements for the Degree of
Doctor of Philosophy at Massey University.

NALLATHAMBY SIVACHELVAN

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This work is dedicated to Dr W J Pryor, Assistant Director, Department of Primary Industry, Australia and former Dean of the Faculty of Veterinary Science, Massey University, who first cleared a way for me in the direction of my aspirations.

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by NALLATHAMBY SIVACHELVAN

The mechanisms involved in the adaptability of muscle to functional changes have been tested using sheep as the experimental animal.

(1) A quantitative study of the antenatal and postnatal development of skeletal muscle was undertaken using the semitendinosus muscle of eighteen fetuses from 60 days gestation to birth and of fifteen sheep from birth to adulthood. The muscle was also used as an experimental model to test antenatal anticipation of postnatal muscle functions. The alkali-stabile myosin ATPase technique was used to identify and quantify the histochemical ATPase low (AL) and ATPase high (AH) fibres and to follow both fibre and fascicular growth within the muscle. Histochemical changes occurring within the developing fibres were also recognized.

Presumptive AL fibres had centrally occupying nuclei up to 80 days. A smaller secondary fibre population formed the presumptive AH fibres during this period of development. From eighty days of gestation onwards, two distinct fibre types could be observed histochemically in the muscle. The AL fibre stained pale while the AH fibre stained dark.

Areas of variable fibre type population density were distinct within the muscle in all stages studied from 80

days of gestation. The highest population density of ATPase low fibres was observed in the craniomedial aspect of the muscle (AL dense area). The lowest AL fibre population density was seen in the caudolateral aspect of the muscle (the AL sparse area). The AL fibre percentage within the AL dense area of the muscle increased from about 10% to about 30% from 80 days of gestation to adulthood whereas in the AL sparse area the AL fibre type population density remained at about 4% throughout this period.

Simultaneous electromyographic studies using the semitendinosus muscle of three adult sheep suggested that the AL dense area is preferentially used for posture and during quiet co-ordinated activity, while the AL sparse area is recruited only intermittently when the hip and stifle joints are less co-ordinated in movement. By using angiographic studies on the muscle, many large and closely spaced blood vessels were seen to run in the AL dense area. Thus, a metabolic and nutritional prerequisite for the mechanically disadvantageous disposition of the AL fibres in the deep part of a muscle with a heterogeneous fibre type distribution has been suggested.

A postural and propulsive involvement of the muscle and an increase in percentage of AL fibres along with increasing body weight suggests a functional adaptatory change. The part of this change occurring antenatally is expected to be the result of genetic anticipation.

A marked reduction in perimysial connective tissue occurred antenatally but no significant changes occurred postnatally. The change involving endomysial connective tissue, on the

other hand, was less marked throughout the development. Also, the number of fibres per fascicle was constant both antenatally and postnatally. The perimysial enclosed fascicles increased in number antenatally but remained constant postnatally. This suggests a role for the connective tissue framework of a muscle in constraining the growth in number of fascicles and fibres.

(2) The function of one hind limb of the sheep was modified to study the growth changes in the musculoskeletal system. Seven lambs treated with one hind limb bound to the body with the hip fully flexed and the stifle and hock fully extended were reared from the day after birth to about three months of age, together with two untreated controls. A carcass dissection study was made of the treated and control lambs. Changes which occurred in the semitendinosus muscle were studied using histochemistry and electron microscopy.

All the supporting limbs of the treated lambs showed growth related changes induced by the treatment, suggesting a diagonal support of body weight. The semitendinosus muscle of the bound limb was heavier than that of the controls and that of the supporting limb, while the quadriceps muscle was heavier on the supporting side and lighter on the bound side. These results from the semitendinosus and quadriceps muscles support the view that stretch is an important requirement of muscle hypertrophy. The semitendinosus muscle, stretched over two joints, hypertrophies to a greater extent than a muscle like the semimembranosus, stretched over one joint. The size increase of other muscles such as the extensors of the stifle joint of the supporting hind limb may also have been due to stretch. A trend was observed for the femur

and tibia to be more mineralised and thicker, but shorter, on the supporting than on the bound side of the body.

The histochemical study of the semitendinosus muscle indicated that both AL and AH fibres are induced to grow by the stretch stimulus. An increase in the AL fibre type population as a percentage of the regional fibre population in the deep part of the semitendinosus muscle suggests that chronic abnormal usage of a muscle can produce an adaptive response. Thus, this exaggerates a similar effect observable during normal growth of an animal because of its increase in body weight. During the growth of a muscle under immobilized but stretched conditions, the shape, the amount of connective tissue and the size of fascicle and fibre components appear to maintain isometric proportions.

Electron microscopically, no abnormalities in the arrangement of the subcellular components and in the proportions of myofibrillar, sarcoplasmic and mitochondrial elements within the fibres of the hypertrophied semitendinosus muscle were revealed.

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1.0 GENERAL INTRODUCTION

A proportionate and orderly distribution and growth of muscles throughout the body of an animal is maintained genetically under normal circumstances. Such a basic growth pattern may be modified applying an intensive selection programme, as used in improving meat production or athletic potential. Differences are seen in both muscle distribution and in growth pattern between species (Berg & Butterfield , 1976), breeds (Davies, 1974; Gunn, 1975) and sex (Lohse, 1973; Berg & Butterfield, 1976), and can be attributed to differences in the functional requirements of genetically different types of animals.

Within a genetic framework, relative growth changes in muscles are essential to accommodate variation in the functional needs imposed on an animal. An increase in body weight or a greater locomotory effort may necessitate growth changes in the appropriate muscles. Also, changes in habitat, nutrition or activity patterns of an animal will make growth changes in muscles desirable. McMeekan (1940) stated that those parts of the body essential for life processes and body function are relatively well developed at birth and, as a consequence, they increase to a small extent postnatally while those organs connected with movement, storage or reserve are poorly developed at birth, but grow more during postnatal life. Fowler (1968) proposed that an animal tends to adjust to environmental changes in such a way that the vital functional relationships between essential body components are preserved, or modified to a form which gives the animal its best chance of survival and successful reproduction. Relative growth rates appear to differ between muscles depending on whether they are primarily postural in function (opposing the gravitational force on the body), or propulsive in function (producing an accelerating force on

the body) (Davies, 1979). If a modification to the growth pattern of muscles is deemed essential, it can be anticipated to occur in such a way that a functional integrity of the whole body is preserved.

Muscles are adaptable to conditions of altered structural integrity or to unexpected functional changes. The postural muscles of an amniote have to support the weight of the animal when it begins its terrestrial mode of life. Various diseases affecting only a part of an animal's musculature can initiate adaptations in healthy muscles to restore function partially or fully. These diseases may be intrinsic to the affected muscles, such as transport myopathy, muscular dystrophy, myositis, the Vitamin E - selenium deficiency diseases and disuse caused by immobilisation, or they may be extrinsic. These latter include neurogenic diseases, for example polio myelitis and multiple sclerosis, or mechanical conditions resulting from arthritis, bone and joint surgery or amputation, whereby disuse of one part of the muscular system results in greater use elsewhere. The changes resulting from training programmes for physical fitness, athletics and body building can presumably also change the nature of muscles. The mechanisms and the types of adaptation are, for most of these conditions, still obscure.

A muscle can be expected to undergo both qualitative and quantitative changes as an adaptive response. The quantitative changes may involve the size of both the whole muscle or the fibres within it. The qualitative changes may be related to muscle metabolism, to the contractile properties of the muscle, or to the myofibrillar and myofilamentous constituents in addition to the nature of the connective tissue framework.

In this thesis, two models of the adaptability of muscle

have been used to identify the changes involved and to suggest the mechanism causing the changes in each case. In Part 1, qualitative changes in muscle occurring before, during and after the amniotic to terrestrial changes at birth are described in one muscle of the sheep, namely the semitendinosus muscle. Contractile properties of the muscle are studied by using histochemistry and electromyography, and changes involved in the connective tissue components and the muscle fibre population are examined. In Part 2, quantitative and qualitative changes occurring as a result of immobilization in an abnormal limb position and by chronic overload have been studied using sheep from birth to 2-3 months of age. Quantitative changes are described for muscles and bones throughout the trunk and four limbs due to the altered function of one limb. Qualitative changes as seen in a chronically stretched muscle have been studied using histochemistry and electron microscopy, again for the semitendinosus muscle. Changes in the fibre population and in connective tissue components of this muscle have also been analysed.

The histochemical and electromyographic aspects of muscle from Part 1 above have been published as Sivachelvan (1979) and Sivachelvan & Davies (1980, 1981).

2.0 PART 1. ANTENATAL AND POSTNATAL DEVELOPMENT OF THE SEMITENDINOSUS MUSCLE OF THE SHEEP

2.1 INTRODUCTION

Growth in skeletal muscle, as for any other tissue in the body, has two naturally separated phases, namely, the antenatal and postnatal periods. The present study examines the antenatal adaptive changes occurring in a muscle performing a postural function postnatally and the changes observed in the myofibre and connective tissue components during both antenatal and postnatal growth.

In early literature, there have been observations on the mechanisms which control the growth of an organism. Aristotle made a general observation that growth can be explained in terms of completeness of function (Nussbaum, 1978). Roux (1905) suggested that the formation of structures in the "embryonic period" is predetermined, while the development of structures formed during the "functional development period" is brought about by their "specific action" (Singer, 1959). Similar observations are also found in the literature relating to the growth of skeletal muscle. Hammond (1932) conducted growth studies in sheep and suggested that the changes in proportions of a muscle with age can be due to a) function b) heredity and c) changes in the shape and relative proportions of the different bones. Bryden (1969, 1973) used the elephant seal to study changes in muscle distribution occurring in different postnatal growth phases and concluded that postnatal growth changes in size occurring in any muscle are governed by a functional adaptatory requirement Berg & Butterfield (1976) suggested, from their observations based on relative muscle growth in cattle, that the antenatal phase of growth in a muscle is almost entirely under the influence of a genetic template, the immediate postnatal

phase largely influenced by function, and the prepubertal and adolescent phase a product of genetic and functional adjustment. Although genetic and environmental influences on growth of skeletal muscle have thus been defined, few earlier studies have attempted to describe the phenotypic expression of the genetic mechanism influencing antenatal muscle growth. Muscular adaptations in animals which walk soon after birth, like the sheep (Hammond, 1932; Pálsson, 1955), must be greater at birth than at any other stage. Nevertheless, little consideration has been given to the mechanism of this perinatal muscular adaptation. Because the fetus of a mammalian quadruped grows as an amniotic aquanaut, its limbs are not used as postural struts. The gravitational force on the fetal body is opposed by a force of buoyancy. The movements (Barcroft, Barron & Windle, 1936) and electrical activity in muscles (Änggård & Ottoson, 1963) of the limbs of sheep can be detected during fetal stages, but such movements are not expected to be involved in posture. After birth, gravitational force is no longer opposed by an effective force of buoyancy, but by the animal's musculoskeletal system. The present study examines the extent of this postural awareness antenatally in a muscle.

Growth changes in fibre size and number within skeletal muscle have been studied by several workers. A constancy of fibre number during postnatal growth has been suggested for the human (Maccallum, 1898) and for the pig (McMeekan, 1940). Joubert (1956a) used the rectus femoris, gastrocnemius and longissimus dorsi muscles of the sheep from birth to 290 days and observed that changes in fibre diameter correspond with changes in muscle weight. He, therefore, suggested that muscular growth is primarily a function of physiological age, and not strictly one of chronological age. Enesco & Puddy (1964) found that, in the biceps

brachii, extensor carpi radialis, gastrocnemius and cranial tibial muscles of the rat, fibre number is constant, while an increase in muscle weight during growth involves an increase in both muscle fibre size and connective tissue amount. They noticed no changes in the relative proportions of the perimysium, the endomysium and the muscle fibres. Rowe & Goldspink (1969) used the cranial tibial, biceps brachii, long digital extensor, soleus and sternomastoideus muscles of the mouse, and noticed a constant fibre number during growth. Their study also suggested that a considerable difference in mean fibre diameter both between muscles within individuals and within a muscle between individuals is possible. Davies (1972) used the longissimus muscle of the pig and suggested that in addition to a constancy in fibre number within postnatal muscles, changes in histochemical fibre type proportions occur with growth to compensate for the strength insufficiency of a muscle relative to the body mass. In contrast to the above studies which indicated a constant number of fibres postnatally, Gunn (1975, 1978) used the semitendinosus, diaphragm and transverse pectoral muscles of the dog and horse and noticed a postnatal increase in total fibre number. His studies also showed that, in each muscle studied, differences in histochemical fibre type proportions can be observed between non-athletic and athletic breeds. There are also research reports to indicate a decrease in total fibre number during postnatal growth. Layman, Hegarty & Swan (1980), for example, used the soleus, plantaris, extensor digitorum longus and the biceps brachii muscles of the rat to show that there is a decrease in muscle fibre number during growth and suggested that fusion of muscle fibres is a possible explanation.

Few workers have studied changes involving the fibre size of fetal muscles. Joubert (1955) used the longissimus,

rectus femoris and gastrocnemius muscles of the sheep, and observed changes in weight, dimensions and histology. His studies indicated that fetal fibres undergo relatively little change in diameter between approximately the 45th and 103rd days of gestation, while during subsequent stages, a considerable increase in fibre diameter occurs. Ashmore, Robinson, Rattray & Doerr (1972) used the semitendinosus muscle of the sheep between 50 days and 140 days of gestation, and observed changes in relative proportions of histochemical fibre types between 70 and 100 days gestation. Although they did not publish measurements, their observations indicated that, as growth progresses, there is an increase in the number of fibres per perimysial enclosed fascicle and in the size of fascicles. Ashmore, Addis & Doerr (1973) conducted a similar study to that of Ashmore *et al* (1972) in the semitendinosus muscle of the pig and noticed differences in growth rates between different histochemical fibre types.

Thus, several studies have concentrated on fibre size changes during growth. Nevertheless, no single study has examined the growth changes occurring in the fascicular, myofibre and the endomysial and perimysial connective tissue components of a muscle covering the early fetal period up to adulthood, in any species. Such a study can determine the relationship between the growth in perimysial and endomysial connective tissue components and the total number of fascicles and fibres. The present study therefore endeavours to achieve this using whole muscle sections of the semitendinosus muscle of the sheep. It covers a period between 60 days gestation up to 5 years of age.

A method is available to study the extent which muscles, used postnatally to maintain posture, 'anticipate' a sudden change

in function at birth. The histochemical reaction for myosin adenosine triphosphatase (ATPase) activity has been shown to indicate the intrinsic speed of contraction of individual fibres. As developed by Padykula & Herman (1955), this method demonstrates a dark staining fibre high in myosin ATPase activity and a light staining fibre low in myosin ATPase activity (Guth & Samaha, 1969). The studies of Bárány (1967), Guth & Samaha (1969) and Bárány & Close (1971) showed that the ATPase high fibres are fast twitch while the ATPase low fibres are slow twitch in contractile properties. This idea was furthered by the histochemical and physiological studies of Burke, Levine, Tsairis & Zajac (1973) in classifying motor units of the gastrocnemius muscle of the cat. Amongst the three types of motor units recognised by Burke *et al, (loc. cit.)*, two groups of fibres with short twitch contraction times are both ATPase high, while the third group (with fibres showing long contraction time) is ATPase low. Within each physiological type of fibre, namely aerobic, anaerobic or combined aerobic and anaerobic, all of the units examined had the same histochemical profiles. In addition, Kugelberg (1973) identified the contractile properties of the motor units of ATPase high and ATPase low fibre types. He demonstrated that the ATPase low slow motor units are the least fatiguable. Within the ATPase high, fast motor units, the muscle fibres with high oxidative activity show tolerance to intermittent bursts of stimuli for longer periods of time without showing fatigue,while the fibres with low oxidative type of activity are most fatiguable and produce brief but strong contractions. Thus, these findings support the concept that the dark staining alkali-stabile myosin ATPase high fibre is suited for high energy demanding, isotonic propulsive activity and that the light staining fibre that is low in myosin ATPase activity is suited for low energy demanding, isometric postural activity (Davies, 1972; Burke

et al. 1973; Kugelberg, 1973).

The alkali-stabile myosin ATPase method has been used previously by Dubowitz (1963, 1965) to follow the differentiation of muscle fibres in the trunk muscles of the guinea pig, hamster, rabbit, rat, mouse and human; by Fenichel (1966) in brachial and thigh muscles of the human; by Ashmore, Addis & Doerr (1973) in the semitendinosus muscle of the pig; by Ashmore, Robinson, Rattray & Doerr (1972) in the semitendinosus muscle of the sheep; by Nyström (1968) in the gastrocnemius and soleus muscles of the cat; and Beermann, Cassens & Hausman (1978) in the semitendinosus muscle of the pig. Of these studies, only that of Beermann *et al*. (1978) compared the population densities of fibre types ante- and postnatally and then only for 21 days after birth. Ashmore *et al*. (1972, 1973) suggested that the fibres which are to become ATPase low fibres are observed prior to the appearance of ATPase high fibres and that these presumptive ATPase low fibres act as a structural framework upon which the ATPase high fibre development subsequently occurs. Beermann *et al*. (1978) noticed histochemically that the induction of acid-stabile myosin ATPase differentiation precedes that of alkali-stabile ATPase and that the primary fibres (alkali-stabile myosin ATPase low) exhibit a positive reaction for acid-stabile myosin ATPase in the deep portion of the semitendinosus muscle but not in the superficial portion. Their studies also suggest that fibre type differentiation is neurally regulated.

The present study accepts the concept that the alkali-stabile myosin ATPase fibres are predominantly postural in activity, by opposing the gravitational force on the body, and the myosin ATPase high fibres are predominantly propulsive in activity, by producing an accelerative force on the body. The semitendinosus muscle of the sheep extends the hip joint against the force of gravity, and also retracts the limb in propulsion. The development of a muscle with such a dual function might therefore

incorporate characteristics relevant to both. Compared to the usual laboratory animals, the gestation period of the sheep is long (about 150 days). Therefore, the successive stages of growth are more easily distinguishable. In addition, the limb muscles of a sheep are functionally prepared to support and propel the body from the day of birth. This model appears, therefore, to be suitable for an investigation of the aspects of growth outlined.

The existence of a heterogeneous distribution of histochemical fibre types within the semitendinosus muscles of the dog and horse has been established by Gunn (1975). The deep parts of these muscles have the highest percentage of alkali-stabile myosin ATPase low fibres. However, the functional significance for such a distribution of histochemical fibre types has not been previously explored. The present study, therefore, incorporates electromyographic and angiographic studies to determine any correlated structural or functional regional differences.

Electrical activity has been associated with muscle contraction ever since Galvani discovered 'Animal electricity' in 1771 (Liddell, 1960). Vrbová (1963) used the soleus, a slow muscle, and the tibialis cranialis, a fast muscle of the rabbit for electromyographic recordings and found that the slow twitch fibres show a continuous type of activity while the fast twitch fibres show bursts of activity during spontaneous movement. In independent work published by Herring, Grimm & Grimm (1979), at about the same time as a preliminary communication by the present author (Sivachelvan, 1979), both the alkali-stabile myosin ATPase technique and electromyography were employed in an investigation of the masseter muscle of the miniature pig. They observed that the region of the muscle having a higher percentage of ATPase low fibre shows activity throughout mastication, whereas the region with the lower percentage of

ATPase low fibres is involved only during a brief portion of the masticatory cycle. In the present study, a qualitative electromyographic investigation has been undertaken to test for differences in activity between the areas of highest and lowest ATPase low fibre type population densities within the semi-tendinosus muscle of the sheep.

There is a close relationship between the metabolic need of individual muscle fibres and their vascularity (Romanul, 1965). The fibres with aerobic metabolic capacity have more blood capillaries than anaerobic fibres. Furthermore, Myrhage & Eriksson (1980) observed that 'slow' muscles have a denser vascular network and a shorter average distance between the individual groups of secondary vessels than 'fast' muscles. They concluded that there is a direct correlation between the density of secondary vascular branches and the percentage of oxidative fibres in skeletal muscle. If this concept is valid, studying the vascular patterns of areas of extreme histochemical fibre types within a muscle could be expected to reveal regional differences between the areas. The possibility of an accumulation of ATPase low fibres close to large and closely spaced blood vessels has been investigated in the present study.

The growth of organs and tissues within an organism is multiplicative (Huxley, 1932). The time independent allometric equation, $y = a.x^b$ (Huxley, 1932), when transformed into a logarithmic form, $\log y = a + b. \log x$, produces a linear graph (Seebeck, 1968), where b is the regression coefficient, x is the independent and y the dependent variable, and a is the intercept of the regression line. In this linear form, it can be used to test a null hypothesis, using a power function set for the b value relating x and y irrespective of their dimensions. The equation can also be used to calculate a

prediction of y at any value of x within the range studied, and the confidence limits of this prediction (Steel & Torrie, 1960). Logarithmic regression has been previously used by many workers to study both carcass composition (Butterfield & Berg, 1966; Seebeck, 1967; Davies, 1974) and changes occurring within individual muscles (Davies, 1972; Gunn, 1975; Tan & Davies, 1980). This method has, therefore, been considered appropriate to study the relative growth of various muscle components.

2.2 MATERIALS AND METHODS

2.2.1 *SOURCE, NUMBER, SEX AND DEVELOPMENTAL STAGES OF ANIMALS USED*

The semitendinosus muscle samples were collected between June 1977 and July 1978 from eighteen fetuses (10 females and 8 males) of ages at or near 60, 70, 80, 100, 120 and 140 days of gestation, and fifteen postnatal animals (12 females and 3 males aged 5 weeks, 5 months and 5 years old (Table 1a). These ages were chosen to provide multiplicative growth increments in body weight appropriate for logarithmic statistical analysis. The ewes and lambs used to provide the muscle samples were reared under controlled conditions at the Department of Scientific and Industrial Research's property adjacent to the University campus. New Zealand Romney ewes grazed on pasture had been mated to Romney rams fitted with harnesses to detect mating. Gestation ages were calculated from the day of successful mating. The pregnant ewes were killed by exsanguination while under barbiturate anaesthesia. Wherever possible, samples of the left semitendinosus muscles (Figs. 1 & 2) from both the dam and the fetus(es) were collected for histological examination. The samples from the lambs were collected in the same manner as that for the ewes.

2.2.2 *PREPARATION OF HISTOLOGICAL MATERIAL*

2.2.2.1 *Collection of samples*

The semitendinosus muscle was obtained from each animal used in the study. As soon as a ewe was killed, the fetus was removed through an abdominal incision and weighed. Removal of the semitendinosus muscle was made *via* a skin incision at the caudolateral aspect of the thigh. The muscle, which could be identified easily because of its caudal position in the femoral region, was separated from its neighbours and its craniodorsal attachment to the gluteobiceps muscle severed. The muscle was then removed *in toto* by detaching its proximal and distal tendons. The distal stump of the nerve and blood vessels entering the hilus of the muscle was left intact. The fully contracted muscle was cleaned of fat and weighed before being placed in a plastic bag prior to further processing.

2.2.2.2 *Sectioning and staining procedure*

Except for samples from three fetal and two postnatal sheep listed below, sections were cut from slices of the semitendinosus muscle on removal from the animal within 2 h of the animal's death. Problems relating to the availability of a cryostat meant that the muscle samples belonging to animals number 7, 16,17,19,29, had to be sectioned after thawing for 1 h following storage at -50°C for up to one week.

A complete transverse slice approximately 1 cm thick was obtained between the middle and distal thirds of the muscle immediately distal to the hilus where the branch of the ischiatic nerve and its accompanying blood vessels entered its craniolateral aspect. The sectioning at this level made the orientation of a slice of muscle easier and the cryostat sectioning of the fibres kept near the same transverse sectional level in all the muscles investigated. When the

slice was too large for complete cryostat sections to be cut, it was divided into six smaller pieces, and Indian ink was injected into the craniomedial aspect of each piece to enable subsequent orientation. Immediately before sectioning, the slices were frozen as rapidly as possible in isopentane cooled to its freezing point (-160°C) with liquid nitrogen. Serial transverse, 10 μm thick, sections were cut from each of them in a cryostat, mounted on cold slides, and then either stained for alkali-stabile myosin ATPase by the method of Padykula & Herman (1955) as modified by Davies & Gunn (1972) (Appendix 1) or stained with PAS- hematoxylin. The unstained, mounted sections were left in a refrigerator for at least 30 minutes before staining for myosin ATPase to prevent the sections separating off the slides at later stages in the staining procedure. Preliminary tests showed that the concentration of calcium chloride is an important criterion for the reaction. Whenever possible, sections of muscles from both a dam and its fetus were stained together for myosin ATPase to facilitate comparison of antenatal and postnatal muscle. Five slides were stained from each sample and the best one was used for quantitative analysis.

2.2.3 *DETERMINATION OF THE PATTERN OF HISTOCHEMICAL FIBRE TYPE POPULATION DENSITY IN SECTIONS OF THE SEMITENDINOSUS MUSCLE*

By using the alkali-stabile myosin ATPase technique, a simple division into ATPase high (AH) or ATPase low (AL) was possible for any fibre relative to the overall activity of adjacent fibres in the semitendinosus muscle belonging to animals, beginning at the 80 days fetal stage and extending up to 5 years postnatally. The semitendinosus muscle had a heterogeneous distribution of AL and AH fibres within it. As the AL fibres formed a smaller percentage population of the two fibre types in any region of the muscle, the AL fibre

was preferable to the AH fibre for statistical analysis of the relative proportions of the two fibre types. The population density of AL fibres, expressed as a percentage of the total number of fibres counted in each region, was estimated over the entire transverse sectional area of the muscle using the method described below.

A projection microscope (Leitz, Wetzlar) was set up in a dark room to display the fibres within the stained sections of the muscle on to tracing paper on the opposite side of a glass screen. The sections were magnified 100 times for postnatal muscles and 250 times for antenatal muscles. In the case of antenatal muscles, fascicles from about 20 regions and in the postnatal ones up to 50 regions were examined. From each of these regions 2 to 3 fascicles were selected for counting a total of about 5000 to 7000 fibres. The regions were changed by shifting the slide in two directions using the mechanical stage control knobs to ensure that the same region was not viewed more than once.

2.2.4 *DETERMINATION OF TOTAL FASCICULAR, TOTAL FIBRE, FASCICULAR AND FIBRE TRANSVERSE SECTIONAL AREAS*

For the study of the total fascicular, total fibre, fascicular and fibre transverse sectional areas (TSA) within the semitendinosus muscle, outlines were traced on paper by means of a projection microscope, cut and weighed. This method is based on Delesse's principle according to which the area density of a profile on section is, on the average, directly proportional to the fractional volume of that component in the original solid body (Weibel, 1979).

A standard paper square of known area and weight was compared with the weight of an unknown area of an outline traced on the same grade of paper (110-115 g/m^2, Carson, France). A slide objective micrometer (Olympus, Japan) of 1 mm length and with 100 subdivisions was used to draw the standard paper square at the desired magnification on the projection microscope.

The outline of a whole stained transverse section of the muscle was traced directly from the slide on to paper and the area (referred to as actual TSA) was obtained by cutting and weighing. In larger muscles, the tracings of sections of pieces were oriented to form a mosaic of the whole muscle.

To count and trace any trait used in this histological analysis, a low magnification of the projection microscope was used for postnatal samples and a high magnification for antenatal samples. For the purpose of the present study, groups of fibres with a mixed population of presumptive or definite AL and AH fibres and circumscribed by large spaces or by definite perimysial connective tissue boundaries, as seen from 70 days gestation onwards, were defined as fascicles. For postnatal muscles, the sections were back projected at a magnification of 250 times and the fascicles and the fibres occupying the interior of a square of 125 x 125 mm (effective area 0.5 x 0.5 mm) were traced and counted. Several squares were traced from different regions using the same sampling method already described (*vide supra*) for establishing histochemical fibre type population density. The total area of the fascicles and fibres occupied within the squares were estimated by cutting and weighing. As both the total number of fascicles and the fibres within these squares were known, an estimate for fascicular area and the fibre area could be obtained. By using the difference between total area of the squares

and the total area of the fascicles, a percentage estimate for the perimysial connective tissue was obtained. Similarly, the difference between the total area of fascicles and the total area of fibres was used to obtain a percentage estimate for the endomysial connective tissue. The percentage estimates of perimysial and endomysial connective tissue components, so obtained, were used to calculate the total fascicular TSA and total fibre TSA respectively.

For antenatal muscles, the same procedure was followed but with following modifications:-
The fascicles and fibres were traced within squares of 100 x 100 mm (effective area 0.25 x 0.25 mm) and at a magnification of 100 in the projection microscope. For 60 days gestation, however, no separate estimates for endomysial and perimysial connective tissue components could be obtained. In muscle samples of the 60 and 70 days gestation periods, approximately 200 clearly observable presumptive AL fibres were traced from each section to assess the size changes involved during this growth period.

In all, 33 muscles were used in the study. About 100 sections were analysed, 400,000 fibres counted, 4000 fascicles and 60,000 fibres traced, cut and weighed in obtaining the data.

2.2.5 *ELECTROMYOGRAPHIC STUDY*

Two rams and one ewe were used in an electromyographic investigation of the possible functional significance of the observed variations in the proportions of AH and AL fibres within the semitendinosus muscle.

2.2.5.1 *Surgical procedure*

Each animal under investigation was anaesthetised using thiopentone sodium solution (Pentothal, Abbot). Using aseptic precautions, a skin incision was made parallel to the long

axis of the femur. The deep part of the semitendinosus muscle was uncovered caudal to the gluteobiceps muscle, taking care to avoid damage to its main blood and nerve supply. Three insulated, multistrand, flexible grade, stainless steel wires (0.08 mm diameter; Cooner Wire Company, Chatsworth), with their bared ends silver-soldered and bent into hooks to form recording electrodes, were implanted close to each other, distal to the hilus of the muscle at a depth of approximately 10 mm. Three other wires were similarly implanted caudolaterally at the same level in the superficial part of the muscle. The two recording sites were thus in areas already established as having, respectively, dense or sparse distributions of AL fibres, and they were approximately 25-35 mm apart in the muscle. The two groups of wires were passed loosely through the subcutaneous fascia of the thigh region of the sheep and their free ends were soldered on to a multi-junction block taped to the gluteal region. In each instance the sheep was fed *ad lib* and looked bright and active from the following day. There was no evidence of distress resulting from the operation and no signs of infection were observed.

2.2.5.2 *Recording procedure*

Each sheep was first used for recording one week after the preparatory operation. The action potentials from within the superficial and deep regions of the muscle were recorded simultaneously by using the two of the three leads in each group which gave the clearest recording and the same two leads were used in recording muscle activities throughout a single session of the experiment. The third lead served as the indifferent electrode. From the multi-junction block, each group of leads was connected *via* a screened multi-cable wire to a preamplifier (Type 122, Tektronix) and thence to one channel of a cathode ray oscilloscope (561B, Tektronix) with a four trace amplifier (3A74). Traces were recorded with a moving film camera (Shackman AC2/25). The two

differential preamplifiers were set to a frequency range (i.e. band pass) of 80 Hz to 10 KHz and an amplification of 1000X. The amplitude setting on the oscilloscope was 200 uV per division and the camera film speed 5 $cm\ s^{-1}$. Events during the experiment were marked on the film by switching a light emitting diode in the camera viewing field.

Recordings were taken with the animal standing, walking and bearing additional weight. The sheep was compelled to use the operated limb by having its contralateral hind limb held off the ground by an assistant. It was induced to bear more weight on the operated limb by having an assistant press on the back of the animal while raising the unoperated hind limb, to walk only on its hind limbs with an assistant holding its two forelimbs in a raised position above the ground, and to kick backward with its operated limb in response to pinching the skin in the metatarsal region of the limb.

The first recording session consisted of three series of trials in each of which each event was replicated five times. A further, similar set of recordings was obtained from each animal one week later.

At the end of each experiment, the sheep was killed to check the actual placement of the electrodes within the muscle, by making a skin incision in the thigh region and following each electrode into the muscle to confirm its depth and the correctness of its position.

2.2.6 *ANGIOGRAPHIC STUDIES*

The distribution of the main vessels within the semitendinosus muscle of the adult sheep was studied by using corrosion casting and angiographic techniques.

The corrosion cast was prepared by following the general guidelines of Tompsett (1970). The sheep was heparinized and then killed with a captive-bolt gun. The hind limb was removed by sawing through the hip bone, and the semitendinosus muscle was exposed. The neighbouring muscles of the thigh region were removed, to leave the semitendinosus muscle and its femoral arterial branch entering its distal part intact. The part of the limb distal to the hock joint was removed and the semitendinosus, with its associated bones, was left overnight. A solution of 1% saline was injected into the artery supplying the muscle to ensure that the vascular passage way was clear.

Batson's 17 anatomical resin compound (data sheet 105, Polysciences, 1978) was injected into the muscle on the following day. The final embedding mixture was composed of 10 ml of monomer base solution A (methyl methacrylate), 1.2 to 2 ml of catalyst B, 1 drop of Promoter C and ½ to 1 ml of red pigment.

2.2.6.1 *Injection of resin*

A polythene tube of internal diameter 0.38 mm and external diameter 1.09 mm (Portex) was cut obliquely, was slid into the remaining portion of the femoral artery entering the muscle and was tied in place. The resin was injected with moderate pressure using a 2 ml hypodermic syringe fitted *via* a 21" gauge needle to the polythene tubing. Leakages through other collateral branches of the main vessels were promptly stopped by using artery forceps. The resin was injected over a 90 to 120 s period, after which the colour of the resin was apparent beneath the surface of the muscle.

2.2.6.2 *Maceration*

The limb was left overnight for the resin to set in the muscle. The muscle was then separated from the limb and

macerated by suspending it on a wire gauze in a solution of potassium hydroxide (340g ℓ^{-1} of water) contained in a small stainless steel tank. The tank itself was left for three days in a water bath maintained at a temperature of 50°C by a tempette (Techne).

2.2.6.3 *Washing and trimming of the cast*

The corrosion cast, lying on the wire gauze, was lifted out of the maceration tank and washed in water by means of rubber tubing from a water tap connected *via* a plastic syringe fitted with a hypodermic needle, to remove any organic material remaining. By using fine tweezers, the cast was trimmed off to show the main vessel and its secondary and tertiary branches.

2.2.6.4 *Preparation of angiogram*

The venous drainage of the semitendinosus muscle was demonstrated radiographically in a sheep, heparinized prior to it being killed. The fresh muscle, with its attachments to the hind limb intact, was exposed and the vein leaving the hilus cannulated and injected with meglumine lothalmate (Conroy 280), a water-soluble radio-opaque fluid. Because of the resistance of the venous valves, the injection and radiography proceeded in three stages using approximately 1.5 ml of the fluid initially and then an additional 1 ml each time to create a progressive back flow through the venous valves.

2.2.7 *STATISTICAL METHODS*

The allometric relationships estimated between variables were calculated for antenatal, postnatal and ante- plus postnatal periods using double logarithmic regressions (Table 2). For any regression obtained, x was the independent variable, y the dependent variable, s_b the standard error and a the intercept.

The various relationships were used to suggest changes in total fibre number, in total fascicular number, in fibre number within fascicles, in the growth of histochemical fibre types and connective tissue components, and in the relative growth and shape of the semitendinosus muscle of the sheep, during the periods investigated. A b value of 1 was used to test a null hypothesis within each pair of variables considered, except for isometric growth changes involving the shape of the muscle where a b value of 0.667 was used to test a 2/3 power relationship between area and weight of the muscle. Predictions for the total fibre number at 60 days gestation, birth and adulthood were made using each possible regression between mean fibre area (x) and total fibre area (y). A confidence limit at a 95% level was set in each case (Table 3). When there was no overlap in these ranges between any two stages, the effect was considered significant.

Estimates of the mean fascicular and mean fibre TSAs were obtained by weighing, together, the traced fascicles or fibres sampled for each muscle and then by dividing the calculated total area by the number of fascicles or fibres included in the sample. Because the fascicles and fibres were not, therefore, weighed individually, an estimate of standard error for the mean could not be obtained.

2.3 RESULTS

2.3.1 *ATTACHMENTS AND ORIENTATION OF THE SEMITENDINOSUS MUSCLE OF THE SHEEP*

Figure 1 shows the attachments of the semitendinosus muscle of the sheep. The muscle originates from the lateral ischiatic tuber and runs caudal to the femur to insert on to the crest of the tibia. The attachment of the muscle to the

gluteobiceps near its origin and its fascial connections to the gastrocnemius muscle are not shown. The semitendinosus muscle can, thus, extend the hip joint and flex the stifle joint. Figure 2 shows a transverse section of the thigh of the sheep and indicates the relative positions of various muscles at the level at which the semitendinosus muscle was transected for histochemical studies. The blood vessels and nerves entered the semitendinosus muscle in its craniolateral aspect (not shown).

2.3.2 *MORPHOGENESIS AND DIFFERENTIATION OF MYOSIN ATPase FIBRE TYPES*

Figure 3 shows the morphological and histochemical changes occurring within the semitendinosus muscle between 60 and 70 days gestation. Two types of fibres are observed within the muscle at 60 days gestation. With PAS-hematoxylin staining, many large fibres with central nuclei are seen to form clusters (Fig. 3a). With alkali-stabile ATPase staining, these large fibres have pale centres which are presumably unstained, nuclei or the spaces between nuclei, and have a darker periphery (Fig. 3b). Some of these larger fibres are closely associated with smaller sized fibres (Figs. 3a, 3b). By 70 days, the large fibres are more separated from each other and surrounded by more small sized fibres than previously (Fig. 3c). Alkali-stabile myosin ATPase staining does not, however, clearly distinguish the two types of fibres (Fig. 3c). Each aggregated group of cells, with large sized fibres located in the midst of many small sized fibres as seen in 70 days gestation, is defined as a fascicle. Fig. 5 shows sections of the semitendinosus muscles, stained for alkali-stabile myosin ATPase, from 80 days gestation (5a,b), 100 days gestation (5c,d) and adult (5e,f) stages in the sheep. At 80 days gestation, the fibres seen within individual fascicles have differentiated into distinct pale and dark types with alkali-stabile myosin ATPase

staining. The pale fibres are identified as ATPase low (AL) and the fibres which appear homogeneously dark by this reaction are identified as ATPase high (AH) fibres. These two distinct myosin ATPase fibre types are observed within the muscle from the eightieth day of gestation onwards.

During the period between 60 to 70 days gestation, the presumptive AL fibres actually decrease in size (Fig. 3b,c). Measurement of a few selected presumptive AL fibres from myosin ATPase stained sections, indicated that the transverse area of these cells at 60 days is approximately 325 μm^2 while at 70 days it is approximately 150 μm^2

2.3.3 *ATPase LOW FIBRE DISTRIBUTION PATTERN WITHIN THE SEMITENDINOSUS MUSCLE*

The ATPase low (AL) fibre population density patterns of the whole muscle at fetal ages 80, 100, 140 days and those of young and adult sheep are represented schematically in Figure 6. This general scheme is based on data from at least three sheep at each stage, and groups the percentage of myosin ATPase low fibres into four categories (3 - 6%, 7 - 9%, 10 - 19%, 20 - 30%). The highest density of AL fibres is observed in the craniomedial aspect (the AL dense area) of the muscle and the lowest density of AL fibres is seen in the caudolateral aspect (the AL sparse area). Representative areas of AL sparse and AL dense regions have been shown at a low magnification in sections of the semitendinosus muscle of a sheep of 30 kg bodyweight (Fig.4a, 4b) and at a higher power magnification in those of 80 days gestation, 100 days gestation and adult (Figs.5a,b,c,d,e,f). Figure 7 shows the relationship between log body weight and ATPase low fibre type density population, in AL dense and AL sparse areas within the semitendinosus muscle of the sheep from 80 days gestation to adulthood. The percentage of AL fibres in

the AL sparse area is within the range of approximately 3% to 6% throughout development (Table 1b, Figs.6 & 7). However, the AL fibre percentage in the AL dense area increases from approximately 10% in the fetal stages to about 20-30% in the postnatal stages (Table 1B, Figs.6-7). The AL sparse areas are seen peripherally in both caudolateral and caudomedial regions of the fetal muscle but are restricted to the caudolateral border in the postnatal muscles. There is a shift in the AL dense area to a more central location during development. A study of the adult semitendinosus muscle proximal to the tendinous intersection in three muscles showed a similar pattern of fibre type distribution to that found distally.

2.3.4 *QUANTITATIVE GROWTH ANALYSIS*

Table 1a summarises the data obtained for the age, sex, and body weight of the animals and the weight of the semitendinosus muscle used. It also shows the quantitative measurements obtained for actual TSA, total fascicular TSA, total fibre TSA, mean fascicular TSA and mean fibre TSA of each muscle analysed. Table 1b shows the data related to the quantitative measurements obtained on fibres reacted histochemically to myosin ATPase classified as high (AH) or low (AL) reacting. The relevant data from these two tables (1a, 1b) have been analysed using logarithmic regressions to obtain information related to
a) growth in fascicles, fibres, perimysium and endomysium
b) relative growth of the semitendinosus muscle and
c) dimensional changes including the shape and mechanics of the muscle (Table 2). The relationship between each pair of variables shown in Table 2, have also been represented graphically (Figs.8-19).

2.3.4.1 *Fascicular growth*

Fascicular growth was analysed using the regression of mean

fascicular TSA on total fascicular TSA (Table 2B, Fig.8). The growth in the mean fascicular TSA is significantly lower than that of the total fascicular TSA of the muscle antenatally (b= 1.25, significantly greater than 1, $P < 0.05$). No significant changes are shown postnatally (b= 0.74, not significantly less than 1, $P > 0.05$). This suggests that there is an increase in total fascicular number antenatally while it may even decrease postnatally.

2.3.4.2 *Growth in fibre transverse sectional area*

The growth in the fibre TSA within both the whole muscle and individual fascicles have been analysed (Table 2,A,C, Figs 9,10). The regression of mean fibre TSA on total fibre TSA shows that the mean fibre TSA grows at a significantly lower rate than the growth of total fibre TSA antenatally(b= 1.70, significantly greater than 1, $P < 0.01$). Postnatally, no significant change is observed (b= 0.82, not significantly greater than 1, $P > 0.05$). Antenatally, therefore, there is an increase in total fibre number, while the number is possibly constant postnatally. The regression of mean fibre TSA on mean fascicular TSA, on the other hand, shows a direct proportionate relationship between these two variables (b= 1.20, not significantly different from 1, $P > 0.05$). This suggests that the fibre number within individual fascicles does not change throughout the growth period studied.

The growth in TSA of the AL fibres is not significantly different from that of the AH fibres in either AL sparse or AL dense areas (Table 2D,E, Figs.11 & 12).

2.3.4.3 *Changes in connective tissue*

Large areas of perimysial connective tissue are present during early fetal life. The regression of actual TSA on

total fascicular TSA (Table 2K, Fig. 17) shows that there is a marked decrease in extrafascicular connective tissue antenatally (b= 1.28, significantly greater than 1, $P < 0.001$) but no significant changes are observed postnatally (b= 1.00, not significantly different from 1, $P > 0.05$). The reduction in endomysial connective tissue (Table 2J, Fig 18) on the other hand is less marked, although this effect is significant during the whole period (b= 1.05, significantly greater than 1, $P < 0.05$). The regression of actual TSA on total fibre TSA also shows that the proportionate changes in connective tissue within the whole muscle is largely dependent on extra-fascicular connective tissue (b= 1.29, significantly greater than 1, $P < 0.001$) than its endomysial components (Table 2L, Fig. 19).

The changes in connective tissue proportions and in fascicular and fibre number during antenatal growth suggests that the formation of new fascicles takes place with a concurrent significant reduction in perimysial connective tissue. This is in preference to an addition of new fibres within existing individual fascicles, for which no significant proportionate changes occur antenatally.

2.3.4.4 *Changes in muscle shape and mechanics*

The actual TSA, total fascicular TSA and total fibre TSA of the muscle and the weight of the muscle show changes antenatally and postnatally which are more proportionate (b is more closely equal to 0.667) when the connective tissue component of the dependent variable is reduced (Table 2G,H,I, Figs. 13,14 & 15). Also the growth rate in the actual TSA is significantly less (b= 0.56, $P < 0.001$), the total fascicular TSA is proportionate (b= 0.67, $P > 0.05$), and the total fibre TSA is significantly greater (b= 0.73, $P < 0.05$) than 0.667, relative to the growth rate of muscle weight throughout

development. The disproportionate growth between the actual TSA and the weight of the muscle appears therefore to be due to a reduction in the connective tissue components, rather than due to changes in the components within the muscle fibres. Rather than a decrease in the strength of the muscle, relative to its weight, as might be inferred from its actual TSA (Table 2G), the strength, as estimated by the total TSA of its myofibres, appears to increase (Table 2I).

The muscle grows faster during early antenatal stages ($b = 1.11$, significantly different from 1, $P < 0.05$) relative to body weight (Table 2F, Fig.16) than postnatally ($b = 0.93$, not significantly less than 1, $P > 0.05$) where growth is maintained at a more proportionate rate.

2.3.4.5 *Estimation of total fibre number*

Table 3 gives predictions of total fibre number in the semitendinosus muscle, at the level of transection, at 60 days of gestation, birth and adulthood. Regressions of the total fibre TSA on the mean fibre TSA for the antenatal, ante- and postnatal and postnatal periods (Table 2A) have been used in calculating the estimates. When the equation involving both antenatal and postnatal periods is used, there is a significant increase in fibre number for both growth stages. However, when the postnatal equation is used, no significant change in fibre number from birth to adulthood is shown. Postnatally, the variability between sheep is too high to establish a definite pattern of changes in fibre number. Antenatally, however, whichever equation is used, there is approximately a three-fold increase in fibre number between 60 days of gestation and birth.

2.3.5 *ELECTROMYOGRAPHIC STUDY*

Simultaneous electromyographic recordings from the superficial

(corresponding to AL sparse area) and the deep (AL dense area) parts of the semitendinosus muscle of a sheep obtained during various events in a single recording session are shown in Figure 20. The upper tracing shows the activity recorded through the electrodes implanted in the superficial part of the muscle and the lower tracing is from that of the deep part. No muscle activity was apparent in the operated limb when the animal was standing on its own (a), but when the operated limb was forced to support weight, there was activity in the deep part but not in the superficial part (b). When more powerful stimuli to muscular effort were progressively applied, activity was shown in both superficial and deep parts (c,d,e,). The results were consistent in all three animals used in the experiment.

2.3.6 *ANGIOGRAPHIC STUDIES*

The corrosion casts of the part distal to the tendinous intersection of the semitendinosus muscle is shown in Figure 21 b,c. A diagrammatic representation of the branching pattern in AL dense and AL sparse areas is shown in Fig.21d. It is apparent that the AL dense area being the most aerobic part of the muscle is situated in proximity to the main arterial branches which are larger and closely placed. On the other hand, only a few widely placed large vessels enter the AL sparse area. The AL fibres, which are aerobically active, are thus accumulated close to an abundant and immediate source of nutrients and oxygen. The angiogram showing the venous drainage (Fig. 21a) also indicates that the vessels become narrower and wider apart as they approach the superficial part from the deeper area of the muscle.

2.4 DISCUSSION

2.4.1 *ANALYTICAL METHODS*

The developmental stages of both the fetuses and postnatal

sheep were chosen in such a way that the successive growth increases in body weight could be effectively represented in statistical logarithmic analysis. An additional stage between 140 days gestation and the 5 weeks early postnatal period would be desirable. However, this material was not available. All the postnatal animals, except for the three at 5 weeks of age, were females. The influence of sex on the measurements made is expected to be small (Lohse, 1973). Five of the 33 semitendinosus muscles used (2.2.2.2) had to be frozen and thawed before cryostat sectioning. These samples were not sufficient to analyse, statistically, whether there was a significant change observable in fibre size of these twice frozen samples, relative to the rest of the samples which were frozen only once before cryostat sectioning. Errors such as shrinkage, prerigor and thaw contracture, variation in response to treatment by different fibre types, and the possible differences between fresh samples and those stored frozen before processing were inherent in the techniques used. These technical variables, together with the actual variation between individuals, contribute to the large overall variation observed (Table 1A). There is, however, little reason to suppose that this variation can bring about an incorrect conclusion from the statistical analysis.

Problems of measuring fibre size have been discussed by various workers (Hegarty & Naude, 1970; Goldspink, Gelder, Clapison & Overfield, 1973; Levine & Hegarty, 1977; Clancy & Herlihy, 1978). Hegarty & Naude (*loc. cit.*) suggested that the peripheral fibres of muscles fixed postrigor are smaller than fixed prerigor. Goldspink *et al.* (1973) indicated, on the other hand, that the type of fixative used is more important in measuring fibre size than the effects of rigor. Gunn (1976) showed that storage of muscles by freezing did

not alter the subsequent TSA of his sections. It is evident that standardization of technique is necessary to reduce errors in measuring fibre size. This was attempted, as much as possible, in the present study. Errors in the measurement of individual fibres were minimized by the estimation of the mean TSA of at least 2000 fibres for each muscle, and the avoidance of fixatives before sectioning.

In the present study, a paper cutting and weighing method was employed to measure total fascicular, total fibre, mean fascicular and mean fibre TSAs of the muscle. Planimetry, line integration or point counting can be used to estimate structure parameters (Weibel, 1979). In planimetry, a direct estimation of the relative areas occupied by profiles is made; in line integration, a test line is applied to measure the intercept length; and in point counting, a random point grid or a systematic lattice is used. Planimetry can be employed by using a planimeter, by circle fitting, or by tracing the profiles, cutting them out and weighing them. Amongst these methods, the cut and weigh method has been shown to have the lowest error although it is the most time consuming (Weibel, 1979). However, in the present study a projection microscope enabled direct tracings at various magnifications of the necessary profiles of stained sections. The sampling of large numbers of areas was therefore easier.

2.4.2 *GROWTH OF FIBRE AND FASCICULAR TRANSVERSE SECTIONAL AREAS*

As discussed previously (2.1.), various reports suggest that the postnatal growth of muscle is due to hypertrophy of fibres present at birth rather than an increase in number of fibres (Maccallum, 1898; Staun, 1963; Enesco & Puddy, 1964; Rowe & Goldspink, 1969; Davies, 1972; Tan & Davies, 1980). However, there are also reports suggesting an increase (Rayne & Crawford, 1975; Gunn, 1979) or a decrease (Layman,

Hegarty & Swan, 1980) in fibre number postnatally. The present study suggests that fibres increase in number during antenatal growth, but provides no evidence that the number of fibres change postnatally. There is a wide variability in fibre size amongst individuals at any particular stage of development (Table 1A) and the estimates of total fibre number for a muscle at any stage depend on the developmental period used in calculating the estimate (Table 3). A wide variability in fibre size in the semitendinosus muscle during postnatal growth, between individuals, has also been observed by Suzuki (1971) in the sheep and Gunn (1979) in the dog and horse. Joubert (1956b) noticed a similarly wide range in fibre size in three different muscles in newborn lambs.

The body weight of the fetal sheep increases approximately 50 times and the mean fibre TSA of the semitendinosus muscle six times, between 60 and 140 days of gestation (Table 1A). A re-analysis of the data of Joubert (1955) for 31 fetuses between 60 days and 129 days, using a double logarithmic regression between the mean fibre TSA and the area of individual muscles, estimated by using a product of their widths and depths, suggested that the longissimus ($b = 1.67$, $s_b = 0.31$) has a smaller antenatal increase in fibre number when compared with the gastrocnemius ($b = 2.20$, $s_b = 0.22$) and rectus femoris ($b = 2.11$, $s_b = 0.35$). The mean fibre area of these three muscles shows a three-fold increase during this period of growth (Joubert *loc. cit.*). The fibre area in the semitendinosus muscle in the sheep also shows a three-fold increase over an equivalent period in the present study. The two studies show a large difference in absolute fibre area, presumably due to the different tissue preparation techniques employed.

As observed in the present study, although the growth ranges in the antenatal sheep are appropriate for studies involving

changes in the fibre number, postnatally the fibre TSA increases only about three-fold and body weight only twenty-fold. A large sample size would to some extent compensate for this small growth range. The pig, with a hundred-fold increase in body weight postnatally (Davies, 1974) is a better model to test the constancy of postnatal muscle fibre number.

In a study involving growth related changes in connective tissue, the fibrous architecture of muscle should be given due consideration. The semitendinosus muscle, being a strap muscle, should not show much variation in the distribution of connective tissue along its length. Yet, it is possible that the most amount of connective tissue is found at the musculotendinous junction with the least amount being deposited at the widest region of the belly. In the present study variation between samples was avoided by obtaining all samples from a fixed region of the muscle in between these two extremes.

Spaces in between myofibres and those outside the fascicles had been considered as connective tissue components, in the present study, to estimate their proportions. But these spaces potentially include, in addition to the normal connective tissue components, sectioning and staining artefacts and other extra-myofibre cells like antenatal myoblasts and postnatal satellite cells. The changes in connective tissue components were studied using the same sections of muscles stained histochemically by the alkali-stabile myosin ATPase technique to examine growth changes in the relevant histochemical fibre types. This technique, however, does not clearly demonstrate the connective tissue components and their boundaries and also does not stain the proliferative myogenic cells. The artefact which was inherent to the technique could have been greater in muscles frozen twice than those frozen once but the significance of this effect could not be ascertained for reasons mentioned in 2.4.1. However, visual examination did not reveal any

obvious differences between sections frozen once and those frozen twice. The overall effect of measuring potential spaces as connective tissue would be that the actual amount of connective tissue would have been overestimated and the total fascicular and fibre areas underestimated. The effect of this overestimation of connective tissue which occurred in all the samples throughout the whole period of investigation is thought to be negligible since this study is concerned with connective tissue proportions.

The present study suggests that there is a significant proportionate reduction in perimysial connective tissue during antenatal growth, while postnatally the proportion appears to be constant. The endomysial connective tissue, on the other hand, shows fewer changes than that of the perimysial connective tissue. These results on postnatal growth of connective tissue elements agree with those of Enesco & Puddy (1964) and Bridge & Allbrook (1970). The present study has extended this work by estimating the size and number of fascicles during their entire development. The number of fascicles increases antenatally while no significant changes occur postnatally. It appears that there is no increase in fibre number within a fascicle once it is enclosed by perimysium. The fascicles, on the other hand, have the potential to grow in number within the muscle antenatally, with a corresponding proportionate decrease in perimysium. This ability is lost postnatally. Therefore, an antenatal increase in number of fibres can be expected to be accompanied by newly forming fascicles rather than an increase in number within already existing fascicles. This observation is the first to suggest a pattern of fibre and fascicular growth within a muscle.

A collagenous matrix is shown to be necessary for 'normal' muscle differentiation in tissue culture (Hauschka & Kŏnigsberg, 1966). The formation of a basal lamina and

associated collagenous connective tissue has been considered to serve as a mechanism regulating the extent of fusion between myofibres and mononucleated myoblasts (Fischman, 1970). An excessive deposition of collagen in the endomysium and perimysium has been shown to be associated with various forms of muscular diseases (Adams, Denny-Brown & Pearson, 1962; Pearson, 1963; Duance, Stephens, Dunn, Bailey & Dubowitz, 1980). These studies and the present one indicate the importance of the connective tissue framework in controlling normal muscle growth.

2.4.3 *DIFFERENTIATION OF MUSCLE FIBRE TYPES*

Wirsen & Larsson (1964) considered that three generations of myotubes occur in the thoracic muscles of fetal mice during prenatal growth between sixteen and nineteen days. A histochemical technique using phosphorylase activity was used in their studies. This was criticised by Dubowitz (1970) who considered that their findings were due to variable staining intensities of the reaction depending on the number of polysaccharide units present in the synthetic glycogen formed. Dubowitz (1965) suggested that early fetal fibres originate as a common pool, differentiating later into adult muscle fibre types. His observations were based on studies using human limb and trunk muscles. However, Ashmore, Robinson, Rattray & Doerr (1972), using the semitendinosus muscle of the sheep, demonstrated that the development of muscle fibres is biphasic. A primary generation of cells is early to develop, and a secondary generation of cells originates later in the antenatal period. The postnatal fibre types evolve from these two basic types. The biphasic theory of development of skeletal muscles has been supported by Swatland & Cassens (1973) for fetal porcine muscles. The myoblasts which are destined to form secondary muscle fibres come to lie on the surface of the existing primary fetal myotubes. Fusion of such

myoblasts results in the formation of secondary fetal myofibres. Autoradiographic studies in chick muscles by Kikuchi (1971), and the present observations, support this theory.

The fibres of the primary generation are larger at sixty days than at seventy days (Figs 3, a,b,c). The photomicrographs of these fibres in developing limb muscles published by Ashmore *et al.* (1972) for the sheep and Beermann *et al.* (1978) for the pig show a similar size decrease, although no reference was made to it. Bridge & Allbrook (1970) suggest that this decrease in fibre size may be caused by fixation artefact, shrinkage due to loss of fluid from fibres as they mature or smaller fibres splitting off the larger ones. This unusual observation of a decrease in size during normal growth is worthy of further investigation.

Using the semitendinosus muscle of the pig, Beermann *et al.* (1978) noticed that fibres in the superficial and deep portions of the muscle show differences in histochemical differentiation. When an acid-stabile myosin ATPase technique was used, only the primary fibres in the deep region, and not the superficial region, are positive at 60 days. Again, only in the deep region and not the superficial region are some secondary fibres later changed to a positive reaction. With alkali-stabile ATPase staining, all fibres in the superficial region are positive at 90 days, whereas in the deep region primary fibres are negative. The present study did not use the acid-stabile myosin ATPase technique. However, both superficial and deep parts differentiate into alkali-stabile myosin ATPase fibre types in a similar manner, beginning 80 days of gestation in the sheep.

2.4.4 *MORPHOGENESIS OF ISOENZYMES OF MYOSIN*

Two suggestions have been made for the morphogenesis of

isoenzymes of fast and slow myosin types. Masaki and Yoshizaki (1974), using a fluorescein-labelled antibody technique on the pectoralis muscle of the chicken, and Gauthier, Lowey & Hobbs (1978), using an immunocytochemical technique on the diaphragm muscle of the rat, showed that fast and slow isoenzymes coexist in all early developing muscle fibres. Rubinstein, Pepe & Holtzer (1977) used immunodiffusion and electrophoretic techniques on the developing pectoralis and anterior latissimus dorsi muscles of the chicken and suggested a second scheme of isoenzyme differentiation in which only fast myosin is present during early development. They suggested that the synthesis of slow myosin is dependent upon exogenous factors such as innervation, while fast myosin formation is endogenous. Either of these suggestions could be appropriate to the present histochemical observations. Since the myosin ATPase reaction reveals a pattern of fetal development consistent with adult differentiation, it appears useful even though the nature of the enzymatic change in fetal AL fibres is in doubt.

2.4.5. *THE SIGNIFICANCE OF FIBRE TYPE DISTRIBUTION WITHIN THE SEMITENDINOSUS MUSCLE*

Previous electromyographic studies of the semitendinosus muscle in the dog (Tokuriki, 1973; Wentink, 1976), the horse (Wentink, 1978) and the cat (Wetzel, Atwater & Stuart, 1976) have shown activity to be confined almost entirely to the retraction phase during locomotion. These studies did not incorporate histochemical investigations. Conversely, Gunn (1978) described a histochemical division within the semitendinosus muscle in both the horse and the dog, with the AL dense part of the muscle closest to the limb axis. He suggested a postural role for this region although no electromyographic studies were undertaken.

Other electromyographic studies on crural muscles of the cat (Smith, Edgerton, Betts & Collatos, 1977; Walmsley, Hodgson & Burke, 1978) suggest that a division of labour exists between fast and slow muscles, that they are recruited during different types and speeds of limb movement, and that the slow muscles are used more readily for postural function and the fast muscles are active when more power is required.

Gollnick *et al*. (1974), using the quadriceps femoris muscle of the human and Sullivan & Armstrong (1978), the forelimb and hindlimb muscles of the rat, showed glycogen depletion during various activities. There was a selective depletion within muscles suggesting a differential recruitment of muscle fibres. According to Sullivan & Armstrong (1978), as the activity of muscles becomes greater, there is a progressively greater reliance on fibres with lower oxidative capacities, situated in the more peripheral muscles or in the more peripheral areas of a single muscle. A combined histochemical and electromyographic study made by Herring, Grimm & Grimm (1979) showed that the electrical activity in different portions of the masseter muscle of miniature pig varies systematically during the various phases of mastication. The rostral portion (which had 35% of AL fibres) is used throughout the masticatory contraction whereas the caudal portion (23% AL fibres) is used only during a brief portion of the cycle.

In the present study, the superficial and deep parts of the semitendinosus muscle were used to obtain simultaneous electromyographic recordings in order to test for a functional difference between the areas confirmed as having the lowest and the highest AL fibre type population densities in this muscle. Although the fibres in the two regions run parallel to one another, no gross division occurs between the two regions. Even though the two regions used for recordings

were about 25-35 mm apart from each other in the muscle, there is no evidence that these two regions contained fibres belonging to separate motor units. The results of the present study are based on qualitative responses which need not be conclusive (Basmajian, 1974). Basmajian (*loc. cit.*) states, however, that two bellies or two heads in parallel within a muscle may show different electromyographic activities. Gans & Gorniak (1980) discussed the repeatability and limitations of electromyographic recordings and indicated that electromyographic recordings from physiologically and histochemically different fascicles of compound muscles can show differences in activity level. They also concluded that the electromyographic recordings obtained from reasonably standardized fine wire electrodes and standardized recording equipment and well-defined sites in major subdivisions of muscles yield repeatable observations and permit predictions for equivalent events in the same muscle in other specimens. In the present study, motor units of the slow, myosin ATPase low and the fast, myosin ATPase high fibres, as shown by Kugelberg (1973) and Herring, Grimm & Grimm (1979), appear to have been recruited in a selective manner during different activities. A functional significance for a regional difference in the myosin ATPase histochemical fibre type density within a muscle can therefore be postulated. The region with highest AL fibre type population density becomes active readily in response to postural needs while the region with lowest AL fibre type population density is used only intermittently as required during propulsive activity.

Fibres located closer to the joints over which a muscle acts have a lower torque about these pivots. Therefore, the AL fibres lying close to the pivot suffer a mechanical disadvantage relative to the majority of the AH fibres. The explanation for the heterogeneous distribution of AL fibres

may involve nutrition and energetics rather than mechanics. First, AL fibres are oxidative and may be expected to be most numerous in immediate proximity to the blood vessel bringing their nutrient and oxygen supply. Electromyographic recordings in the present study and that of Herring *et al.* (1979) suggest that the regions with high proportions of AL fibres are active in almost every function of a muscle and this would require them to be in need of an abundant and continuous supply of nutrients and oxygen. Secondly, during the production of a decelerating force, energy is transformed into heat (Feng, 1932; Carlson & Wilkie, 1974). The production of heat rather than mechanical work must therefore be a feature of the posturally active AL fibres. Not only metabolic waste, therefore, but also heat must be dissipated into adjoining blood vessels.

In earlier studies, Romanul (1965) demonstrated the capillary supply of individual muscle fibres within the gastrocnemius, plantaris and soleus muscles of the rabbit and showed that an aerobic fibre is associated with a larger number of capillaries than an anaerobic fibre. Myrhage & Eriksson (1980) showed that the 'slow' soleus and medial heads of the gastrocnemius muscles of the cat have a denser vascular network with shorter average distance between the individual groups of secondary vessels compared to the lateral head of gastrocnemius, biceps femoris and tenuissimus muscles. The appearance of the corrosion cast of the semitendinosus muscle of the sheep (Fig 21c) suggests that in the AL dense area, the vascular branches are thicker and more closely placed than in the AL sparse area, where the vessels are narrower and wider apart. It may be possible that AL fibres occupy the part of the muscle where there is provision for ample blood supply to meet their nutritional and heat dissipation requirements. The semitendinosus muscle of the sheep was used in the present study for corrosion casting as this

muscle was already employed to study various other aspects. However, this muscle in the sheep is not suitable to carry out an extensive study on vascular patterns and the dynamics of blood flow between the AL dense and AL sparse areas within the muscle, because many AH fibres are also aerobic; they show a strong reaction for the aerobic metabolic enzyme succinate dehydrogenase (Fig.22).

Previous studies indicate that blood flow patterns differ remarkably between slow and fast muscles. In the cat, the resting blood flow of the slow contracting soleus is approximately four times higher than that of the fast contracting medial head of the gastrocnemius, cranial tibial and digital extensor muscles (Hilton, Jeffries & Vrbová 1970). During contraction, the blood flow through the fast muscles increases about seven times, while the change in the soleus muscle is much less. Hilton (1972) deduced that inorganic phosphate, released by fast muscles much more than the slow muscles during contraction, is responsible for the greater functional vasodilation in these muscles. It is possible, therefore, that even within one muscle with distinct regions of ATPase low oxidative and ATP high glycolytic fibre types, marked differences in the vascular pattern and blood flow might be observed. The semitendinosus muscle of the pig should be suitable for such a study because it has visually distinct 'red' and 'white' fibre areas which run parallel to each other for the length of the muscle and have the same origin and insertion (Tarrant, Hegarty & McLoughlin, 1972). Techniques involving radioactive microspheres (Ramsey & Donner, 1980) can be used to study the blood flow pattern between the red and white regions. In the same model, corrosion casting to show both arterial and capillary distribution in the two regions could be carried out. The validity of the hypothesis advanced in the present study for the preferential accumulation of AL fibres in a definite

region of a muscle could thus be tested. Electromyography could also be employed.

2.4.6. *CHANGES IN MYOSIN ATPase LOW FIBRE PROPORTION*

An increase in the percentage of AL fibres with the postnatal increase in body weight had been reported by Cardinet, Tunell & Fedde (1971) in the pectineus muscle of the dog, by Karpati & Engel (1967) in the soleus muscle of the rat and cat, by Davies (1972) in the costal part of the diaphragm and in the thorocolumbar portion of the longissimus muscle of the pig and by Gunn (1975) in the semitendinosus and pectoralis muscles of the dog. Kugelberg (1976), working on the soleus muscle of the rat and Swatland (1977), using the longissimus muscle of the pig, also reported a growth-related shift in ATPase activity. Holmes & Ashmore (1972) saw no significant increase in the percentage of AL fibres in biopsies of the semitendinosus muscle of cattle, but neither was such a change seen in the superficial part of this muscle in the present study. The observation of Swatland (1978) that the proportion of AL fibres decreases postnatally in the dark red area of the vastus medialis muscle of the pig does not concur with the above studies. An increase in the percentage of AL fibres is theoretically necessary postnatally if both isometric growth and postural ability are to be maintained (Davies, 1974). Thus it is the deeper postural part of the muscle that changes its AL fibre proportion during growth. Wherever possible, entire sections of muscle should be analysed if overall changes involving histochemical fibre types are to be observed. Biopsies are liable to give insufficient information.

2.4.7. *DEVELOPMENT OF FIBRE TYPE PATTERNS IN THE FETUS*

In the present study, regions of different proportions of ATPase low and ATPase high fibre types, within the

semitendinosus muscle of the sheep, are seen from the 80 days gestation period onwards. There is an increase in the percentage of ATPase low fibres in the deep part of the muscle. Ashmore *et al*. (1972) noticed an antenatal increase in the ratio of AH fibres relative to AL fibres within the semitendinosus muscle of the sheep but they did not recognise differences between superficial and deep parts of the muscle. Beermann *et al*. (1978), on the other hand, carried out a study on the semitendinosus muscle of the pig independent of that of the present study of the sheep (Sivachelvan, 1979) and observed an antenatal increase in the AL fibres in the deep but not in the superficial part of the muscle. The observations in the present study concur with those of Beermann *et al*. (1978).

Activity of fetal limb muscles has been recorded in studies such as those of Änggård & Ottoson (1963) in the sheep and Bekoff, Stein & Hamburger (1975) in the chicken. Änggård & Ottoson (1963) noticed that electrical and mechanical responses in the gastrocnemius muscle increase with increasing fetal age and that these changes coincide with the myelination of nerve fibres. Bekoff *et al*. (1975) recorded activities of the gastrocnemius, peroneus and tibialis muscles electromyographically and noticed that the flexor and extensor muscles are activated at different times while the activity of the synergists occur at the same time. They suggested that the neural pattern generating circuits for selective activation of muscles are established in the central nervous system without reliance on functional reflexes. The activities in the limb muscles of an 'amniotic aquanaut' cannot be postural. The gravitational force acting on the fetal body is largely opposed by a force of buoyancy. Thus, a precocious histochemical fibre type differentiation and a regional distribution of the fibres within a fetal limb muscle are not due to a special mechanical

requirement. Both the fibre type differentiation and the AL fibre type distribution pattern suggests an antenatal genetic influence on the semitendinosus muscle for its functional requirement at birth.

3.0 PART 2: EXPERIMENTAL INDUCTION OF RELATIVE GROWTH CHANGES IN THE MUSCULOSKELETAL SYSTEM OF THE SHEEP.

3.1 INTRODUCTION

A muscle may be altered in size by experimental conditions. Increased work load and consequent increased size has been produced by surgical incapacitation of synergists by using denervation, tenotomy or removal methods by Crawford (1961) in the cranial tibial muscle of the rabbit, by Denny-Brown (1961) in the soleus muscle of the cat, by Reitsma (1969) in the rectus femoris, plantaris and soleus muscles of the rat, by Binkhorst (1969) in the plantaris muscle of the rat, by Jablecki & Kaufman (1973) in the soleus and plantaris muscles of the rat and by Vaughan & Goldspink (1979) and Hofmann (1980) in the soleus muscle of the mouse. Crawford (*loc. cit.*) noticed that the increase in size of the cranial tibial muscle involves only the girth of the muscle and not its length. Denny-Brown (*loc. cit.*) observed that the fibre diameter of the hypertrophied soleus muscle varies between 51 to 93 μm while that of the control soleus muscle ranges between 38 to 87 μm. An increase in both the myofibrillar and sarcoplasmic components within hypertrophied fibres has also been suggested in his studies. Reitsma (*loc. cit.*) observed evidence for fibre splitting, an increase in the number of nuclei, the formation of new capillaries and an increase in connective tissue within muscles hypertrophied by surgical removal of their synergists. Gutmann & Hajek (1971) induced hypertrophy in the extensor digitorum longus muscle of the rat by tenotomy of its synergist, the cranial tibial muscle, and showed that the hypertrophied muscle has a prolongation of contraction time, a decrease in ATPase activity, and a relative increase of sarcoplasm but a decrease of contractile proteins. Vaughan & Goldspink (1979) noticed fibre splitting within the soleus muscle hypertrophied by tenotomy of its synergists. In

contrast to these experiments involving muscle hypertrophy, disuse of a muscle following its denervation, tenotomy or immobilization was used by Knowlton & Hines (1936) to demonstrate size decrease in the gastrocnemius, soleus and plantaris muscles of the rat, by Goldberg & Goodman (1969) and Rifenberick & Max (1974) in the soleus and plantaris muscles of the rat and Gauthier & Dunn (1973) in the semitendinosus muscle of the rat.

Many denervation and immobilization studies, however, indicate that either an increase or a decrease in size can be produced in a particular muscle depending on the state of stretch at which the muscle is kept during the period of disuse. This has, for example, been shown by Thomsen & Luco (1944) in the soleus and cranial tibial muscles of the cat, by Chang & Feng (1962) and Stewart, Sola & Martin (1972) in the latissimus dorsi muscles of birds, by Stewart (1968) in the gastrocnemius, plantaris and soleus muscles of the rat and by Williams & Goldspink (1978) in the soleus muscle of the mouse. Thomsen & Luco (*loc. cit.*) used a plaster cast to immobilize the tarsal joint of cats and noticed that when the joint is in full extension there is an increase in the weight of the cranial tibial muscle while the soleus muscle decreases in weight. On the other hand, when the joint is fully flexed, an opposite effect with an increase in the weight of the soleus muscle and a decrease in that of the cranial tibial muscle is seen. Summers & Hines (1951) immobilized the hind limb of the cat in neutral, shortened or stretched positions and noticed that the extent of atrophy in the muscles immobilized in the shortened position is greatest or least in the stretched or extended position respectively. Tabary, Tabary, Tardieu, Tardieu and Goldspink (1972) immobilized one hind limb of the cat in lengthened, normal and shortened positions with a plaster cast, and measured muscle fibre length, sarcomere length and total number of sarcomeres within the soleus muscle in each instance. Contralateral muscles

were used as 'controls' and no muscle weights were reported. Although fibre length was similar between treated and supporting sides, there were more, shorter, sarcomeres in the soleus muscle of the plastered limb. Williams & Goldspink (1978) noticed that in the soleus muscle of the adult mouse, sarcomeres are lost in response to immobilization in the shortened position, whereas in the lengthened position, an addition of sarcomeres occurs. Sola, Christensen & Martin (1973) showed that the cranial latissimus dorsi muscle of the chicken increases in size, whether the muscle is innervated or not, provided it is stretched. In an *in vitro* model using mechanical stretch on embryonic chicken muscle in culture, myotubes showed some of the biochemical changes seen in skeletal muscle hypertrophy (Vandenburgh & Kaufman, 1979). Thus the stimulus of stretch has a role in producing either an increase or a decrease in the size of a muscle (Thomsen & Luco, 1944; Stewart, 1972; Goss, 1978; Holly, Barnett, Ashmore, Taylor & Mole, 1980).

Of the experimental models used, only immobilization techniques maintain the anatomical integrity of the animal. The results obtained by immobilization alone are therefore the easiest to interpret, provided the positions of joints over which affected muscles act are fixed in a consistent manner. Although the stimulus of stretch to growth will presumably be greatest for a muscle acting over two joints when both joints are fixed in such a way as to stretch the muscle, no earlier study has compared a single-joint muscle with a two-joint muscle to show such an effect. Because immobilization of a limb in a quadruped alters the stance of the animal, there can be relative growth changes throughout the musculoskeletal system of the trunk and limbs induced by alteration of both muscle length and altered work load. Again, no earlier study on muscle hypertrophy using an immobilized limb has attempted to examine the overall effects on the components of the musculoskeletal system.

The present study was carried out on the sheep, a species of appropriate size for total and detailed musculoskeletal dissection and quantification. One hind limb was immobilized shortly after birth to ensure stretch of some muscles and contraction of others, for a period of 2-3 months. A possible differential response, due to the influence of stretch, between single joint and two joint muscles was examined. An attempt was also made to analyse the relative growth changes throughout the musculoskeletal system of the trunk and limbs. Alterations to growth patterns were studied by a comparison of treated and untreated sides of the animals, and by the study of untreated control animals. Changes in weight and ash content of bones have been shown to be associated with changes in the usage of muscles (Pottorf, 1916; Gillespie, 1954; Kharmosh & Saville, 1965). Thus, measurements of length, transverse sectional area and ash percentage of the femur and tibia bones of the experimental lambs were undertaken to verify the repeatability of these observations.

Changes in histochemical fibre types during muscle hypertrophy have been examined by earlier workers. Schiaffino & Bormioli (1973) induced hypertrophy in the extensor digitorum longus and extensor hallucis longus muscles of the rat by surgically removing the synergistic cranial tibial muscle and noticed that the succinate dehydrogenase activity increases remarkably in the hypertrophied muscles, that the 'white' or mitochondria-poor fibres are altogether absent compared to a proportion of about 40% in their control counterparts and that the population of fibres with low myosin ATPase activity increases in proportion. Yellin (1974) used the hypertrophied, denervated hemidiaphragm of the rat and observed that the ATPase low fibres hypertrophy to the greatest extent, while within the ATPase high fibres, the ones which are purely anaerobic hypertrophy the least and for the briefest interval, and the ones which have combined aerobic and anaerobic capabilities respond in an intermediate

fashion. Melichna & Gutmann (1974) induced hypertrophy in the extensor digitorum longus muscle of the rat using, in the same model, both immobilization of the limb in extension and denervation of the muscle. A decrease in myosin ATPase activity and an increase in succinate dehydrogenase activity occurred. Holly *et al.* (1980) used a spring-loaded metallic tubular assembly to induce stretch hypertrophy in the wing muscles of the chicken and observed that the oxidative enzyme activities increased substantially with stretch in the patagilis, a twitch muscle, but are relatively unchanged in the slow tonic cranial latissimus dorsi muscle.

No previous histochemical study of fibre types has used hypertrophy induced by stretching an immobilized muscle without other interference. The present study, therefore, used the semitendinosus muscle, stretched in this way, to examine changes in the myosin ATPase low and high fibre types. Changes in the shape of the muscle and in the number of its fascicles and muscle fibres, and the amount of connective tissue were studied in a manner similar to that employed in the study of this muscle during normal growth (Part 1). In this way, it was ascertained whether this type of hypertrophy occurs without alteration to the proportions of its constituents.

Electron microscopic studies by earlier workers have indicated that there can be disarranged sarcomeres and streaming of Z lines (Tomanek, 1976; Vaughan & Goldspink, 1979). The studies of Vaughan & Goldspink (1979) and Ho, Roy, Tweedle, Heusner, van Huss & Carrow (1980) also indicated that splitting of fibres occurs in muscle hypertrophy. However, their studies did not use immobilization. A limited electron microscopic investigation was included in the present study to observe the occurrence of these features in the semitendinosus muscle hypertrophied by immobilization technique. It is known that the tension developed by a muscle ultimately depends on the transverse

sectional area of the myofibril content (Helander & Thulin, 1962). Therefore, an estimation of the proportions of the mitochondrial, sarcoplasmic and myofibrillar components was undertaken to assess whether there were any changes at a subcellular level to indicate that hypertrophy is associated with altered intrinsic properties.

3.2 MATERIALS AND METHODS

3.2.1 *SOURCE OF MATERIAL*

Three male and six female, two-day old, Romney or Romney-Border Leicester cross lambs were used (Table 4). The lambs were obtained during two lambing seasons, four in the first and five in the second season. Each lamb was separated from its dam two days after birth and its body weight was recorded. Two lambs from the second lambing season were used as untreated controls. The right hind limb of each of the remainder was bound to the sternum for a period of two to three months.

3.2.2 *BINDING OF LIMBS*

Binding of the right hind limb of each lamb with adhesive tape was done immediately on its arrival in the experimental pen. One end of the tape was loosely wrapped around the metatarsal region of the limb, which was placed ventral to the sternum between the forelimbs, and secured by the tape around the thorax (Fig. 23). Under these conditions, the lambs were able to support their body weights and walk on three limbs. The bindings were changed every seven to ten days.

3.2.3 *MANAGEMENT AND FEEDING*

Foster milk (Glaxo) was introduced to each of the treated and control lambs on the day of their arrival to the pen. The quantity of milk fed was gradually increased while reducing the

frequency of feeding from five times to twice daily. During the third week of the experiment, grass and hay were introduced to supplement the milk diet. The animals were maintained in this manner throughout the rest of the experimental period.

All lambs were vaccinated against enterotoxaemia. The lambs from each season were reared indoors, together in a pen 9 m^2 in area. The pen was kept clean and warm. The wooden slatted floors were not slippery. The animals were released from the pen at each feeding time and were made to walk or run around a larger area for at least 30 minutes. The body weights of the lambs were recorded once a week.

3.2.4 *SAMPLING PROCEDURE*

Each lamb (except Nos. 3 & 4, Table 4) was killed when it reached about 3 months of age. Lambs No. 3 (weight gain 75.9 g/day) and No. 4 (67.7 g/day) showed a slow weight gain and were killed after a shorter period at lighter weights than the others. Because of these two lambs, the mean weight gain for the experimental lambs was 104±23 g/day, less but not significantly so ($P > 0.05$) from that of the controls, 141±39 g/day. The lambs were killed by stunning with a captive bolt and exsanguination. They were killed at five day intervals to allow time for carcass dissection to be performed and for histochemical and, in the case of the animals belonging to the second lambing season (Nos.5 to 9), for electron microscopic specimens to be processed.

Following stunning but before exsanguination, the semitendinosus muscle on each side was exposed and small strips from superficial and deep parts corresponding to AL dense and AL sparse areas of the muscle were obtained for electron microscopic studies. The strips were immediately transferred into separate vials containing modified Karnovsky's fluid (Appendix 3). They were left in the refrigerator for at least one hour before further processing.

Following slaughter, each lamb was skinned and eviscerated and its carcass weight (less head) was recorded. The semitendinosus muscle from each side was dissected out. A complete transverse slice was obtained and stored at -50°C for later histochemical studies. The remaining carcass was stored in a chiller for about three hours before muscles and bones from the limbs and trunk were dissected.

3.2.5 *ELECTRON MICROSCOPIC STUDIES*

The muscle strips, fixed in modified Karnovsky's fixative (pH 7.2), were transferred to petri dishes containing Karnovsky's fixative. With the aid of a dissection microscope, they were cut into 1 mm cubes using two halves of a blade, making cutting strokes parallel to each other but in opposite directions. About ten selected cubes were then left overnight in a change of Karnovsky's fixative. The cubes were then washed in 0.1 M phosphate buffered sucrose (pH 7.2, Appendix 3) and left in this solution at 4°C for about four hours, changing the solution at least three times within this four hour period. The cubes were post-fixed in 1% Osmium tetroxide solution (Appendix 3) for one hour. Following post-fixation, they were washed again in phosphate-buffered sucrose solution, and left in a change of that solution for about 30 minutes. An ascending series of alcohol of strengths 70%, 90% and 100% concentrations were used to dehydrate the specimens, which were agitated throughout the process. The specimens were left for 10 minutes each in 70% and 90% alcohol and were changed three times in absolute alcohol allowing 10 minutes for each change. The specimens were infiltrated first with two changes of propylene oxide, 10 minutes each, and then with 75% epoxy-resin (Appendix 3) in propylene oxide overnight. The caps of the specimen vials were removed in order to aid evaporation of the propylene oxide during infiltration by the resin. The specimens were then transferred into fresh 100% resin and left 6-8 hours.

They were finally orientated at the bottom of a gelatine capsule, embedded in freshly prepared epoxy-resin and left in an oven for about 48 hours for polymerization.

The specimen blocks were trimmed in an ultratome (LKB). Semithin sections of about 1 µm thickness were obtained and stained with 1% toluidine blue. The sections were examined under a light microscope and the best regions, with fibres cut either longitudinally or transversely, were selected. The specimen block was finally trimmed to use the best region for thin sectioning. Thin sections of about 70-90 nm thickness were cut and picked up on both supported and unsupported grids.

When the specimen grid was dry, it was washed in distilled water by holding with curved forceps, face downwards. It was blotted carefully with filter paper and immersed in a drop of uranyl acetate solution (Appendix 3) placed on parafilm, and stained for six minutes. The specimen was then washed in 50% alcohol and then in distilled water before counter-staining with a drop of lead citrate solution (Appendix 3) for six minutes. The specimen was washed and dried before it was viewed under an electron microscope.

The thin sections were viewed under a Philips 200 electron microscope. Each section with about 3 to 5 fibres was examined for possible occurrence of a disorganisation of myofibrils, streaming of Z lines and splitting of fibres. Electronmicrographs were taken at various magnifications from appropriate regions. A point counting method was used to analyse the electron-micrographs. A square of 12.5 x 12.5 cm with 625 points was used to calculate the proportions of the sarcoplasmic, myofibrillar and mitochondrial components within fibres of the semitendinosus muscle from both sides of the control lambs, and from the supporting and bound limb sides of the experimental lambs. A test point distance of 5 mm was found to be best to

analyse the electronmicrographs of 12.5 x 12.5 cm area taken at a magnification of 17,500. Electronmicrographs from transverse, longitudinal and oblique sections were used in determining the proportions of the subcellular components (Weibel, 1979).

In total, four semitendinosus muscles from 2 control animals and 3 muscles each from supporting and bound limbs of the experimental group were used for electron microscopy. Approximately 75 fibres were examined under the microscope to observe ultrastructural characteristics. A quantitative analysis was carried out in a total of 22 fibres chosen from 52 electron micrographs. 2 or 3 fibres were examined from each of the muscles from the left and right sides of the 5 sheep.

3.2.6 *HISTOCHEMICAL AND QUANTITATIVE STUDIES*

Stored, transverse slices of the semitendinosus muscle were thawed, sectioned and stained for alkali-stabile myosin ATPase following methods already described in Part 1 of this thesis. The transverse sectional area of the muscle, total fascicular area, total fibre area, mean fascicular area, mean fibre area and the mean fibre TSA of the AL and AH fibres in both AL dense and AL sparse areas were obtained (Table 9).

3.2.7 *CARCASS DISSECTION STUDIES*

The carcass dissection study began about three hours after slaughter. The muscles were separated individually or in groups (Table 5), cleaned of extraneous fat and connective tissue, and weighed. Bones were also cleaned and weighed.

3.2.8 *BONE MEASUREMENTS*

The length of each femur, from intertrochanteric crest to intercondyloid fossa, and tibia, from its intercondyloid area

to distal extremity, was measured. The individual bones were cut in the mid-shaft region using a band saw. The cut surface of the shaft was painted with ink and imprinted on paper. The outlines were traced and used to calculate the transverse sectional area (TSA) of both the shaft and cortex using a paper weighing method. The individual bones were then wrapped in aluminium foil and weighed. Contralateral bones from the same animal were placed together in a muffle furnace and heated at 500-550°C for 10 to 15 hours until there was no significant further weight loss. Each aluminium foil, with and then without its contents, was weighed on removal from the furnace.

3.2.9 *STATISTICAL METHODS*

The difference between each sampling pair (left and right sides of the body) of muscles and bones within each of the treated lambs was tested using the Student's t-test (tables 6, 8). This comparison between supporting and bound sides within an individual excluded variations between animals. It was necessary to estimate the extent to which these changes seen in the supporting and bound sides deviate from the normal. For this purpose, control animals were used. Each control lamb provided two sets of data (left and right). This enabled a mean to be compiled for control samples comparable in number with those of the treated lambs (Table 7). An analysis of the means, however, includes the variation between animals. Comparisons of the weights were expressed as a percentage of the carcass weight instead of the slaughter weight in order to eliminate the variable contributions of wool, skin and gut contents to slaughter weight (Table 7). When a consistent trend could be observed between the muscles and bones of the treated and untreated sides of the experimental lambs, this effect was considered to be due to the treatment regardless of the level of statistical significance (Tables 6,7, and 8).

The measurements obtained for the semitendinosus muscle were subjected to logarithmic regression analysis to determine changes in the muscle of the bound side of the lambs relative to those in the muscle of the supporting side. The rationale for the use of double logarithmic regression has already been outlined (2.1., 2.2.7). Both individual slopes for each animal and a common slope to include every experimental animal were incorporated in the analysis to test the significance of the changes (Table 11). This method of analysis recognised separately first, the variation within individuals due to the treatment, and secondly the variation between individuals due to the possible effect of such factors as sex, birth weight, slaughter weight and the length of restraint. The difference between absolute area measurements of each sampling pair of the semitendinosus muscles in the experimental lambs was analysed using the paired t-test (Table 10). The mean values of these area measurements for the treated and untreated sides of the experimental lambs were compared with those for the controls to observe any significant decrease or increase in relative sizes of these components.

3.3 RESULTS

3.3.1 *CARCASS DISSECTION STUDIES*

Figure 23 is a schematic representation of a lamb with altered hind limb function. Each of the seven treated lambs had its right hind limb bound beneath its sternum. The hip joint was fully flexed and the stifle and hock joints were extended. In some respects, each treated lamb behaved differently from the controls. It was less active, and when in sternal recumbency, lay on the supporting hind limb side. When standing, its supporting hind limb was kept retracted and abducted.

Table 4 gives data related to the breed, sex, age at slaughter, live weight, carcass weight, total carcass muscle and total carcass bone for the seven experimental and two control lambs used in the experiment. Although many variables are observable between individuals, the mean growth rate of the treated lambs, calculated as weight gain per day, was similar to those of the controls.

Table 5 includes the measured values for weight of muscles and bones of the experimental and control lambs used. Table 6 shows the muscles and bones of the experimental lambs ranked according to the mean weight differences between the supporting and bound sides. The t value and its level of significance, for each sampling pair, obtained by using the paired t test, is also indicated. In the hind quarter, the quadriceps femoris, crural muscles, tensor fasciae latae, deep hip muscles and tibia are significantly larger on the supporting side while the sartorius and gracilis, gluteus medius, semimembranosus and semitendinosus muscles and the hip bone are significantly larger on the bound side. In the forequarter, no muscles or bones are significantly larger on the supporting side, while the scapula, humerus, and ribs and sternum are significantly larger on the bound side. In the forequarter, there is a trend for muscles and bones to increase in weight on the bound limb side relative to the contralateral side. The exceptions to this trend are the latissimus dorsi, neck and brachiocephalicus muscles which are expected to have minimal role in supporting the body weight of the animal. In the hind quarter, on the other hand, there is a trend for muscles and bones to increase in weight on the supporting limb side, relative to the contralateral side. This effect is shown, in particular, in the extensors of the stifle joint. The exceptions are the adductor femoris, gluteus medius, semimembranosus, semitendinosus (all of which are extensors of

the hip joint), sartorius and gracilis (which adduct the hip and flex the stifle) and the hip bone.

The mean percentage of weights of pectoral, brachial, antebrachial, femoral and crural muscles and bones of the treated lambs, relative to the carcass weight, were tested for significance against those of the controls (Table 7). In the treated lambs, the scapula and the brachial muscles of both left and right forelimbs show a significant increase relative to those of the controls. The bones and muscles of the antebrachial region also show a significant increase on the bound side. The trend for all the muscles and bones of both supporting and bound side forelimbs to increase in weight relative to the control is more marked on the bound limb side. In the hind limb, neither the femur nor the total side muscle on either side shows significant changes with respect to the controls. In the crural region, however, the total muscles of the supporting side are heavier than the controls. The semitendinosus muscle is lighter, although not significantly, than the control on the supporting side, while it is significantly heavier on the bound side. This is in marked contrast with the quadriceps group which is significantly heavier on the supporting side and significantly lighter on the bound side. Fig. 24 illustrates the semitendinosus muscles obtained for the supporting and bound limb sides of an experimental lamb.

3.3.2 *BONE MEASUREMENTS*

Mean differences calculated for length and transverse sectional area of both the cortex alone and the whole mid shaft, together with the weight of ash and the percentage of ash, of the femur and tibia bones of the supporting and bound limbs of the experimental lambs are given in Table 8. The samples were tested for significance using the paired t test. The ash weight of the tibia is significantly greater in the supporting side. There is a general trend, for hind limb bones to be heavier,

thicker and shorter on the supporting side than the corresponding bones of the bound limb.

3.3.3 *QUANTITATIVE ANALYSIS OF THE SEMITENDINOSUS MUSCLE*

The semitendinosus muscle maintains the heterogeneous AL fibre type population density pattern in both the supporting and bound limbs, as for normal muscles (2.3.3). The AL fibre type population density, expressed as a percentage, in the AL dense area of the bound limb muscles is significantly greater ($t = 3.64$, $P < 0.05$) from that of the supporting limb muscles as tested by the paired t test. There is no significant difference in the AL sparse area ($t = 0.08$, $P > 0.5$). The mean percentage of the AL fibre type population density in both the AL dense area (control 23.7 ± 1.8, supporting 20.9 ± 2.8, bound 28.4 ± 5.8 and the AL sparse area (control 3.80 ± 0.89, supporting 4.35 ± 1.81, bound 4.17 ± 1.44) do not differ significantly between control, supporting and bound muscles.

Table 9 shows the weight and area measurements of the semitendinosus muscle of the supporting and the bound sides of the treated lambs and the left and right muscles of the controls. The paired t test was used to test a null hypothesis, between the supporting and bound sides, for various components of the muscle in the experimental lambs (Table 10). The actual TSA, total fascicular TSA, total fibre TSA, mean fascicular TSA, mean fibre TSA, mean area of AL fibres in both AL dense and AL sparse areas and the mean area of AH fibres in the AL dense area show a significant increase on the bound side. The means of each of these components for the supporting (n=7) sides were compared with the means for the controls (n=4). No significant changes occur in any of these components in the experimental animals relative to the controls, although there is a general trend for the muscle components on the bound side to be greater and those on the supporting side to be smaller than those of the controls in all these cases.

Double logarithmic analyses comparing transverse sectional areas and weights from the semitendinosus of the supporting and bound sides of the experimental lambs were used to examine the relative changes in proportions due to the treatment (Table 11). These changes are graphically represented (Figs. 25-34) to indicate variations both within individuals due to treatment (as shown by the slope of each line), and between individuals (as shown by the intercept of each line) due to various factors previously mentioned (3.2.9.). The control animals (Nos. 8 and 9) show, in each of these graphs, the nature of the variations found between the right and left muscles in normal animals. No significant changes in connective tissue proportions, in fascicle number, in fibre number or in fibre size dimensions occur within the muscle due to an increase in size (Table 11). Thus, the almost two-fold increase (Table 7) in weight in the semitendinosus muscle is due to isometric growth of the muscle; the area of fascicles and individual fibres increases proportionately with those of the surrounding connective tissue components. Both AL and AH fibres are larger in the bound side when compared with the control or supporting side muscles (Fig. 34, Table 10).

3.3.4 *ELECTRON MICROSCOPIC STUDIES*

By an inspection of electronmicrographs, the sarcomere assembly, position of triads and the Z lines were considered normal in their general arrangement (Figs. 35 and 36). The proportions of sarcoplasm, mitochondria and myofibrils were not significantly altered by the treatment (Table 12). Thus, no ultrastructural component appears affected by the size increase due to the stretching of the muscle.

3.4 DISCUSSION

3.4.1 *INCREASED GROWTH OF MUSCLES AND BONES DUE TO INCREASED WORK LOAD*

There have been several reports indicating that an increased

work load on the hind limb muscles of the rat produces an increase in muscle size. The work load was increased in muscles either by enforced exercise, or by the denervation, removal or tenotomy of their synergists. Thus, Goldberg (1967) used tenotomy of the gastrocnemius muscle and showed hypertrophy in the soleus and plantaris muscles; Parizkova & Koutecky (1968) used treadmill exercise and showed hypertrophy of the soleus muscle; Binkhorst (1969) used denervation of the gastrocnemius and soleus muscles and treadmill exercise, and showed hypertrophy of the plantaris muscle; and Reitsma (1969) used removal of synergist muscles and showed hypertrophy in the rectus femoris and plantaris muscles.

In the present study, the quadriceps femoris and tensor fasciae latae muscles increased in the supporting limb in relation to the muscles of the bound limb. These muscles are extensors of the stifle joint. They support the body weight by preventing collapse of this joint. The extensors of the shoulder, elbow and tarsus and flexors of the carpus could presumably also have been shown to enlarge, had the extensor and flexor muscles acting over these joints been separately analysed. The changes in the musculoskeletal components of both the forelimbs and the supporting hind limbs suggest that the abnormal three-legged stance of the animal stimulates growth of muscles and bones of the supporting limbs. In particular, the pattern of increase suggests a diagonal support of body weight using the contra-lateral hind limb and ipsilateral forelimb. There was a trend for the muscles of the crural limb segment of the supporting hind limb to show a greater increase than the thigh region. This may be due to the stance of the treated lambs, with their supporting limb retracted and abducted, the contribution of thigh muscles and the femur being less than those of the crural components in supporting the body weight.

The mechanism of work load induced hypertrophy has been discussed by Stewart (1972), who suggested that the hypertrophy

induced by enforced exercise, or by denervation, removal or tenotomy of the synergists of a muscle could be due to a 'continuous active tension' developing within the muscle. Nevertheless, in experiments involving tenotomy in the rat, Mackova & Hnik (1973) showed that the hypertrophy so produced in the muscles is due to a mechanical stretch of these muscles rather than an excessive use of the muscles themselves. When the gastrocnemius muscle was tenotomised, the soleus muscle of the same side underwent hypertrophy. When the nerve supply of the antagonists of the soleus was cut along with tenotomy of the gastrocnemius, the soleus failed to hypertrophy. They obtained the same results when tenotomy of the cranial tibial muscle was performed along with denervation of the antagonists of the long digital extensor muscles. Hofmann (1980) claimed that such a hypertrophy involved both a stretching effect due to antagonists and a neurogenic effect. However, he used the values obtained for hypertrophy of soleus following unilateral tenotomy of synergists to evaluate the effects of bilateral tenotomy of synergists but, since normal control animals were not used, the results are difficult to interpret. The muscle hypertrophy produced in these experiments may be due to the stretching rather than the chronic stimulation.

3.4.2. *GROWTH OF MUSCLES DUE TO STRETCH AND RESTRICTED CONTRACTION*

In various studies, it has been found that a muscle can undergo weight increase irrespective of intact innervation, provided it is kept stretched by immobilization or an elastic tensile apparatus (Thomsen & Luco, 1944; Chang & Feng, 1962; Sola, Christensen & Martin, 1973; Williams & Goldspink, 1978; Holly *et al*. 1980).

In the present study, the hip extensors of the bound hind limb were heavier than those of the supporting limb. All these muscles were kept stretched due to the flexed position

of the hip during the experiment. Since the semitendinosus muscle is also a stifle flexor, it was maximally stretched and this could well explain why its hypertrophy was greater than that of the other hip extensors. Thus, the present study shows that the stretch influence is greater for a muscle acting over two joints than for muscles acting over a single joint. All the extensors of the hip joint in the immobilized limb had an intact nerve supply. The frequency of stimulation was not measured, but it is expected that in such a bound position, an attempt by the sheep would be frequently made to extend the hip in order to place the limb in a normal position. Neither stretch nor such a stimulation would be expected in the atrophied quadriceps muscle. This muscle group was kept in a shortened position in the immobilized limb. A significant reduction in its growth rate may be due to a lack of stretch stimulus in it. By relating the treated lambs to the control lambs in the experiment, it has been possible to demonstrate that the differences between the semitendinosus and the quadriceps muscles, due to the treatment, arise from a hypertrophy of the semitendinosus in the bound side, and a combined atrophy on the bound side and hypertrophy on the supporting side for the quadriceps muscle.

Experiments involving chronic electrical stimulation (Pette, Smith, Staudte & Vrbová, 1973; Pette, Ramirez, Müller, Simon, Exner & Hildebrand, 1975; Pette, Müller, Leisner & Vrbová, 1976; and Salmons & Sréter, 1976) and cross innervation (Buller, Eccles & Eccles, 1960; Close, 1969; Sréter, Gergely & Luff, 1974; and Salmons & Sréter, 1976), show that the activity pattern of a muscle can change according to the type of nervous stimulus it receives. As well as producing changes in the contractile characteristics of a muscle, a nerve can also modify its metabolic and histochemical properties, as demonstrated in cross innervation experiments (Dubowitz, 1967; Bárány & Close, 1971; Salmons & Sréter, 1976) and by chronic electrical stimulation (Pette *et al.* 1975; Salmons & Sréter, 1976).

Only a few of the above mentioned reports demonstrate quantitative changes in the muscles. Sréter *et al*. (1974) noticed loss of weight in the cross innervated extensor digitorum longus muscle and in both the self innervated and cross innervated soleus muscle of the rat, Pette *et al*. (1975), loss in mean fibre size in the intermittently stimulated cranial tibial muscle of the rabbit, Pette *et al*. (1976), loss in weight in the chronically stimulated long digital extensor muscle of the rabbit, and Bárány & Close (1971), loss of weight in the cross innervated soleus muscle of the rat. Thus, there is no evidence to suggest a positive growth influence induced by a special pattern of nerve activity.

Thus, stretch appears to be the mechanism producing size changes in skeletal muscles kept in either a lengthened or a shortened position under immobilized conditions. The weight increase in muscles due to an excessive weight load may also be due to the same mechanism as observed in the extensors of the stifle joint of the supporting limb. The muscles preventing the collapse of the joint under gravity could have been hypertrophied due to stretch. It is possible that the changes induced in the forelimbs of the sheep in the present experiment were also due to this mechanism because an excessive weight load or abnormal stance may have changed the position of various joints. A detailed analysis of extensors and flexors of the shoulder and elbow joint would perhaps substantiate this. Stretch may also be an essential requirement for body building in man (Tanner, 1952), exercises for which are more successfully applied to the shoulder and arms than for the pelvic limb, in which joint angles are more regulated by their weight supporting function.

3.4.3 *GROWTH CHANGES IN BONES*

Bones have been shown to lose weight and ash content with loss of muscle activity (Pottorf, 1916; Gillespie, 1954; Kharmosh

& Saville, 1965). In the present study, a similar trend was seen in the femur and tibia of the bound limbs. These bones tended to be lighter, thinner and less mineralised than those of the supporting limb. Both these bones, however, showed a tendency to be longer than those of the supporting limb. Compression may, therefore, affect growth in bone length.

3.4.4 *HISTOLOGICAL STUDIES*

The increase in size of the semitendinosus muscle, as observed in the model used, involved for many criteria only changes that would be expected during the normal growth of the muscle. First, the shape of the muscle is isometrically maintained; the relation between weight and transverse sectional area is similar to that observed during normal postnatal growth (Table 2). Holly *et al.* (1980), using the patagilis, biceps brachii, long head of triceps brachii and cranial latissimus dorsi muscles showed that stretch hypertrophy is a result of both increased longitudinal and circumferential growth. Longitudinal growth of the muscle is maintained by an addition of sarcomeres both in normal growth (Williams & Goldspink, 1971) and muscles stretched in hypertrophy (Williams & Goldspink, 1978).

Secondly, no significant change in fibre number occurs within the muscle under stretched conditions. Again, these effects are the same as those observed in normal postnatal growth (Table 2). Vaughan & Goldspink (1979) also noticed that there were no significant changes in the fibre number of the soleus muscle following stretch. However, Reitsma (1969), using histological studies on the rectus femoris, plantaris and soleus muscles of the rat hypertrophied by surgical removal of synergists, and Sola, Christensen & Martin (1973) using both denervation and stretching on the cranial latissimus dorsi muscle of the chicken, claimed that an increase in fibre number in addition to a fibre size increase occurs under these conditions. They described an existence of newly

forming fibres with centrally positioned nuclei in these muscles. No such fibres were observed in the present study (Fig. 34).

Thirdly, the connective tissue architecture shows no changes other than those commensurate with those seen during normal postnatal growth (Table 2). Thus, the normal shape of a stretched muscle appears to be maintained by an increase in transverse sectional area due to a harmonious increase between the fibre, fascicular and connective tissue components and by an increase in length probably due to an addition of sarcomeres in series (Williams & Goldspink, 1978).

In the present study, both AL and AH fibres of the hypertrophied muscle show increase in size compared to those of the semitendinosus muscle of the supporting limb (Table 10) and the same trend when compared with those of the control muscles. Yellin (1974) noticed that, in the stretched hemidiaphragm of the rat, AL fibres increase to a greater extent than the AH fibres. Holly *et al*. (1980) also noticed that the growth response of the slow, cranial latissimus dorsi muscle is more vigorous than that of the patagilis, a fast muscle. Although the present study does not provide conclusive evidence for such a preferential response of the AL fibres, this possibility is not excluded (Table 11). Ho *et al*. (1980) used adult rats in a weight lifting exercise programme and noticed hypertrophy in the long adductor muscle. Their studies suggested that the mean size of both AL and AH fibres are significantly smaller than those of the controls and that there is an increase in the number of fibres per unit of transverse sectional area. These results do not concur with those of the present study. The response to the same stimulus may differ between an actively growing muscle and a mature muscle.

As mentioned previously (3.4.2), a change in contractile properties in muscle has been shown to occur as an adaptive

response when there is a change in frequency of stimulation. This was demonstrated by cross innervation (Buller *et al.* 1960; Dubowitz, 1967; Close, 1969; Sréter, Gergely & Luff, 1974; Salmons & Sréter, 1976) and by chronic electrical stimulation to produce a change in pattern of activity (Pette *et al.* 1973; Pette *et al.* 1975; Salmons & Sréter, 1976). Fast muscles subjected to a continuous low frequency discharge acquire slow muscle properties, and slow muscles relieved of such a pattern of activation undergo reciprocal changes (Salmons & Sréter, 1976). Accompanied by these changes in contractile properties, a change in myosin ATPase activity is also observed. An increase in speed of contraction parallels an increase in myosin ATPase activity. When the muscle contracts more slowly the myosin ATPase activity is lower (Bárány, 1967; Bárány & Close, 1971; Sréter, Gergely & Luff, 1974; Vrbová, Gordon & Jones, 1978). These changes depend on the duration of stimulation (Salmons & Sréter, 1976; Pette *et al.*1976; Vrbová, Gordon & Jones, 1978). Pette *et al*(*loc. cit.*) used long intermittent (8h daily) or continuous (24h daily) stimulation with a frequency pattern resembling that of a slow motoneurone to activate the fast twitch cranial tibial and extensor digitorum longus muscles of the rabbit. With intermittent stimulation, changes in the patterns of myosin light chain or alterations in the distribution of slow and fast fibres, as identifiable by histochemical myosin ATPase techniques, do not occur within 40 days. However, these changes are observed after intermittent stimulation period exceeding 40 days or continuous stimulation periods longer than 20 days.

Chronic abnormal usage of a muscle, as utilized in the present study, may also produce changes in contractile properties. There is a significant increase in AL fibre type proportions seen in the AL dense area of the semitendinosus muscle in the bound limb side. Changes in the activity pattern of this muscle could have been either due to a change in frequency of the stimulus applied or due to long term activity of

the muscle. Since the nerve supply was not interfered with in this experiment, it is unlikely that the frequency pattern of the muscle stimulation was altered by the treatment. However, the muscle might well have, over a large part of the treatment interval, been stimulated to generate a force always opposed by the bindings, in an attempt by the sheep to restore its bound limb to its normal position. The present experiment has shown that an increase in AL fibre percentage within a muscle occurs even without the muscle being involved in the support of body weight. This postural function was invoked to explain the effect of an increase in AL fibre percentage during postnatal growth (2.4.6). The method of strapping up the limb appears to have exaggerated the stimulation and the response that occurs during normal growth of an animal.

3.4.5. *ELECTRON MICROSCOPIC STUDIES*

Earlier workers have shown that in muscle hypertrophied by tenotomy or by weight lifting exercises, 'disarrangement' of sarcomeres, 'streaming' of Z lines and 'splitting' of fibres are possible abnormalities expected in the muscles (Tomanek, 1976, Vaughan & Goldspink, 1979, Ho *et al*. 1980). The present study provided no evidence for these changes.

Morphometric analysis of skeletal muscle fibres imposes many limitations because of an ordered organisation of the fibres and a periodicity shown by the sarcomeres (Williams, 1979). In using the point counting method for obtaining absolute measurements, the sampling method and the number of samples used, in addition to the magnification of the electron-micrographs, the number of test points, the distance of the test point in the square lattice employed and the orientation of the lattice have to be given careful consideration (Weibel & Bolender, 1973, Williams, 1977, Weibel, 1979).

In the present study, the proportionate changes involving the myofibrillar, sarcoplasmic and mitochondrial subcellular components were considered quantitatively. In spite of the above limitations, sufficient material was inspected to determine that the intracellular components of the fibres examined are essentially normal in this type of hypertrophy. Stretch hypertrophy need not be produced by a process other than normal myofibrillar growth.

4.0 GENERAL CONCLUSIONS

The changes involved in skeletal muscle during both normal and altered growth have been studied. The results suggest that the qualitative changes seen antenatally in a muscle are governed genetically. The growth pattern of fascicles and fibres within the semitendinosus muscle of the sheep suggests that endomysial and perimysial connective tissue control the number of fascicles and fibres during normal growth of the muscle. The study also supports the idea that, within a muscle with a heterogeneous fibre type distribution, the region which shows a more frequent postural type of activity has a high proportion of alkali-stabile myosin ATPase low (AL) fibres while the region which is involved in a more intermittent propulsive type of activity has a low proportion of this fibre type. A hypothesis is advanced that an accumulation of AL fibres in the deeper part of a muscle with a heterogeneous fibre type population is due to the special metabolic and heat dissipation requirements of AL fibres.

By studying the regions of extreme fibre type distribution, the histochemical properties of a particular region of a muscle has been shown to be altered during normal growth and by abnormal use. The transformation appears to be due to chronic stimulation rather than to the frequency of motoneurone disharge, since the latter should not have been affected by the altered conditions.

Stretch appears to be a stimulus for muscle growth. The present study provides evidence that a muscle acting over two joints shows greater growth response to a stretch stimulus than a single joint muscle. The model used has provided further evidence that a muscle can change its normal growth pattern, when the necessary stimulus is applied to it, irrespective of whether the muscle is immobilized or not. The increased growth of the muscle under immobilized but stretched

conditions appears to occur isometrically, and without a change in intracellular composition. An immobilized muscle without stretching becomes slower growing despite its intact innervation.

The semitendinosus muscle of the sheep has therefore been a useful model in demonstrating new aspects of muscle growth. By the use of certain extensions and modifications, the model can be used to explore muscle growth further. Chemical analysis involving dry matter, hydroxyproline and intramuscular fat of the semitendinosus muscle will extend the results related to both normal growth changes and in hypertrophy. Electromyographic studies can be used in various muscles and in different species to establish a general concept distinguishing the postural and propulsive involvement of myosin ATPase low and high fibre types. Electrical activity of muscles kept immobilised can also be recorded to ascertain the behaviour of muscles during abnormal usage. Other extensions to the present model such as long term but intermittent immobilization, restoration of limb activity after long term immobilization, or using a series of lambs at different time intervals, will establish the time sequence of stretch hypertrophy. Adult sheep can be used for immobilization to ascertain whether there are differences in stretch response between an actively growing muscle and one in which normal growth has been completed. As an alternative procedure, the hind limb may be immobilised with both the hip and stifle joints flexed. In this instance, the semitendinosus muscle remains at about normal length while the semimembranosus muscle, acting over the hip alone, lengthens as in the present model. Thus, the relative hypertrophy between the two muscles should be different from that already shown.

It was found in the present study that the experimental lamb transmitted more weight on to the forelimbs than on to the supporting hind limb. An immobilised forelimb may, therefore, demonstrate better than an immobilised hind limb

the effects on muscle weight distribution and histochemical fibre type composition in the supporting contralateral limb. One forelimb can be immobilised with both shoulder and elbow joints flexed. Changes in muscles such as biceps brachii and triceps brachii can be expected. These experiments can be extended further to create immobilization of limbs *in utero*, in order to demonstrate whether stretch also acts as a stimulus to the antenatal growth of muscles.

Table 1a: Data related to the semitendinosus muscle of 33 sheep. 18 from 60 days gestation to birth, and 15 from birth to adulthood. TSA = Transverse sectional area.

Sheep No.	Age	Sex	Body weight (kg)	Muscle weight (g)	Actual TSA (cm^2)	Total Fascicular TSA (cm^2)	Total Fibre TSA (cm^2)	Mean Fascicular TSA ($x10^3 \mu m^2$)	Mean Fibre TSA (μm^2)
Antenatal period									
1	60 days	F	0.08	0.18	0.15	-	0.04	-	110
2	60 days	F	0.05	0.06	0.12	-	0.02	-	113
3	61 days	F	0.04	0.05	0.19	-	0.05	-	98
4	70 days	M	0.12	0.18	0.24	0.10	0.08	2.61	100
5	70 days	F	0.18	0.30	0.21	0.06	0.05	2.35	66
6	70 days	M	0.20	0.29	0.25	0.12	0.10	4.45	70
7	80 days	M	0.34	0.72	0.92	0.55	0.33	6.35	186
8	80 days	F	0.35	0.78	0.44	0.25	0.15	4.73	115
9	80 days	F	0.33	0.69	0.63	0.35	0.20	5.14	138
10	102 days	F	1.04	2.75	1.48	0.81	0.52	10.30	202
11	102 days	M	1.06	2.97	1.02	0.80	0.53	11.38	257
12	104 days	M	1.24	2.40	0.96	0.51	0.31	10.71	171
13	119 days	F	3.04	5.50	2.91	2.12	1.42	26.19	309
14	122 days	M	2.98	5.81	3.02	2.16	1.05	26.60	263
15	122 days	M	3.42	5.14	2.37	1.96	1.21	42.74	735
16	140 days	F	1.69	5.90	1.66	1.30	1.18	20.65	464
17	140 days	F	1.73	7.30	1.44	1.22	1.02	18.31	468
18	137 days	M	5.01	10.50	1.73	1.39	1.08	20.59	515
Postnatal period									
19	5 weeks	M	10.90	24.00	2.85	2.68	2.32	55.99	932
20	5 weeks	M	11.81	34.00	4.22	3.78	3.35	55.40	1053
21	5 weeks	M	13.18	33.70	4.73	4.14	3.45	52.80	815
22	5 months	F	26.80	58.00	7.06	6.57	5.79	137.02	2426
23	5 months	F	29.96	60.50	9.75	8.58	7.91	149.80	2830
24	5 months	F	30.00	85.91	8.56	7.94	6.45	172.89	1573
25	5 months	F	35.30	156.30	9.19	7.80	7.10	182.84	2700
26	5 months	F	37.50	86.04	5.40	4.86	4.46	134.43	2085
27	5 months	F	39.30	68.70	8.36	7.95	6.85	181.76	2293
28	5 months	F	39.50	100.10	7.12	6.20	6.03	160.47	1847
29	5 years	F	45.40	93.50	8.93	8.25	7.38	208.01	3042
30	5 years	F	49.00	119.50	8.91	8.41	6.56	175.67	1630
31	5 years	F	52.27	137.00	11.69	10.52	9.62	202.35	2705
32	5 years	F	59.00	124.00	12.06	10.98	9.22	174.58	3066
33	5 years	F	63.00	122.00	9.45	9.07	8.28	139.40	2525

Table 1b: Data related to the semitendinosus muscle of 27 sheep. 12 from 80 days gestation to birth, and 15 from birth to adulthood. Measurements are made on fibres reacted histochemically to myosin ATPase, classified as high (AH) or low (AL) reacting.

Sheep No.	FIBRE TYPE MEAN TRANSVERSE SECTIONAL AREA (μm^2)				AL FIBRE NUMBER AS PERCENTAGE OF TOTAL FIBRES	
	AL dense area		AL sparse area		AL sparse area	AL dense area
	AL	AH	AL	AH		
7	168	137	288	187	4.4	12.8
8	156	131	178	140	4.1	10.8
9	152	148	168	125	5.8	10.8
10	224	280	178	268	6.2	9.6
11	224	256	164	229	4.4	11.0
12	286	266	185	253	3.8	12.2
13	240	390	220	370	4.2	13.5
14	350	553	257	329	2.8	15.5
15	600	960	520	780	2.5	19.1
16	482	423	454	415	5.5	14.1
17	454	415	482	423	5.8	13.6
18	404	667	653	882	6.4	16.2
19	996	1334	804	984	5.5	21.5
20	831	1175	761	1395	4.8	19.3
21	695	1015	657	820	3.7	17.5
22	2358	2687	2696	2776	5.5	23.5
23	2488	2788	2234	2990	6.3	22.8
24	1380	1840	1590	1960	3.6	26.0
25	2730	2740	2690	2930	3.9	24.7
26	1660	2240	1580	1940	4.1	25.0
27	2023	2673	1878	2948	6.9	26.6
28	2223	2154	2353	1914	2.8	24.2
29	2153	2666	1871	3095	4.3	26.8
30	2590	2750	2320	2280	4.1	25.3
31	2569	3706	2160	3040	3.6	30.0
32	2400	2732	2546	3057	3.5	29.0
33	1865	1849	2275	2600	4.4	27.8

Table 2: Regressions of the form log y = a + b log x, comparing transverse sectional areas (TSA) and weights from M. Semitendinosus of the sheep, with ages ranging from 60 days antenatally to five years postnatally. n = number of animals included in calculating each regression: s_b = Standard error of the regression coefficient b. Values of b significantly different ($P < 0.05$) from 1 or 0.67 (G,H,I), as appropriate, are marked with an asterisk.

	VARIABLES	ANTENATAL PERIOD				ANTE AND POSTNATAL PERIOD				POSTNATAL PERIOD			
		b	s_b	log a	n	b	s_b	log a	n	b	s_b	log a	n
A	Total fibre number x = mean fibre TSA (μm^2) y - total fibre TSA (μm^2)	1.70**	0.23	+3.55	18	1.37***	0.08	+4.28	33	0.82	0.12	+6.08	15
B	Total fascicular number x = mean fascicular TSA (μm^2) y = total fascicular TSA (μm^2)	1.25*	0.10	+2.77	15	1.16	0.10	+3.01	30	0.74	0.11	+5.02	15
C	Fibre number within fascicle x = mean fibre TSA (μm^2) y = mean fasicular TSA (μm^2)	1.12	0.13	+1.40	15	1.20	0.06	+1.18	30	0.95	0.15	+1.99	15
D	Fibre types in AL sparse area x = mean TSA of AL fibres (μm^2) y = mean TSA of AH fibres (μm^2)	1.01	0.20	+0.03	12	1.02	0.05	+0.01	27	0.81	0.11	+0.71	15
E	Fibre types in AL dense area x = mean TSA of AL fibres (μm^2) y = mean TSA of AH fibres	1.25	0.17	-0.55	12	1.02	0.04	+0.01	27	0.84	0.13	+0.58	15
F	Relative growth of M. Semitendinosus x = body weight (g) y = muscle weight (mg)	1.11*	0.05	+0.01	18	1.05*	0.02	+0.14	33	0.93	0.11	+0.71	15
G	Muscle shape and mechanics x = muscle weight (mg) y = Actual TSA (μm^2)	0.58*	0.05	+6.10	18	0.56***	0.02	+6.14	33	0.61	0.10	+5.18	15
H	Muscle shape and mechanics x = muscle weight (mg) y = total fascicular (TSA (μm^2)	0.76	0.08	+5.29	15	0.67	0.03	+5.55	30	0.61	0.10	+5.87	15
I	Muscle shape and mechanics x = muscle weight (mg) y = total fibre TSA (μm^2)	0.75	0.05	+5.14	18	0.73*	0.02	+5.20	33	0.63	0.10	+5.65	15
J	Endomysial connective tissue x = total fascicular TSA (μm^2) y = total fibre TSA (μm^2)	0.95	0.04	+0.23	15	1.05*	0.02	-0.56	30	1.04	0.07	-0.42	15
K	Perimysial connective tissue x = Actual TSA (μm^2) y = total fascicular TSA (μm^2)	1.28***	0.05	-2.46	15	1.21***	0.02	-1.88	30	1.00	0.02	-0.05	15
L	Perimysial and endomysial tissue x = Actual TSA (μm^2) y = total fibre TSA (μm^2)	1.26***	0.06	-2.46	18	1.29***	0.03	-2.71	33	0.98	0.27	+0.10	15

Table 3: Predictions of total fibre number in the semitendinosus muscle at the level of transection, from the equations in Table 2A.

Age	Equation for period	Prediction	95% confidence limits
60 days	Antenatal	89.1×10^3	56.1×10^3 - 141×10^3
	Antenatal & Postnatal	105×10^3	75.8×10^3 - 146×10^3
birth	Antenatal	348×10^3	169×10^3 - 716×10^3
	Antenatal & Postnatal	217×10^3	177×10^3 - 265×10^3
	Postnatal	368×10^3	275×10^3 - 498×10^3
Adult	Antenatal & Postnatal	372×10^3	268×10^3 - 516×10^3
	Postnatal	283×10^3	243×10^3 - 333×10^3

Table 4: Data related to the lambs used. Lambs 1 to 7 had their right hind limbs bound beneath their sternum. Lambs 8 and 9 were controls.

Lamb No.	Breed	Sex	Age at slaughter (Days)	Live weight (kg)	Carcass weight (kg)	Total carcass muscle (kg)	Total carcass bone (kg)
1	Romney	Male	95	17.4	8.73	6.52	1.64
2	Romney x Border-Leicester	Male	86	16.5	7.30	5.51	1.41
3	Romney	Male	63	10.0	4.08	2.74	1.04
4	Romney	Female	68	10.1	4.27	3.18	1.03
5	Romney x Border-Leicester	Female	86	14.3	5.96	4.24	1.29
6	Romney	Female	99	13.8	5.76	3.94	1.27
7	Romney	Female	79	13.3	5.18	3.87	1.14
8	Romney	Female	79	16.3	6.80	4.64	1.40
9	Romney	Female	92	15.5	6.38	4.59	1.28

Table 5: Measured values for weights in grammes of muscles and bones of the lambs used. Lambs 1 to 7 had their right limbs bound beneath their sternum. Lambs 8 and 9 were controls. L = left; R = right.

	1		2		3		4		5		6		7		8		9	
	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R
Neck muscles	129	121	97.5	81.7	88.9	87.0	106	123	143	118	132	131	126	134	158	156	161	143
Brachiocephalicus	46.4	50.4	47.5	43.2	29.3	29.1	20.6	23.4	25.5	26.0	27.0	21.5	24.5	27.0	35.0	30.0	28.5	30.5
Trapezius	27.4	31.1	22.7	24.4	18.0	18.4	21.4	18.5	16.5	15.5	17.5	21.0	12.0	14.0	15.0	13.0	16.5	15.5
Latissimus dorsi	63.4	64.2	46.8	45.1	24.3	23.2	34.0	28.4	39.0	34.5	37.5	36.0	28.0	28.0	37.0	32.0	32.0	31.5
Serratus ventralis Rhombideus	144	164	105	118	57.9	68.3	71.9	69.9	88.0	92.0	87.0	93.5	81.0	79.0	105	102	96.5	95.0
Vertebral & rib muscles	201	232	222	273	50.8	61.8	56.0	47.0	43.5	28.5	108	120	111	123	153	147	116	123
Pectoral muscles	144	174	101	109	49.0	50.1	73.6	69.0	58.5	100	80.0	92.0	59.0	72.0	88.0	89.0	75.5	81.0
Brachial muscles	392	410	339	365	156	160	180	170	238	248	235	248	222	234	245	243	238	238
Antebrachial muscles	95.5	101	95.7	97.2	48.2	51.2	58.8	52.2	67.0	69.0	64.0	69.0	54.0	59.0	70.0	72.5	65.0	66.0
Diaphragm	45.1	36.4	30.5	31.7	17.0	15.7	28.4	30.2	26.5	30.0	29.0	26.0	28.0	29.0	31.5	30.0	26.5	30.0
Longissimus system	260	284	253	256	87.4	92.4	111	126	161	149	140	136	145	151	202	191	186	177
Sublumbar muscles	87.8	90.2	72.5	71.3	33.2	24.3	48.1	37.0	45.0	49.0	39.0	37.5	43.0	38.0	50.5	52.5	48.0	47.0
Abdominal muscles	206	208	166	174	93.5	81.6	119	108	135	118	119	129	133	134	156	159	180	164
Gluteus medius	120	126	93.8	110	43.0	56.5	51.5	58.5	73.5	81.0	57.0	66.0	58.0	58.5	81.5	68.0	77.5	77.0
Deep hip muscles *	47.2	41.3	27.4	25.9	19.2	15.4	23.6	21.0	49.0	40.0	40.0	25.5	37.5	28.0	41.0	46.0	45.0	43.0
Tensor fasciae latae	63.6	25.8	30.4	23.0	15.9	5.80	17.8	8.80	26.0	10.0	19.0	11.0	22.0	29.0	16.0	15.0	16.0	14.0
Gluteobiceps	162	144	125	135	57.2	62.4	69.7	73.3	101	77.0	82.0	71.5	82.5	64.5	108	108	104	199
Quadriceps femoris	191	139	177	126	97.1	64.0	97.6	62.9	168	77.0	137	77.5	138	61.0	136	136	151	150
Semitendinosus	48.2	83.1	41.9	59.8	23.0	39.0	25.7	45.5	36.5	71.0	35.0	66.0	26.0	47.5	46.0	44.5	42.0	42.5
Semimembranosus	138	162	106	111	43.9	68.7	64.7	81.9	86.0	112	75.0	95.0	70.5	77.0	128	122	114	112
Adductor femoris	68.4	64.6	52.2	50.1	20.3	21.6	30.3	29.0	35.0	44.0	35.0	39.0	35.0	29.0	52.5	52.5	53.0	52.0
Sartorius & Gracilis	41.2	39.8	26.6	33.1	12.1	21.9	20.1	24.3	23.5	31.5	17.0	23.5	20.0	24.0	24.0	24.5	23.0	24.5
Crural muscles	178	148	165	129	74.6	60.7	99.4	82.0	114	92.0	104	84.5	112	76.0	124	120	110	110
Cutaneous & scrap muscles	333	346	307	266	140	262	144	216	291	341	234	269	240	343	259	228	272	247
Total side muscle	3232	3285	2751	2758	1300	1441	1573	1605	2190	2053	1950	1989	1908	1959	2362	2281	2277	2312
Scapula	53.6	54.1	39.3	41.8	29.7	31.1	27.5	26.8	35.0	40.0	35.5	36.5	28.0	30.5	36.5	38.0	29.0	30.0
Humerus	60.5	63.7	51.3	52.8	41.1	43.2	39.3	39.3	52.0	56.0	49.0	52.0	42.0	43.5	54.5	55.5	51.0	50.0
Radius & ulna	50.1	52.4	43.1	44.9	40.2	40.6	35.1	35.6	43.5	46.0	41.5	44.0	35.5	35.5	44.5	44.0	39.0	39.0
Rib & sternum	153	173	129	144	97.7	100	92.8	92.8	99.0	116	131	130	105	111	145	155	137	132
Cervical vertebrae	85.0	85.0	76.0	76.0	60.0	60.0	56.1	56.1	67.5	67.5	67.0	67.0	61.0	61.0	70.5	70.5	75.5	75.5
Thoracic vertebrae	79.0	79.0	81.0	54.5	54.5	54.5	52.0	52.0	62.0	62.0	70.5	70.5	57.0	57.0	78.5	78.5	75.0	75.0
Lumbar vertebrae	84.0	84.0	80.0	80.0	47.0	47.0	52.0	52.0	56.5	56.5	61.5	61.5	49.0	49.0	67.5	67.5	53.5	53.5
Sacrum	31.0	31.0	19.5	19.5	13.6	13.6	15.0	15.0	19.5	19.5	19.0	19.0	16.0	16.0	20.5	20.5	19.0	19.0
Hip bone	63.6	68.5	50.7	57.0	35.7	38.5	37.4	40.8	50.0	59.0	41.0	44.5	33.0	34.0	45.0	45.0	42.5	41.0
Femur	79.2	77.1	66.1	66.3	50.4	50.2	52.5	55.2	67.0	67.0	63.0	59.5	55.5	50.0	74.0	71.0	68.5	67.5
Patella	5.5	5.2	4.7	4.0	3.7	3.4	4.4	3.9	3.5	5.5	3.0	2.9	2.0	2.0	4.0	4.0	1.0	1.5
Tibia	63.9	57.3	56.3	47.7	43.0	38.8	44.8	41.3	59.0	52.0	54.0	47.5	47.5	40.0	56.5	52.5	52.5	52.5
Total side bone	808	830	697	715	517	521	509	517	632	662	633	634	532	530	696	701	643	636

* Deep hip muscles include Pectineus, Deep gluteal, Obturator and Gemelli muscles.

Table 6: Muscles and bones ranked according to the mean weight difference (supporting side - bound side), for seven lambs with bound right hind limbs.

	MUSCLES	Mean difference (g)	t+	BONES	Mean difference (g)	t+
↑ Larger on left (supporting) side	Quadriceps femoris	+ 56.90	+ 7.11***			
	Crural muscles	+ 25.00	+ 7.35***			
	Tensor fasciae latae	+ 14.20	+ 3.48*			
	Gluteobiceps	+ 7.31	+ 1.44			
	Deep hip muscles	+ 6.86	+ 4.09**			
				Tibia	+ 5.91	+ 8.30***
	Sublumbar muscles	+ 3.04	+ 1.44			
	Abdominal muscles	+ 2.70	+ 0.68			
	Latissimus dorsi	+ 1.94	+ 2.22			
	Diaphragm	+ 1.30	+ 0.88			
				Femur	+ 1.20	+ 1.17
	Neck muscles	+ 0.91	+ 0.15			
				Patella	+ 0.56	+ 2.18
	Brachiocephalicus	+ 0.17	+ 0.12			
Larger on right (bound) side. ↓	Trapezius	- 1.06	- 1.17			
				Radius & Ulna	- 1.43	- 1.41
				Scapula	- 1.74	- 2.52*
	Adductor femoris	- 1.87	- 1.06			
				Humerus	- 2.19	- 4.34**
	Antebrachial muscles	- 2.20	- 1.39			
	Brachial muscles	- 3.00	- 0.51			
				Hip bone	- 4.41	- 4.46**
	Longissimus system	- 5.29	- 1.19			
	Sartorius & Gracilis	- 5.37	- 3.93**			
	Serratus ventralis & Rhomboideus	- 7.13	- 2.34			
	Gluteus medius	- 8.53	- 4.40**			
				Ribs & Sternum	- 9.29	- 3.41*
	Vertebral & rib muscles	- 13.50	- 1.57			
	Pectoral muscles	- 14.40	- 2.36			
	Semimembranosus	- 17.64	- 5.38**			
	Semitendinosus	- 25.82	- 9.52***			

+ Values of Student's t for null hypothesis that supporting side weight - bound side weight = 0
Significant values are marked with an asterisk (* = P<0.05; ** = P < 0.01; *** = P < 0.001)

Table 7: Comparison of the weights of various limb regions. The supporting side and bound side of seven treated lambs are expressed as a percentage of the carcass weight, and compared with that of the two controls. Each control lamb provided two sets of data (right and left). Values showing significant differences from the controls ($P < 0.05$) are shown with asterisks.

		Control lambs (n=4)		Treated lambs (n=7)			
				supporting side		bound side	
		Mean	s.d.	Mean	s d.	Mean	s.d.
	Scapula	0.210	0.019	0.261*	0.032	0.273*	0.028
PECTORAL	Muscles	0.524	0.023	0.585	0.142	0.679	0.149
	Humerus	0.332	0.006	0.356	0.034	0.363	0.037
BRACHIAL	Muscles	1.516	0.015	1.812*	0.230	1.877*	0.266
	Radius & Ulna	0.262	0.010	0.310	0.046	0.320*	0.045
ANTEBRACHIAL	Muscles	0.430	0.010	0.505	0.062	0.518*	0.047
	Femur	0.442	0.008	0.460	0.040	0.453	0.054
	Total muscle	3.324	0.226	3.264	0.383	3.091	0.419
FEMORAL	Semitendinosus	0.274	0.004	0.245	0.0 24	0.430*	0.055
	Quadriceps	0.903	0.069	1.045*	0.071	0.626*	0.113
	Tibia	0.337	0.009	0.384	0.049	0.342	0.047
CRURAL	Muscles	0.729	0.016	0.879*	0.113	0.697	0.106

Table 8: Values for mean differences and Student's t obtained by using the paired t test for various measurements of the femur and tibia bones for seven lambs with bound right hind limb.

	Femur		Tibia	
	Mean difference	t^+	Mean difference	t^+
Length of bone (cm)	-0.30	-1.22	-0.16	-0.33
TSA of cortex (cm^2)	+0.09	+0.41	+0.01	+0.45
TSA of shaft (cm^2)	+0.16	+0.71	+0.003	+0.06
Weight of ash (g)	+0.36	+0.32	+2.20	+3.16*
Percentage of ash	+0.34	+0.22	+1.14	+0.77

+ Values of Student's t for the null hypothesis, supporting side - bound side = 0. Significant value is marked with an asterisk (* = $P < 0.05$).

TSA = Transverse sectional area.

Table 9: Weight of the semitendinosus muscle and area measurements of its structural components, of supporting (left) and bound (right) sides, of lambs used in experimental induction of altered hind limb function. Lambs 1 to 7 are treated animals and 8 and 9 are controls. TSA = Transverse sectional area.

Sheep number		Muscle weight (g)	Actual TSA (cm^2)	Total fascicular TSA (cm^2)	Total fibre TSA (cm^2)	Mean fascicular TSA (μm^2)	Mean total fibre TSA (μm^2)	AL dense area: AL fibre TSA (μm^2)	AL dense area: AH fibre TSA (μm^2)	AL sparse area: AL fibre TSA (μm^2)	AL sparse area: AH fibre TSA (μm^2)
1	L	48.2	5.60	5.21	4.37	70700	1100	1690	1500	1060	1170
	R	83.1	8.52	7.84	6.90	73100	1820	2110	2000	2950	2070
2	L	41.9	5.08	4.37	3.45	61400	1190	1680	1380	1010	1240
	R	59.8	6.07	5.33	4.78	68300	1260	2400	1300	2890	1380
3	L	23.0	4.43	3.63	2.97	44800	494	462	579	480	435
	R	39.0	5.07	4.45	3.63	50300	583	889	698	782	580
4	L	25.7	3.35	2.87	2.27	39000	764	826	1010	631	682
	R	45.5	4.20	3.78	3.02	50500	1181	1220	1300	989	1000
5	L	36.5	3.71	3.01	2.38	76800	1420	1340	1510	1390	1780
	R	71.0	7.46	6.88	5.18	96100	1980	3650	1910	2360	1540
6	L	35.0	5.12	4.00	3.37	37300	750	608	695	611	885
	R	66.0	7.05	6.46	5.52	68500	1110	1630	1390	1550	1330
7	L	26.0	3.57	3.07	2.33	43100	778	733	830	694	785
	R	47.5	5.81	5.13	3.96	55100	1100	1680	1020	1100	1050
8	L	46.0	4.62	4.04	3.31	36400	773	913	857	869	928
	R	44.5	4.51	3.74	3.18	38300	843	938	1020	926	1060
9	L	42.0	4.89	4.29	3.54	54900	1150	1190	999	1430	1030
	R	42.5	4.74	4.31	3.70	52700	1040	1110	1070	1040	920

Table 10: Values for mean differences and Student's t, obtained by using the paired t test for structural components of the semitendinosus muscle, for seven lambs with bound right hind limbs.

	Mean difference	t value+
Actual TSA (cm^2)	-1.90	-4.33**
Total fascicular TSA (cm^2)	-1.96	-4.56**
Total fibre TSA (cm^2)	-1.69	-5.33**
Mean fascicular TSA (μm^2)	-12685	-3.42*
Mean fibre TSA (μm^2)	-363	-4.08**
AL dense area		
AL fibre TSA (μm^2)	-891	-3.49*
AH fibre TSA (μm^2)	-302	-3.11*
AL Sparse area		
AL fibre TSA (μm^2)	-964	-3.72**
AH fibre TSA (μm^2)	-282	-2.15

+ Values of Student's t for the null hypothesis that supporting side area - bound side area = 0. Significant values are marked with an asterisk (* = $P < 0.05$, ** = $P < 0.01$).

TSA = Transverse sectional area.

Table 11: Double logarithmic regression analysis[+] comparing M. semitendinosus of the supporting and bound sides of seven lambs with bound right hind limbs.

	b	s_b	t
a) Changes in shape x = muscle weight (g) y = actual TSA (cm^2)	0.63	0.21	0.16 (0.67)
b) Changes in connective tissue			
i) perimysium x = actual TSA (cm^2) y = total fascicular TSA (cm^2)	1.18	0.40	0.45 (1.00)
ii) perimysium and endomysium x = actual TSA (cm^2) y = total fibre TSA (cm^2)	1.21	0.39	0.54 (1.00)
iii) endomysium x = total fascicular TSA (μm^2) y = total fibre TSA (μm^2)	1.06	0.34	0.17 (1.00)
c) Changes in fascicle number x = mean fascicular TSA (μm^2) y = total fascicular TSA (μm^2)	1.82	0.56	1.46 (1.00)
d) Changes in fibre number			
i) within muscle x = mean fibre TSA (μm^2) y = total fibre TSA (μm^2)	1.35	0.42	0.83 (1.00)
ii) within fascicle x = mean fibre TSA (μm^2) y = mean fascicular TSA (μm^2)	0.72	0.45	0.64 (1.00)
e) Changes in fibre size dimensions			
i) AL sparse area x = AL fibre TSA (μm^2) y = AH fibre TSA (μm^2)	0.39	0.33	1.83 (1.00)
ii) AL dense area x = AL fibre TSA (μm^2) y = AH fibre TSA (μm^2)	0.40	0.32	1.88 (1.00)

[+] The rate of increase of y with respect to x for each sheep was calculated and the value of the common slope b was tested for a significant difference from 1.00 or 0.67, as indicated, using Student's t test. s_b = standard error of b. None of the values is significant. TSA = Transverse sectional area.

Table 12: Measurements + related to ultrastructural components of the semitendinosus muscles of the control lambs and of the supporting and bound sides of experimental lambs with altered limb function.

	Control			Supporting side			Bound side		
	Mean	s.e	n	Mean	s.e.	n	Mean	s.e.	n
Sarcoplasm	19.1	4.2	8	19.4	4.2	7	17.3	2.1	7
Mitochondria	2.1	1.6	8	3.9	2.4	7	3.8	2.1	7
Myofibrillar component	78.7	5.0	8	76.7	5.2	7	80.1	3.5	7

+ Proportionate area of the sarcoplasm, mitochondria and the myofibrillar component expressed as a percentage of the area examined. n = number of fibres analysed. s.e. = standard error of the mean. None of the values is significant.

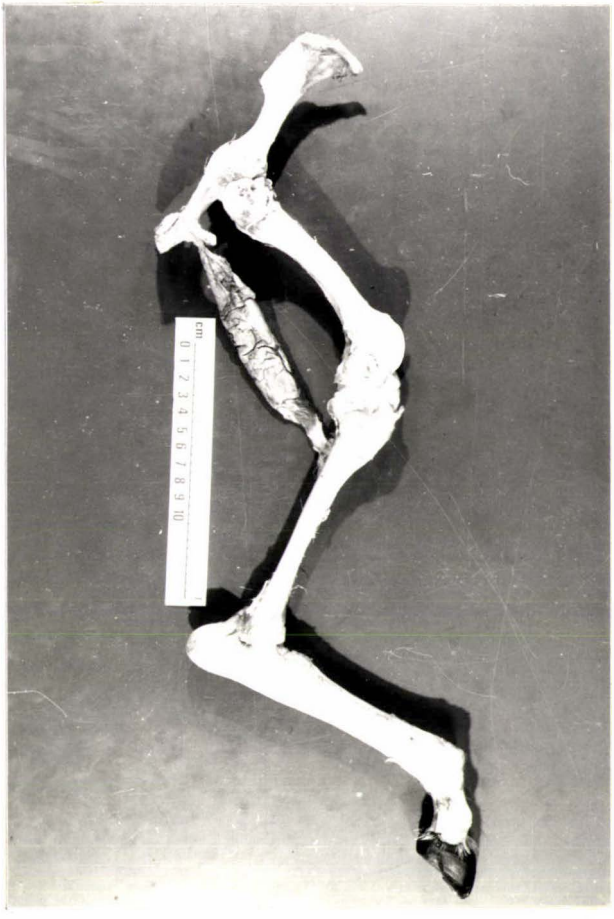


Figure 1: Attachments of the semitendinosus muscle of the sheep.

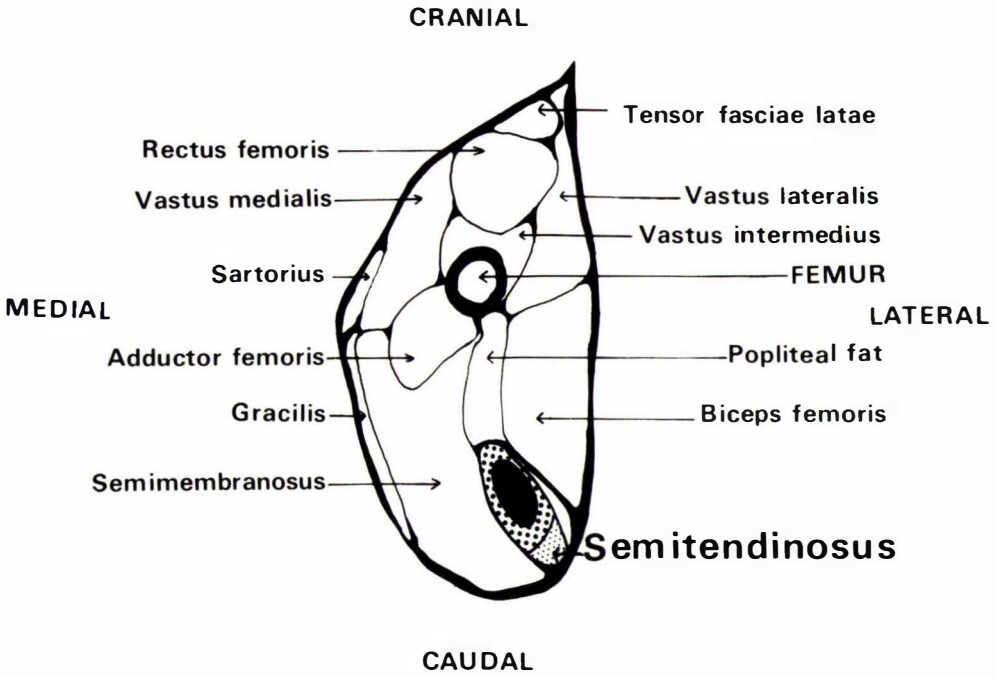


Figure 2: Schematic representation of the transverse section of the thigh of the adult sheep. The proportionate distribution of myosin ATPase low fibres as a percentage of total fibre population, in different regions of the semitendinosus muscle, is indicated by different shades. White represents an AL fibre population density of 3-6% of all fibres; small dots a density of 7-9%, large dots a density of 10-19%; and black, an AL fibre density of 20-30% of all fibres.

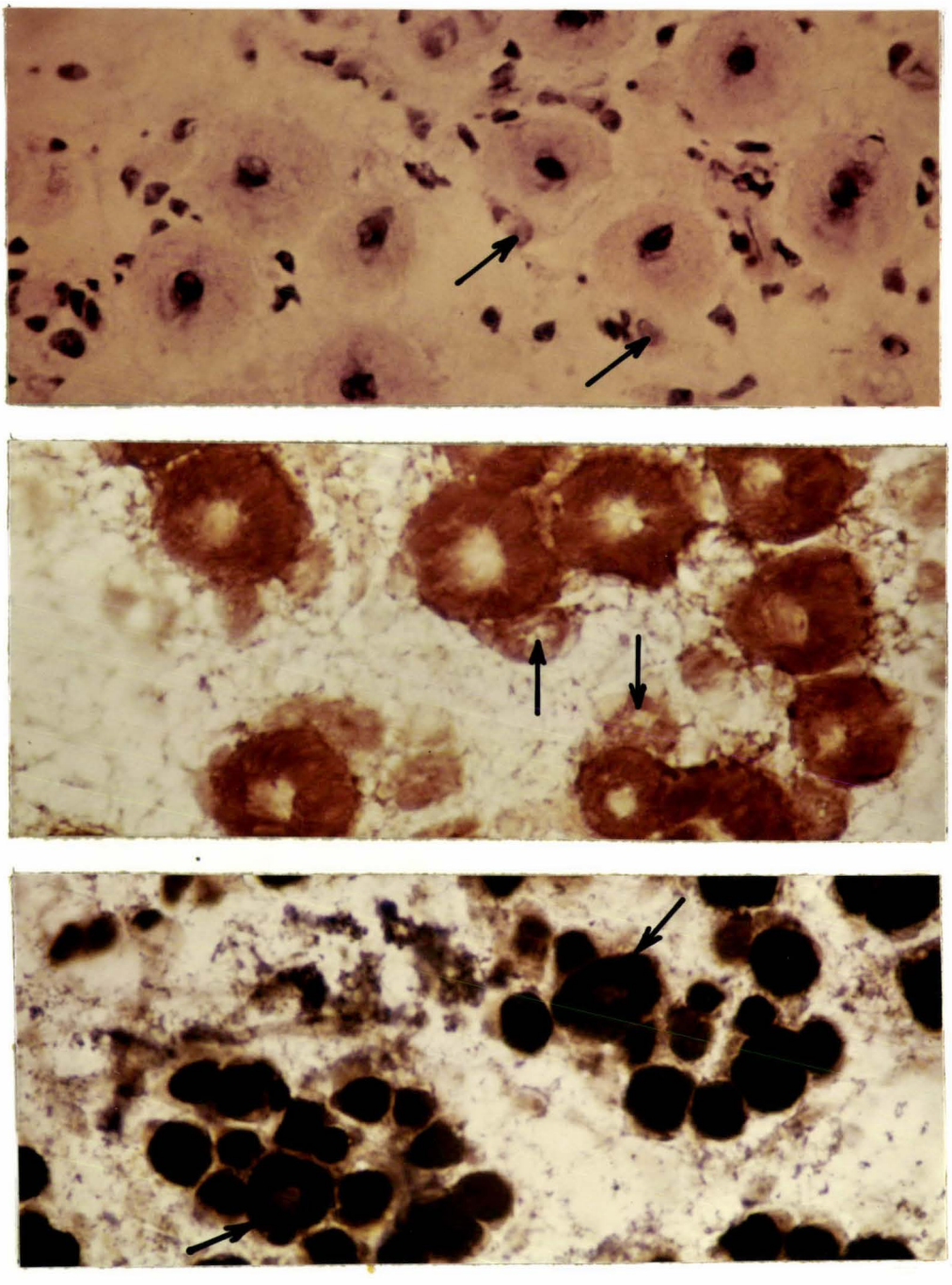


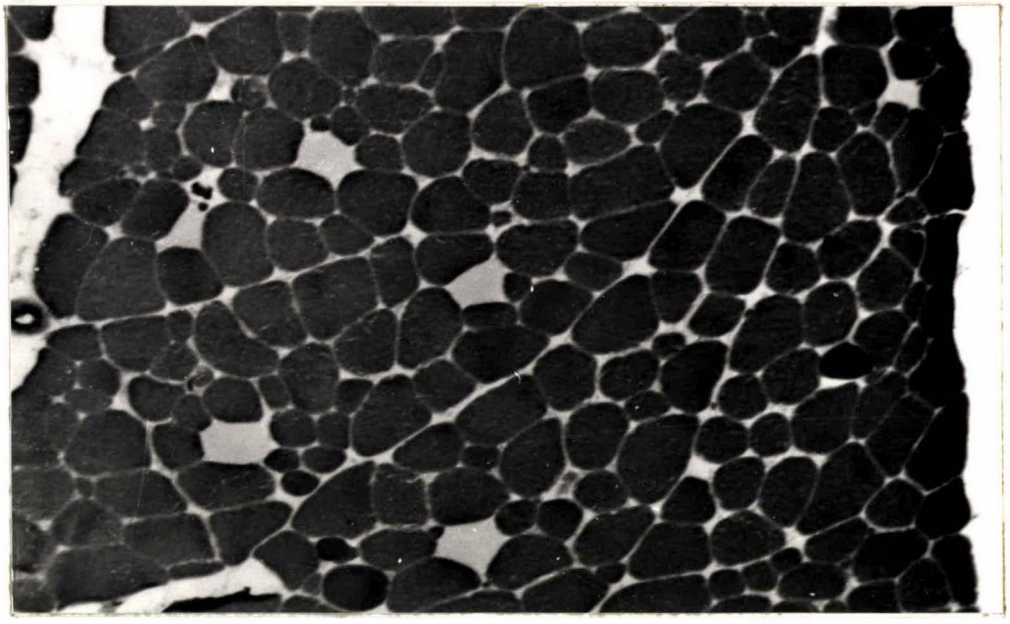
Figure 3. Frozen transverse sections of the semitendinosus muscle of the sheep, showing early differentiation of fibre types . x 650

a. PAS-hematoxylin staining of 60 days fetal muscle. Clusters of, large, primary generation cells with central nuclei are seen. Arrows indicate small sized secondary generation cells.

b. Alkali-stabile myosin ATPase staining of 60 days fetal muscle. The large cells show a pale central area. Arrows indicate the secondary generation cells.

c. Alkali-stabile myosin ATPase staining of 70 days fetal muscle. The large primary generation cells are widely separated and have a paler centre and a darker periphery (arrows). Many smaller cells are seen to surround individual primary generation cells.

a



b

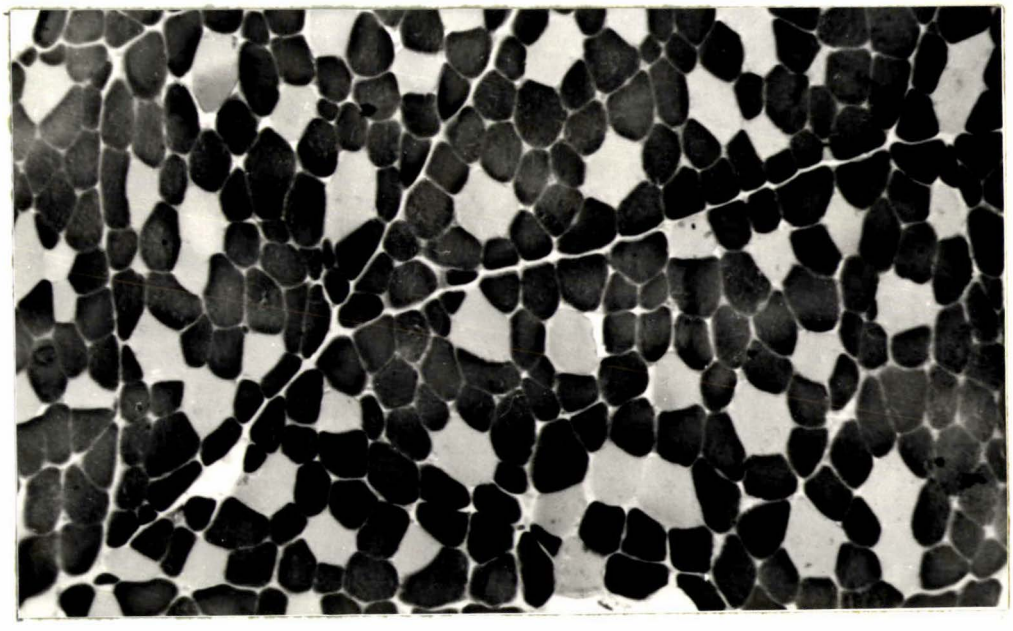


Figure 4. Frozen transverse sections of the semitendinosus muscle of a 30 kg live weight sheep, stained for myosin ATPase in regions of extreme difference in ATPase low (AL) fibre population density within the muscle. x 160.

a. AL sparse area, including the superficial lateral border of the muscle.

b. AL dense area from the deep medial part of the muscle.

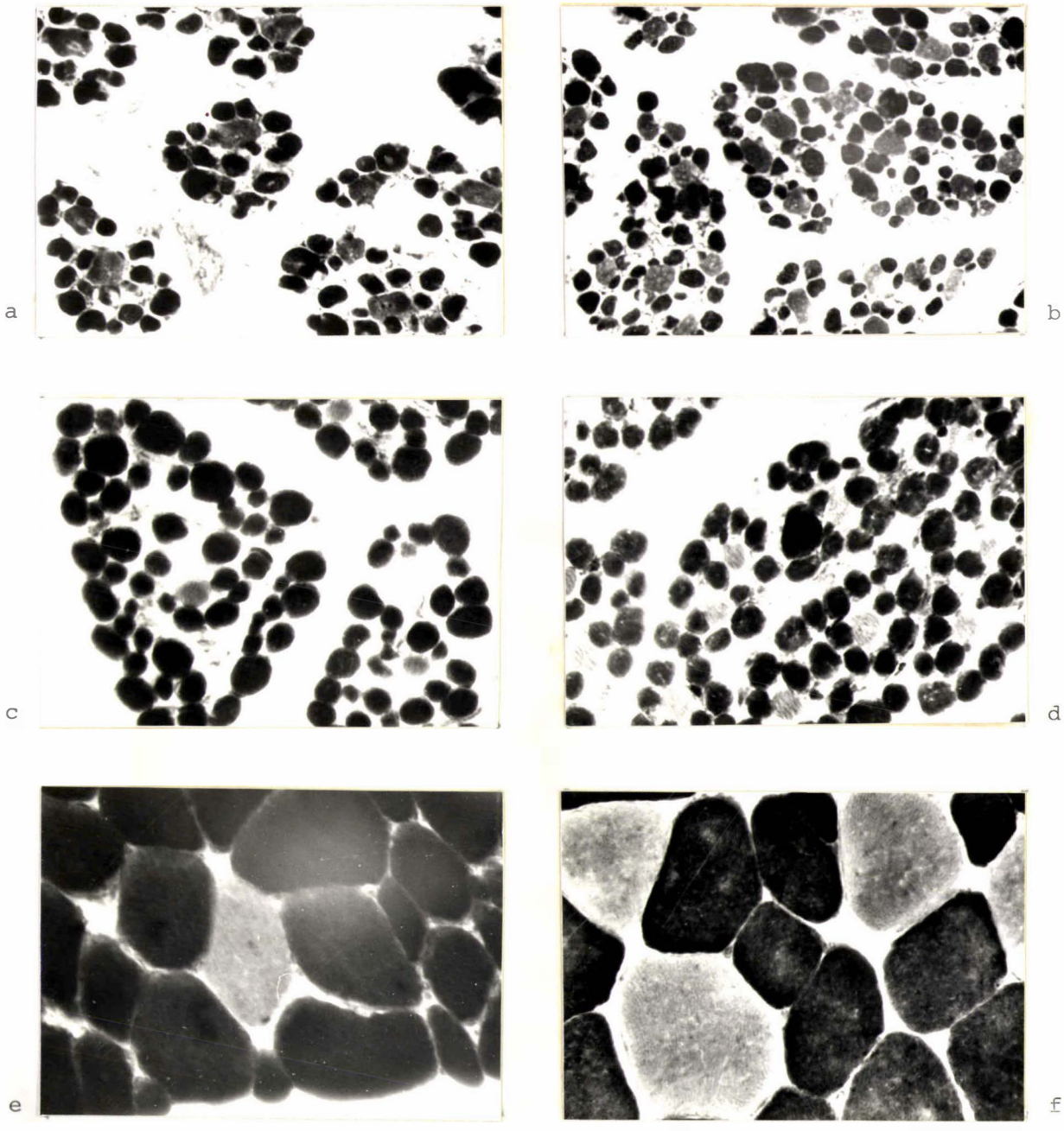


Figure 5: Frozen transverse sections of the semitendinosus muscle of the sheep, stained for myosin ATPase in regions of extreme difference in myosin ATPase low (AL) fibre population density within the muscle at three developmental stages. Top row, 80 days gestation; middle row, 100 days gestation; bottom row, adult. Left side: AL sparse area; right side: AL dense area. x 350.

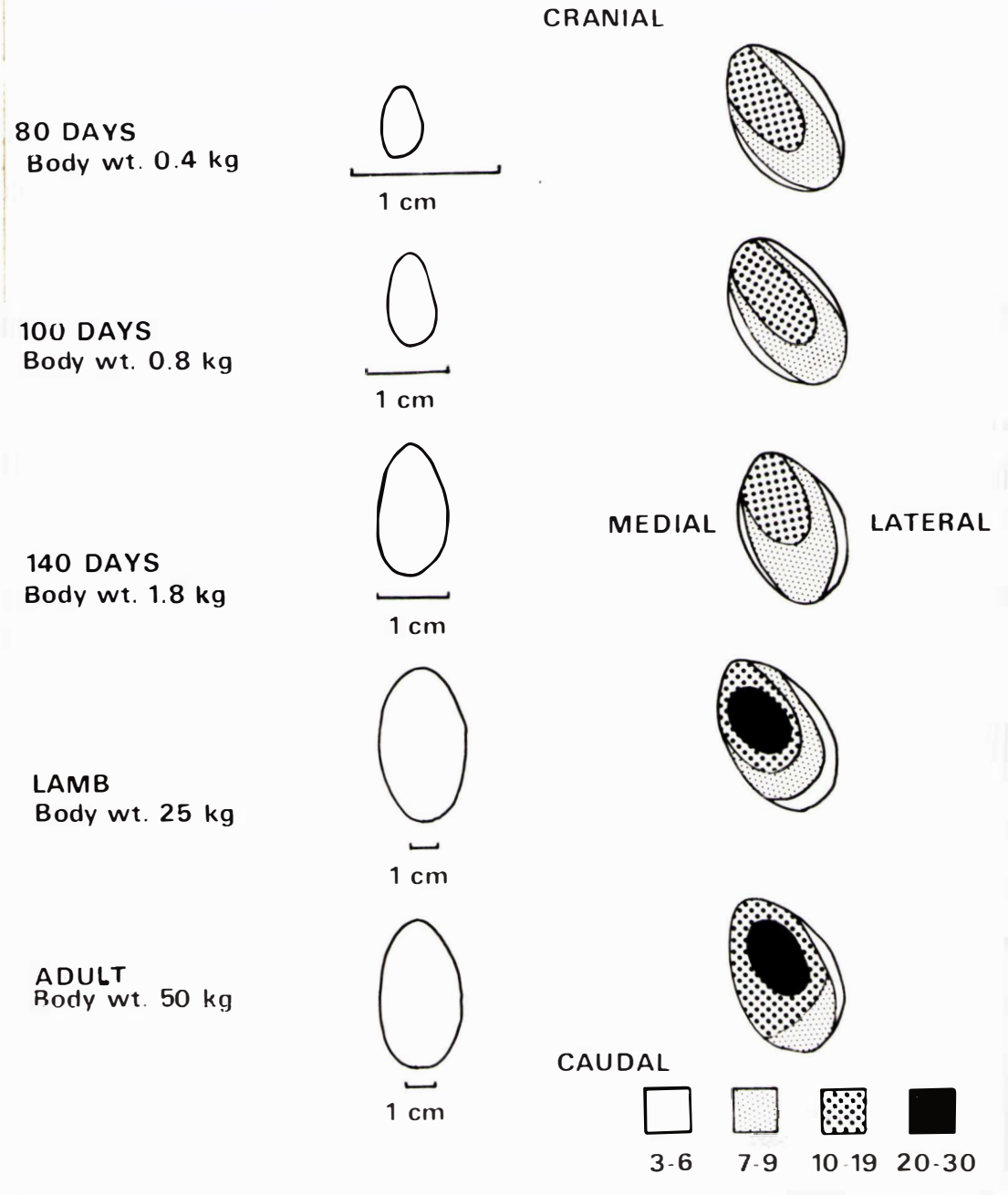


Figure 6: Schematic representation of regional changes involving ATPase low fibre density within the semitendinosus muscle of the sheep at 80, 100, 140 days gestation, and postnatally at 5 months and adult stages based on data from at least three sheep at each stage. Left column represents the transverse sectional size of the muscle between the middle and distal thirds. Right column represents the patterns of ATPase low fibre density, shaded as in Figure 2.

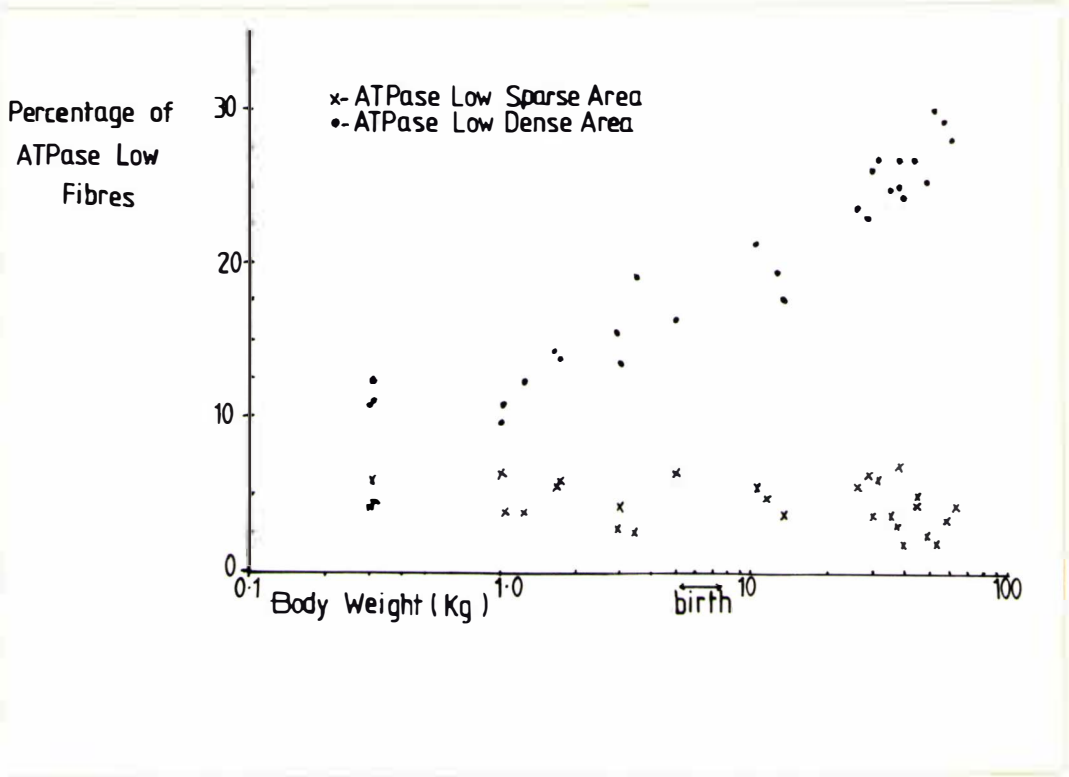


Figure 7: Relationship between log body weight and ATPase low fibre type density population, expressed as a percentage, in AL dense and AL sparse areas within the semitendinosus muscle of the sheep from 80 days gestation to adulthood.

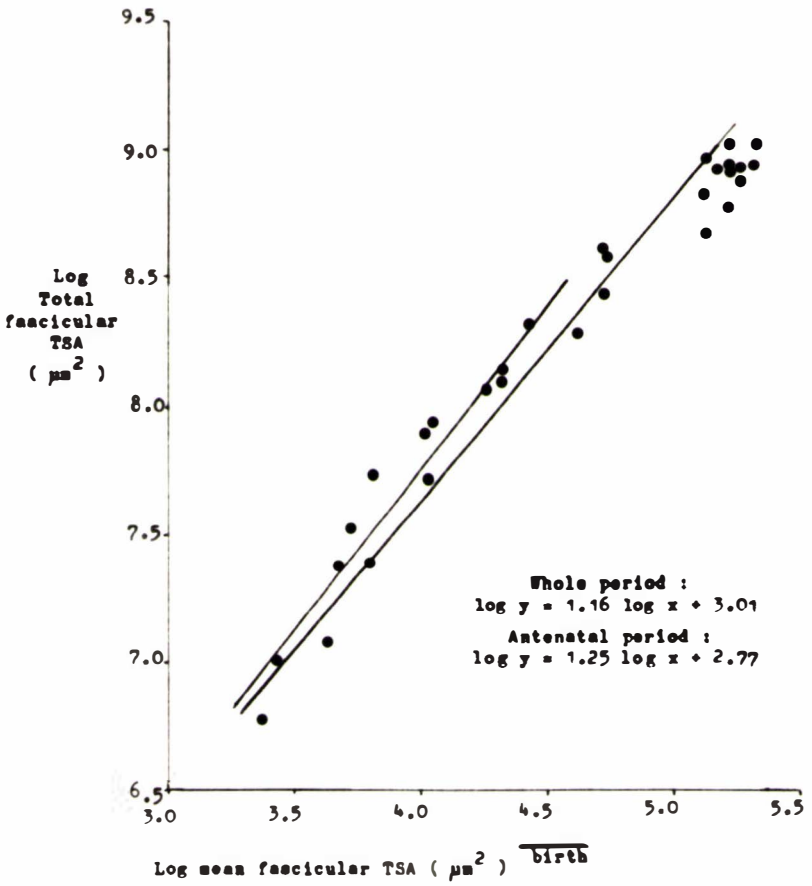


Figure 8: Changes in total fascicular number within the semitendinosus muscle of the sheep.

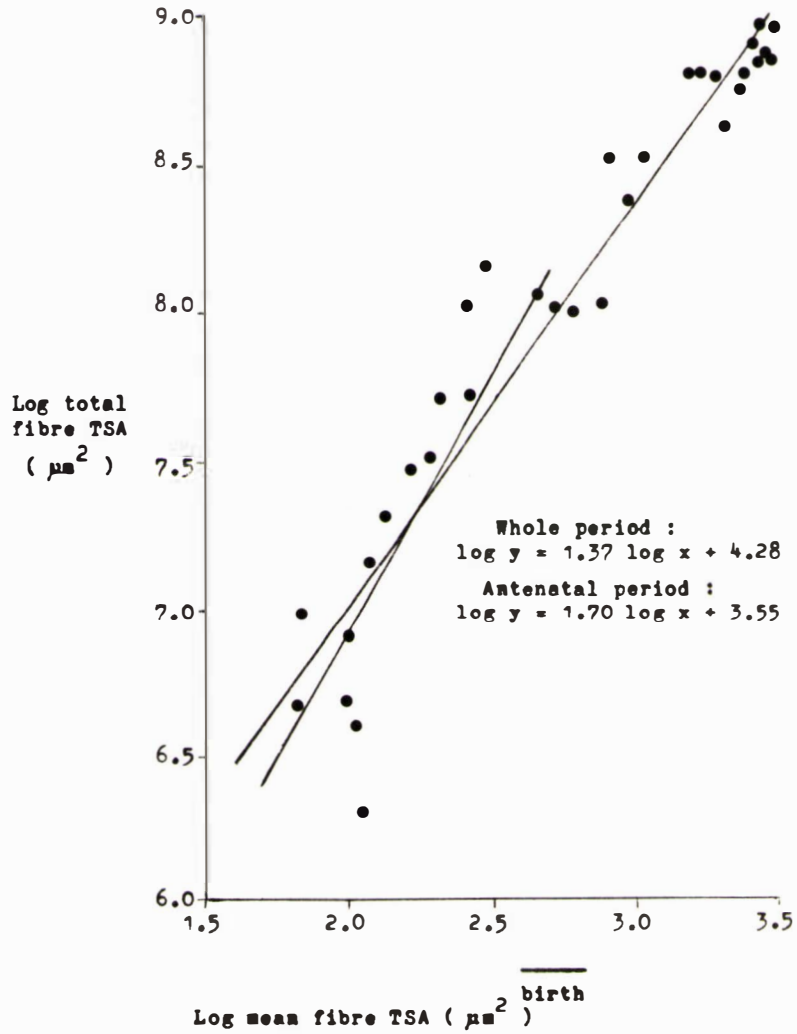


Figure 9: Changes in total fibre number within the semitendinosus muscle of the sheep.

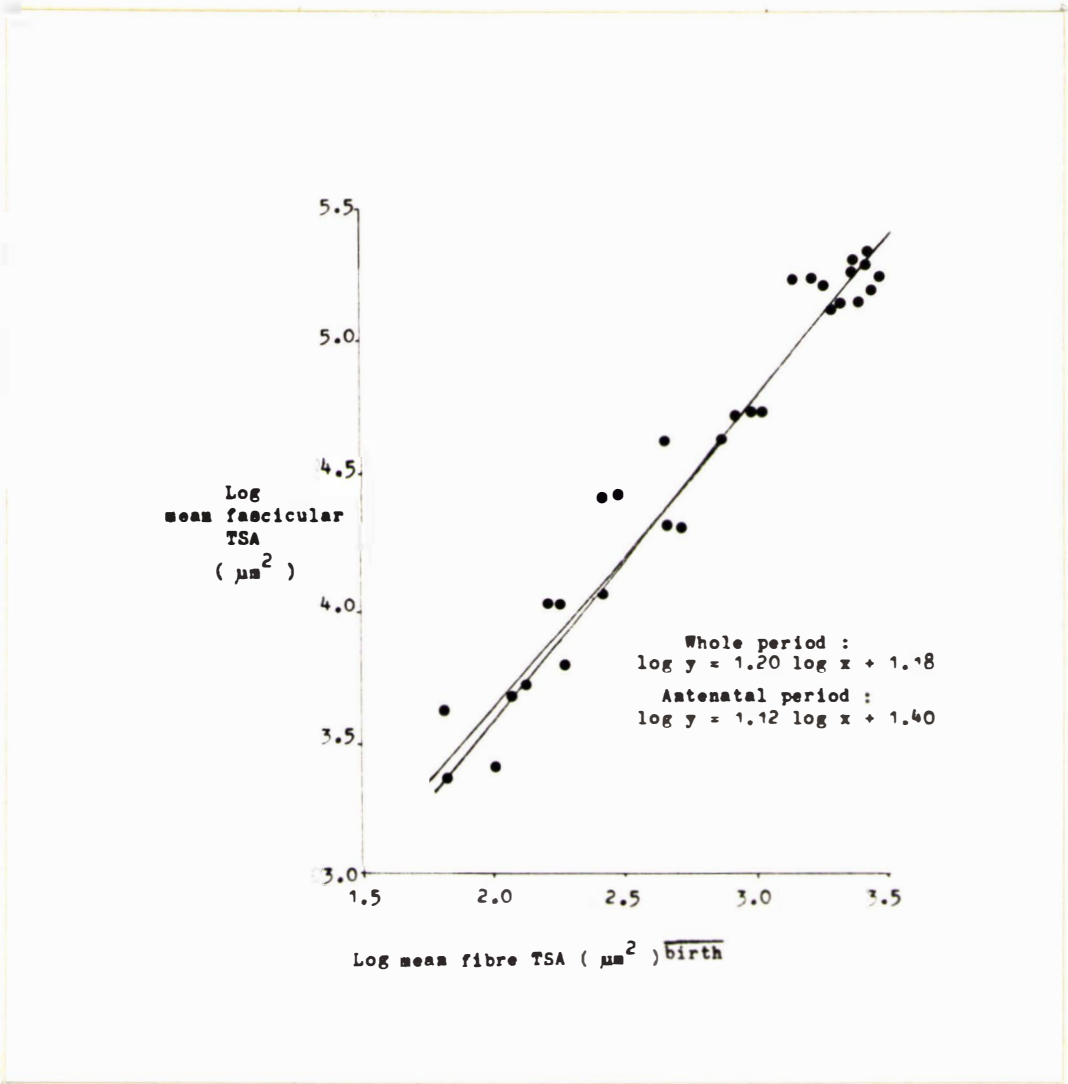


Figure 10: Changes in fibre number within individual fascicles of the semitendinosus muscle of the sheep.

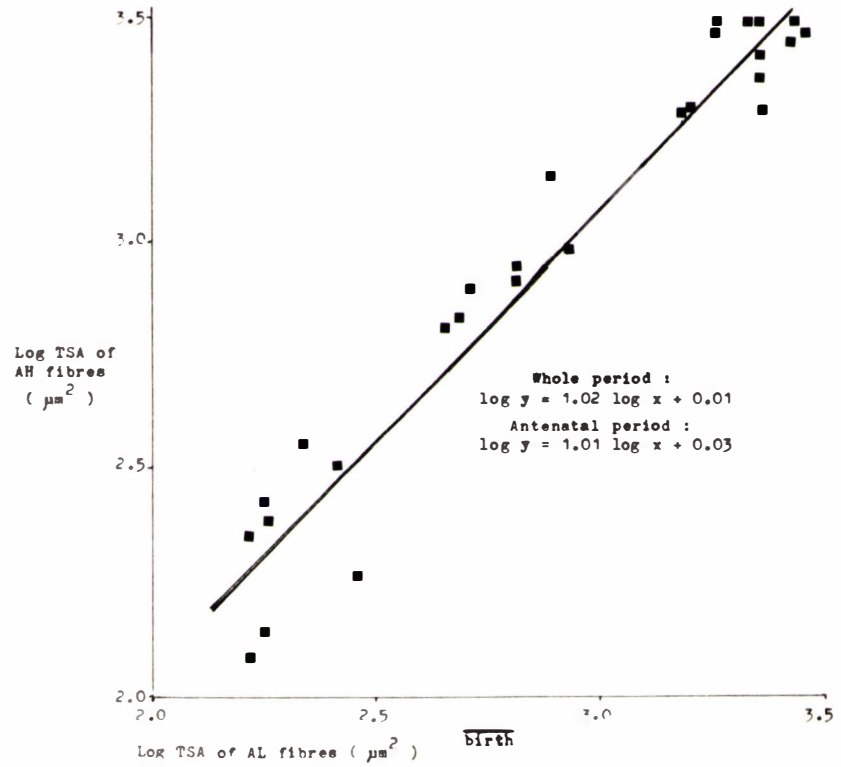


Figure 11: Relative changes in transverse sectional area of fibre types : AL sparse area.

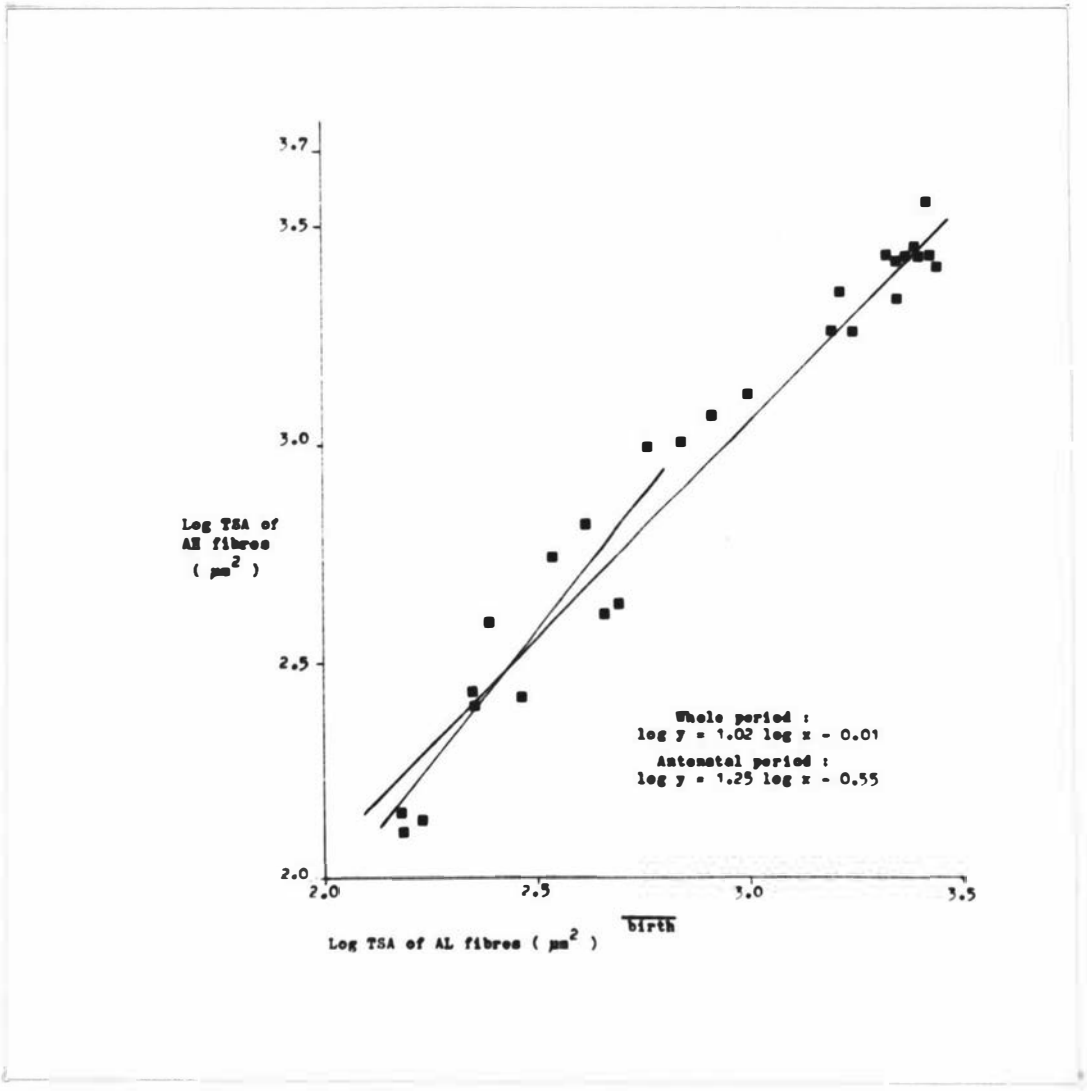


Figure 12. Relative changes in transverse sectional area of fibre types : AL dense area.

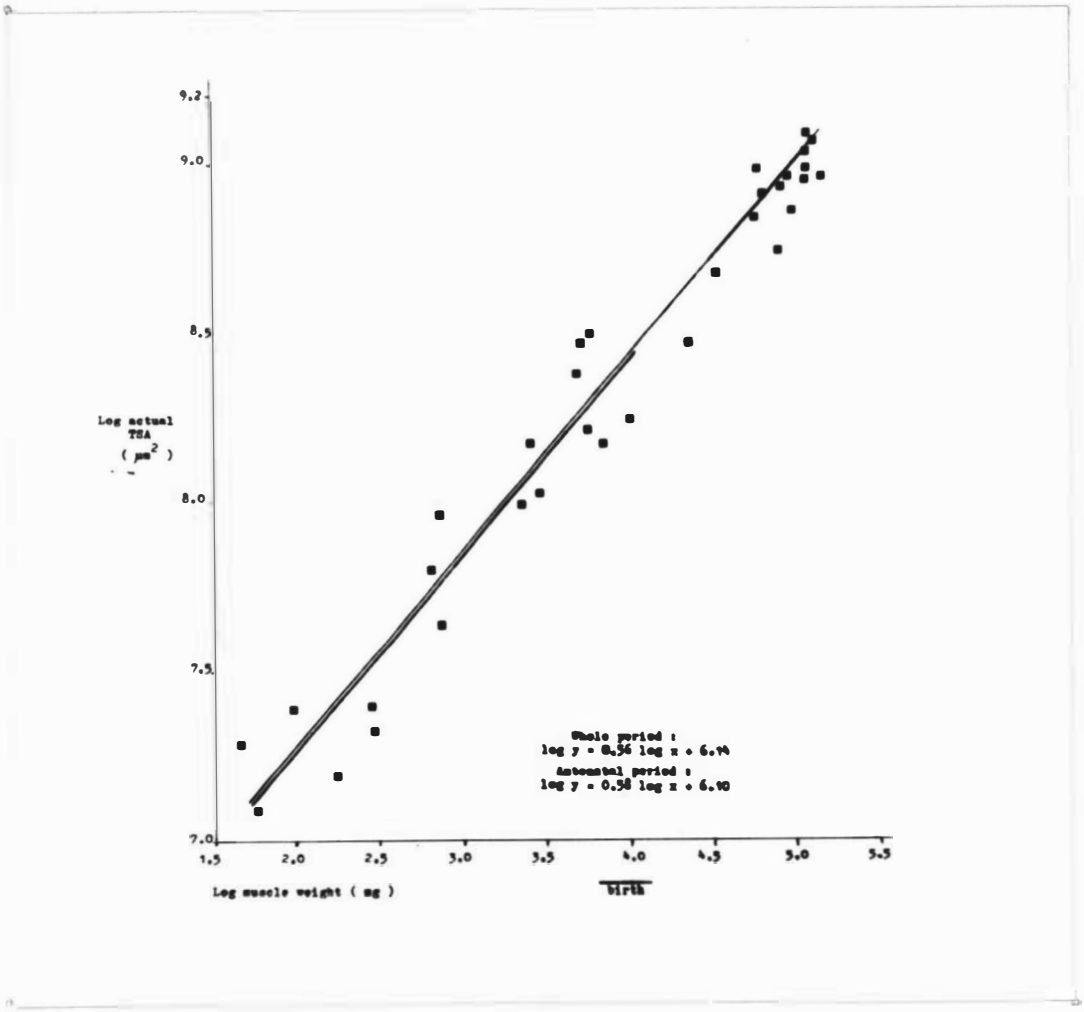


Figure 13: Changes in shape of the semitendinosus muscle of the sheep.

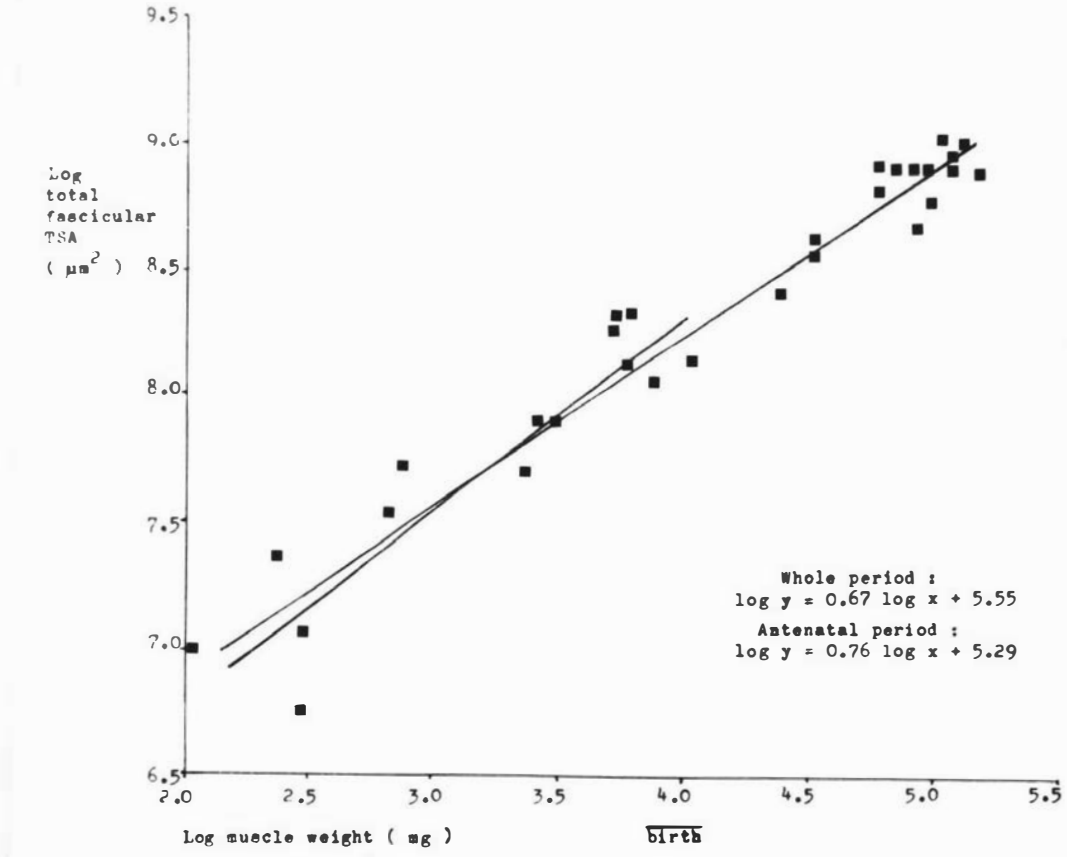


Figure 14: Changes in shape of the semitendinosus muscle of the sheep.

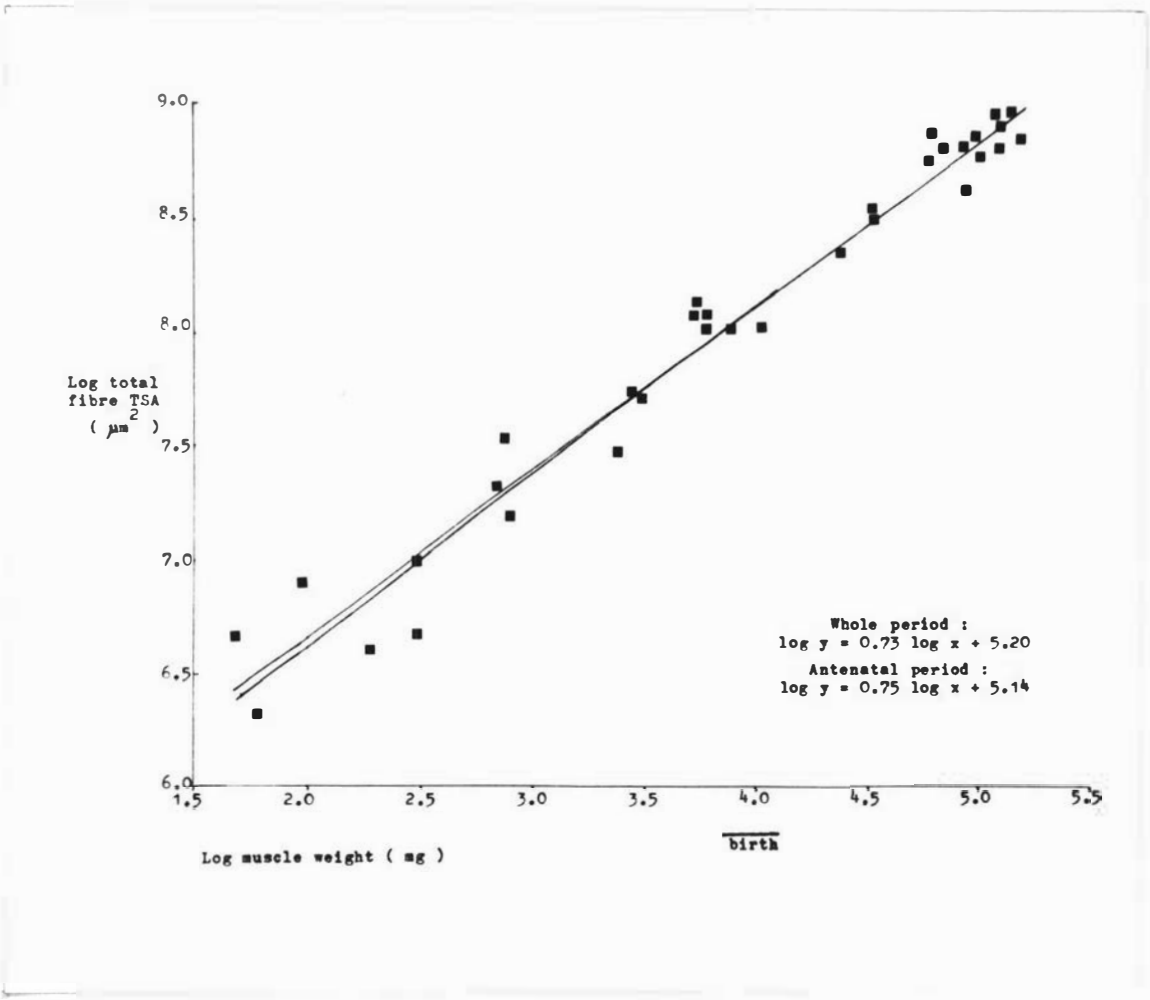


Figure 15: Changes in shape of the semitendinosus muscle of the sheep.

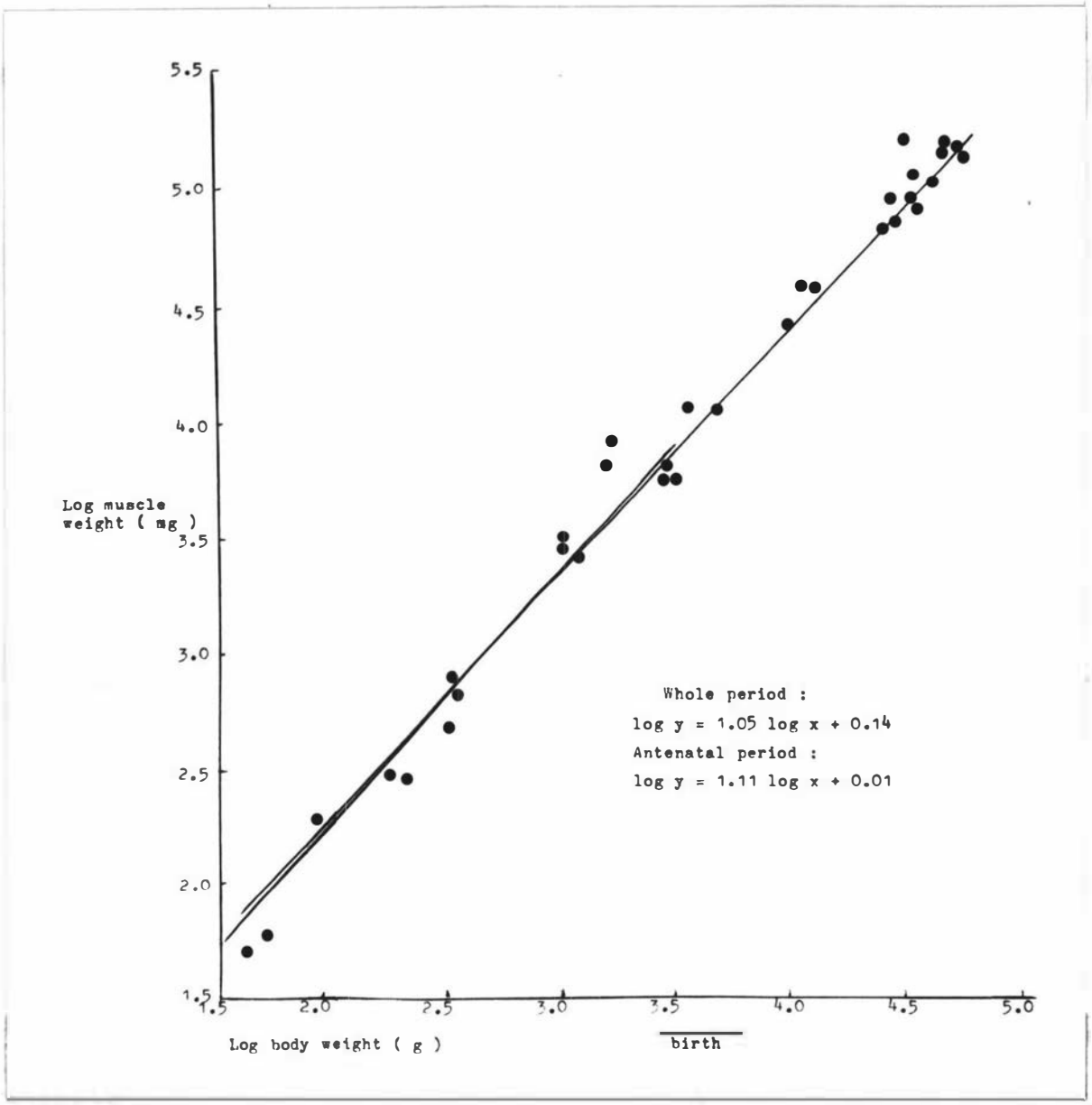


Figure 16: Weight changes with growth of the semitendinosus muscle of the sheep.

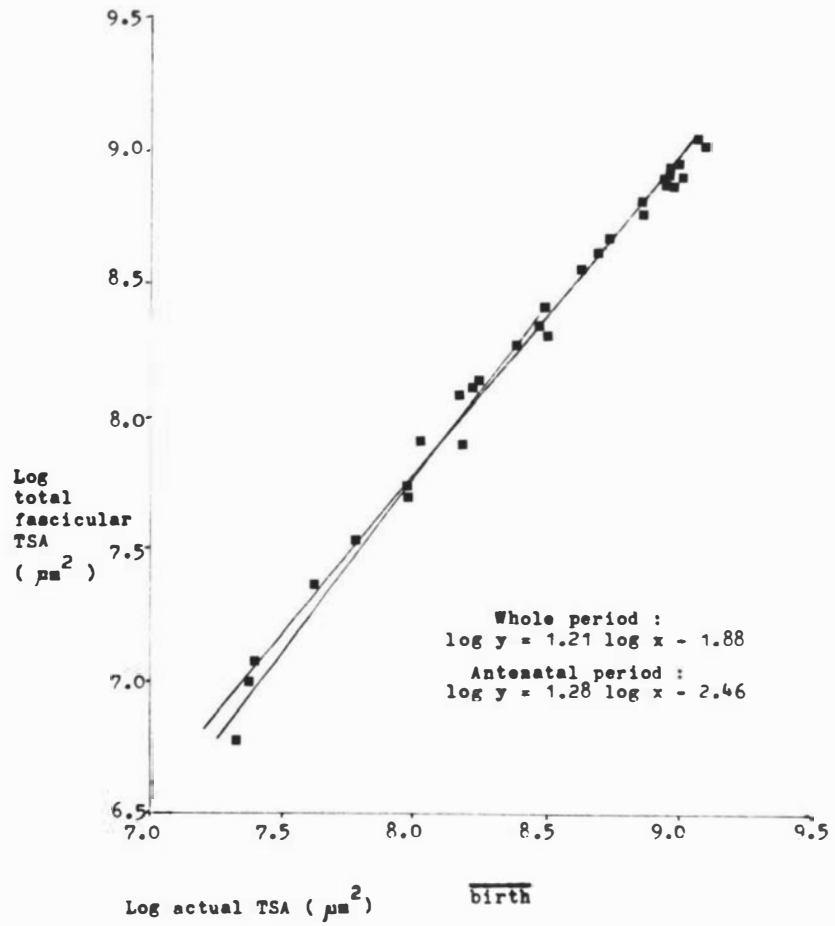


Figure 17: Changes in connective tissue skeleton : Perimysium.

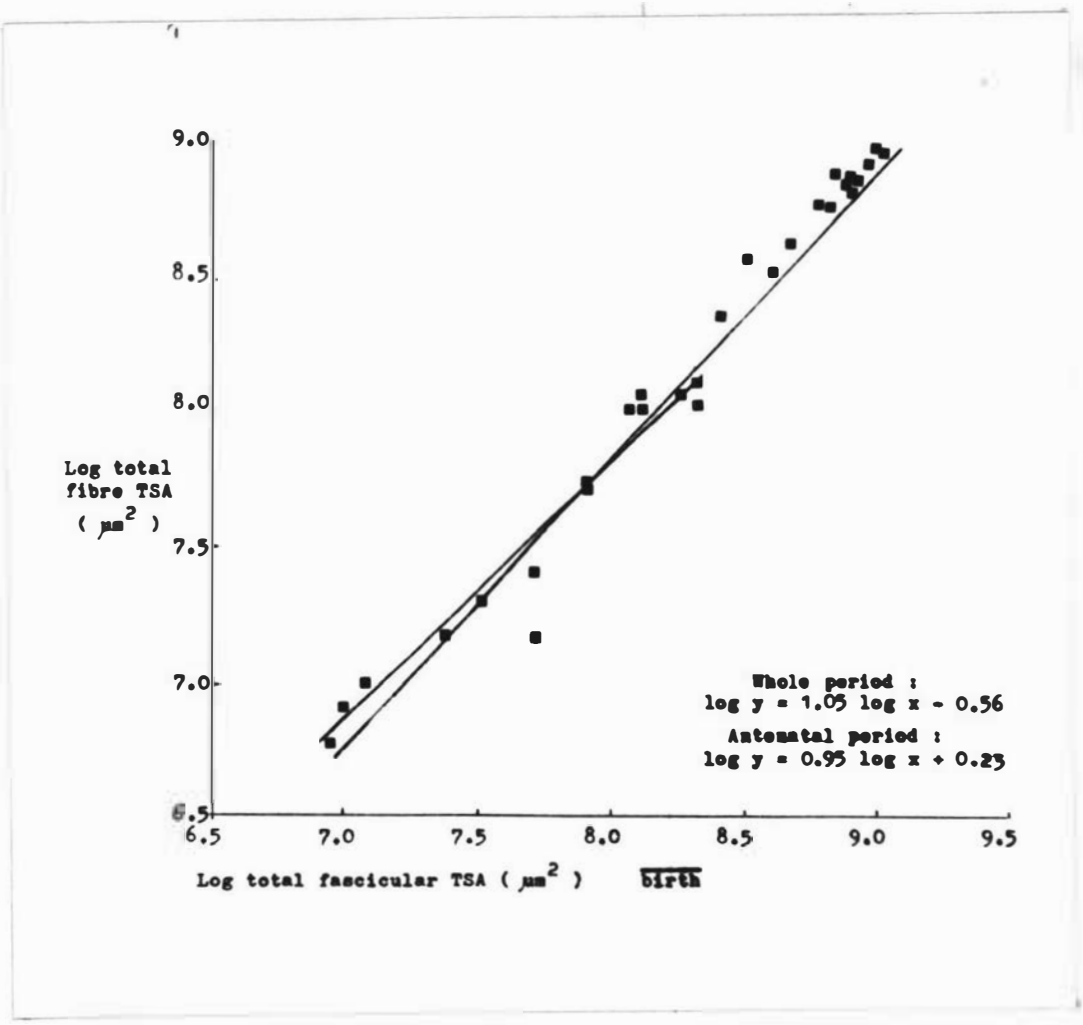


Figure 18: Changes in connective tissue skeleton : Endomysium.

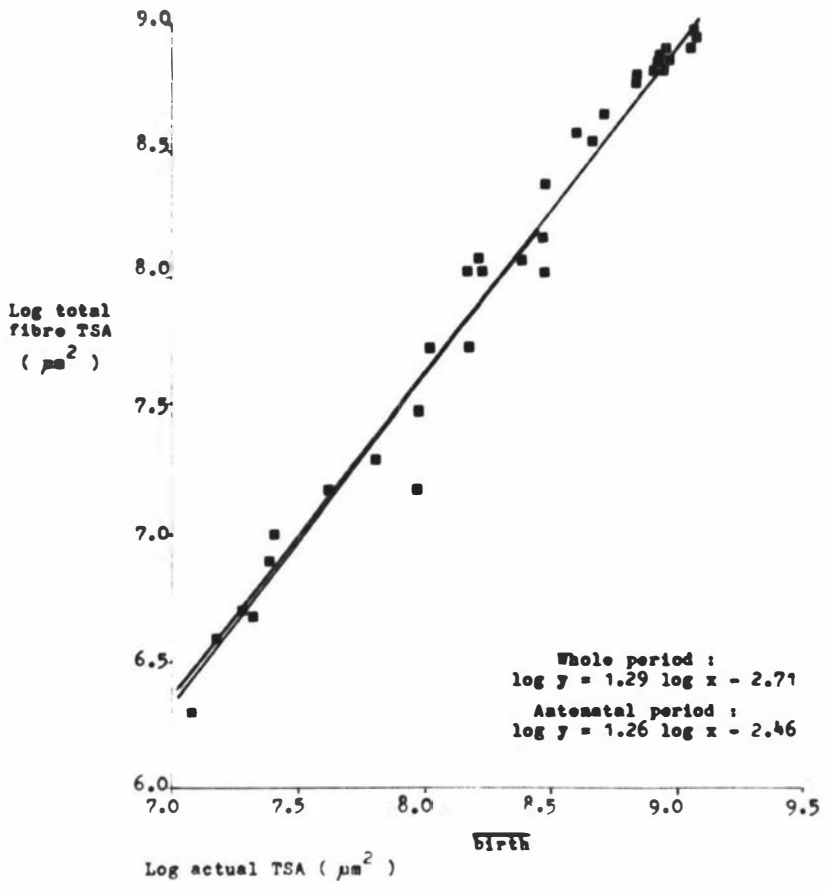


Figure 19: Changes in connective tissue skeleton : Perimysium and Endomysium.

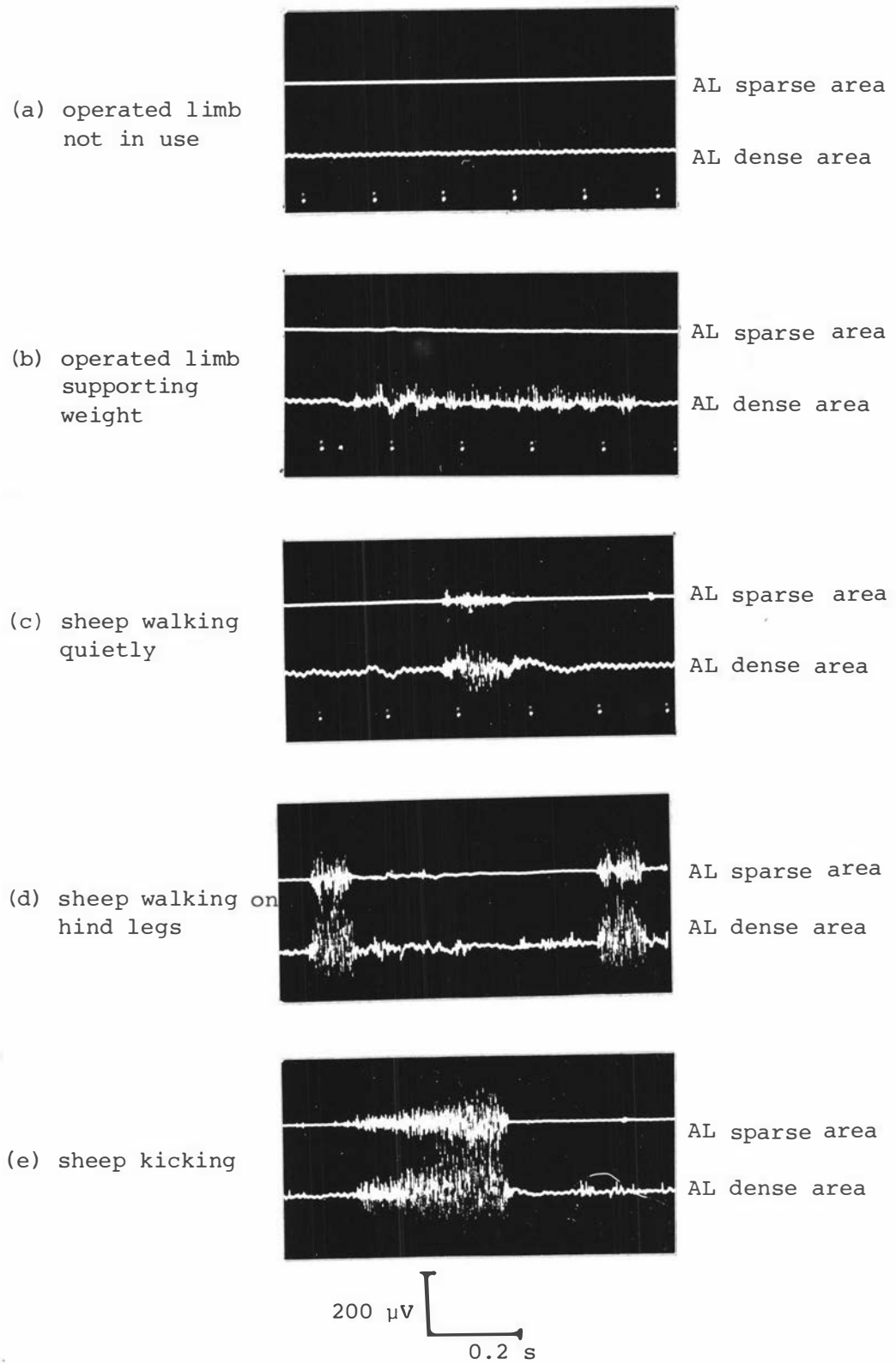


Figure 20: Simultaneous electromyographic recordings in extreme regions of fibre type population density in the semitendinosus muscle of adult sheep during varied limb activities.

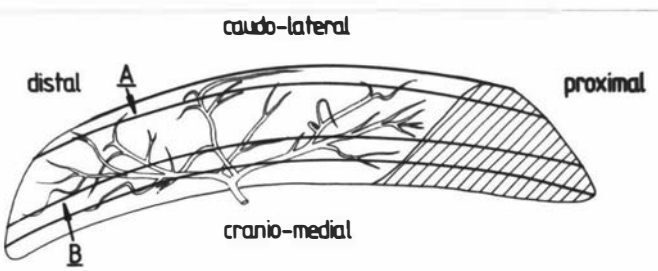
a. Sheep standing quietly. Operated limb not favoured.
b. Sheep made to use operated limb. Only the AL dense area is active.
c. Sheep walking quietly. Activity now extends to the AL sparse area.
d. Sheep made to walk on hind legs. Both areas show enhanced activity.
e. Sheep kicking. Both areas are active.

a

b

c

d



A - AL Sparse Area
B - AL Dense Area

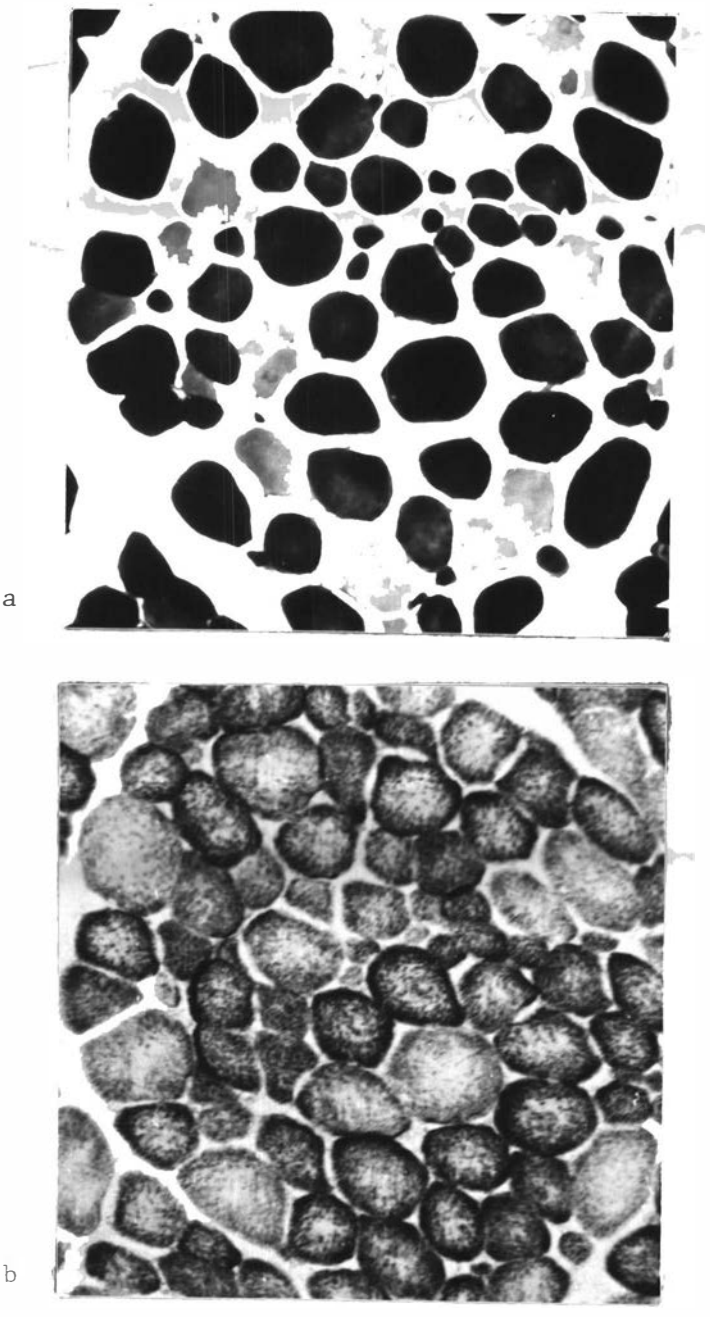
Figure 21: Blood supply to the portion distal to the tendinous septum of the semitendinosus muscle of the sheep.

a. Angiogram showing the venous drainage.

b. Corrosion cast showing the arterial supply.

c. Corrosion cast sculptured to show the main vessels.

d. Diagramatic representation of AL sparse and AL dense areas of the muscle in relation to the arterial supply. The portion of the muscle proximal to the tendinous septum is indicated by shading.



a

b

Figure 22: Frozen serial sections of the semitendinosus muscle of the adult sheep stained for alkali-stabile myosin ATPase and succinate dehydrogenase according to the method of Nachlas M.M. *et al*. 1957 (Appendix 2). x 160.

About 30% of the fibres within the fascicle have a low myosin ATPase reaction (a) while about 60% of them show high SDHase activity (b).

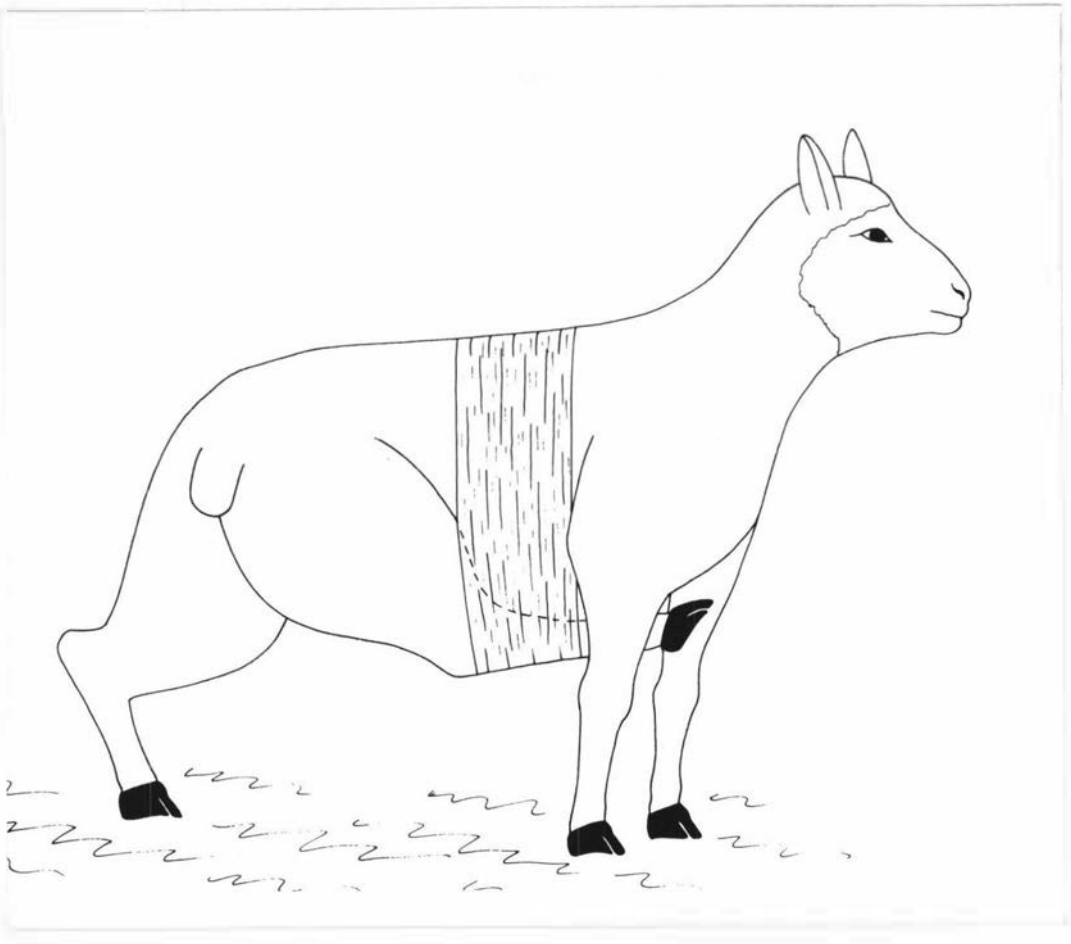


Figure 23: A schematic representation of a lamb with altered hind limb function. Each of the seven treated lambs had its right hind limb bound beneath its sternum with fully flexed hip and extended stifle and hock joints, from the second day after birth to about 3 months of age.

Figure 24: The semitendinosus muscles from the supporting (left) and bound (right) limb sides of a lamb with altered hind limb function. The muscle of the bound hind limb is larger than the one of the supporting limb.

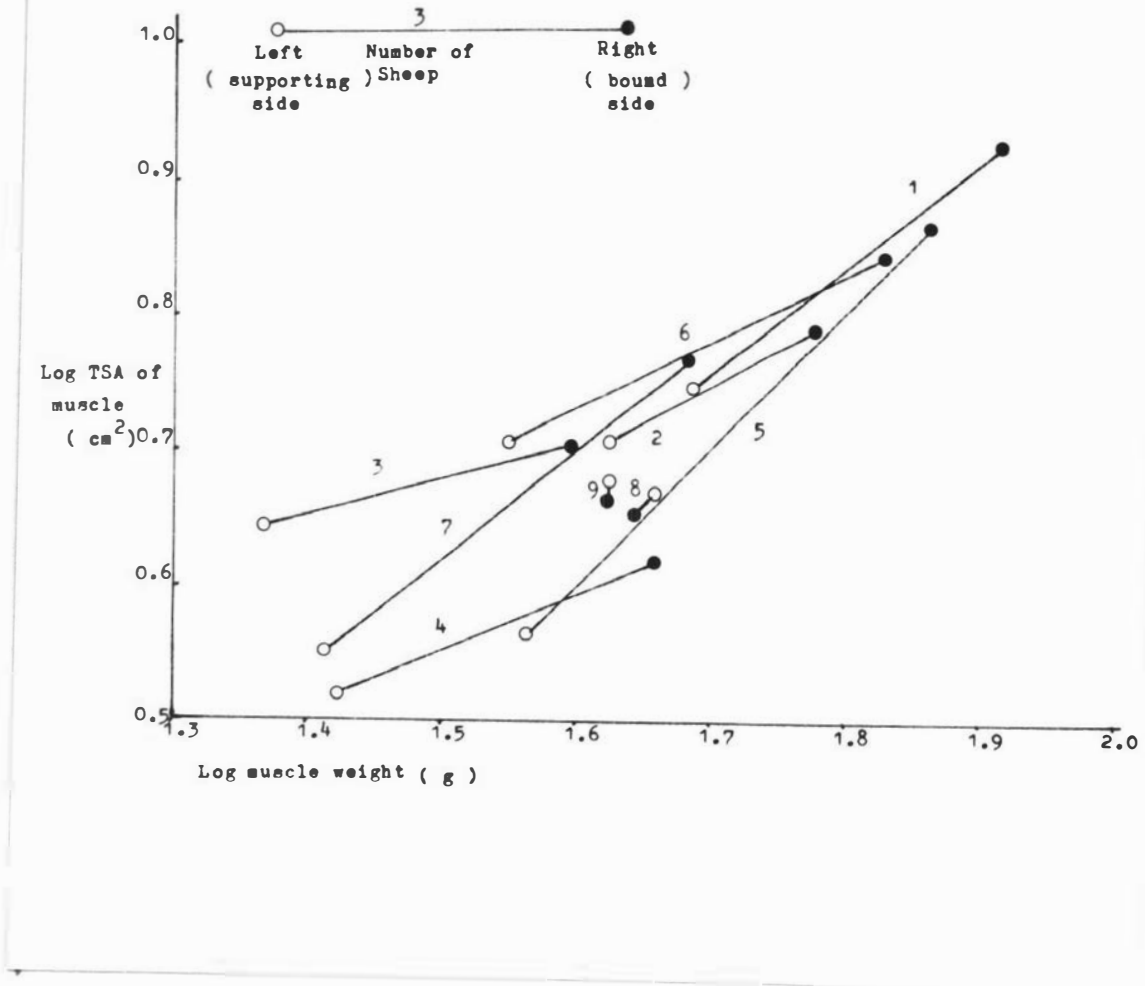


Figure 25: Shape changes in the semitendinosus muscle of the sheep due to chronic stretching.

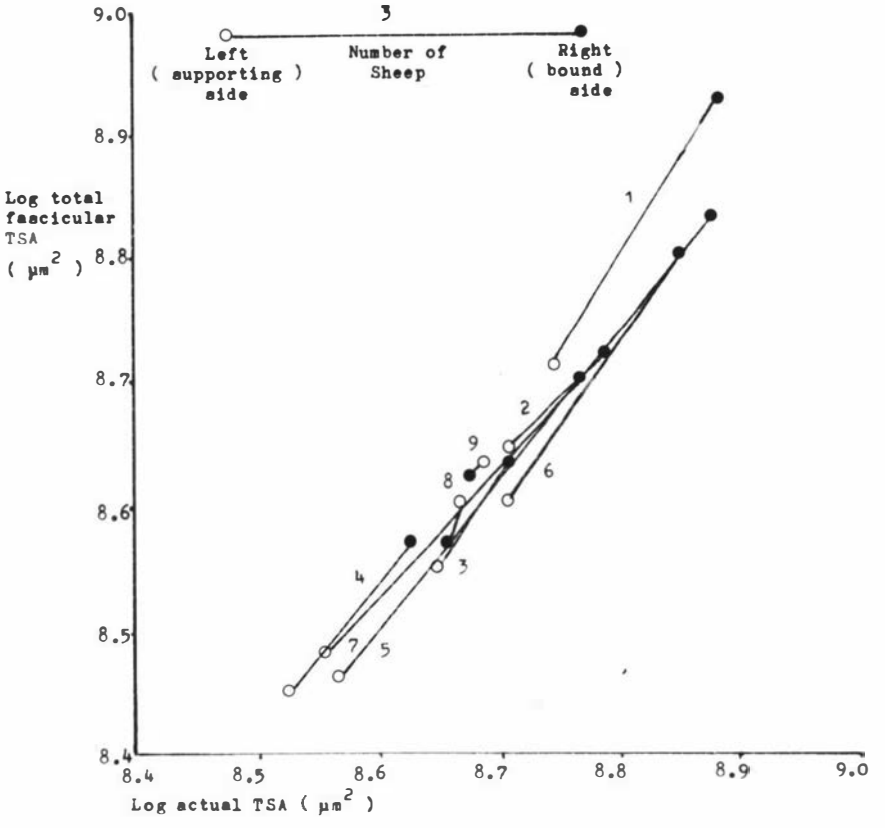


Figure 26: Changes in connective tissue content of the semitendinosus muscle of the sheep due to chronic stretching : Perimysium.

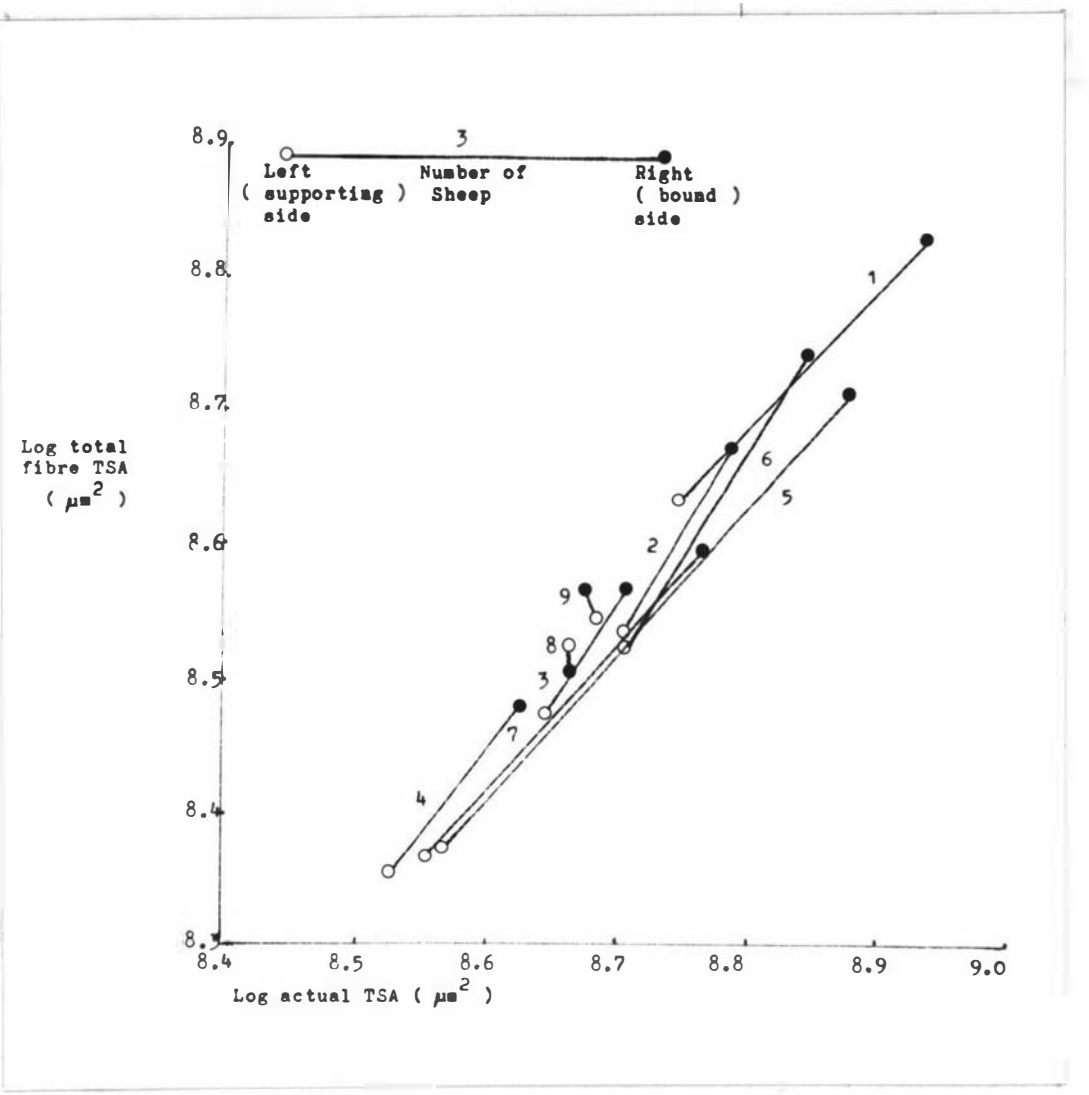


Figure 27: Changes in connective tissue content of the semitendinosus muscle of the sheep due to chronic stretching : Perimysium and Endomysium.

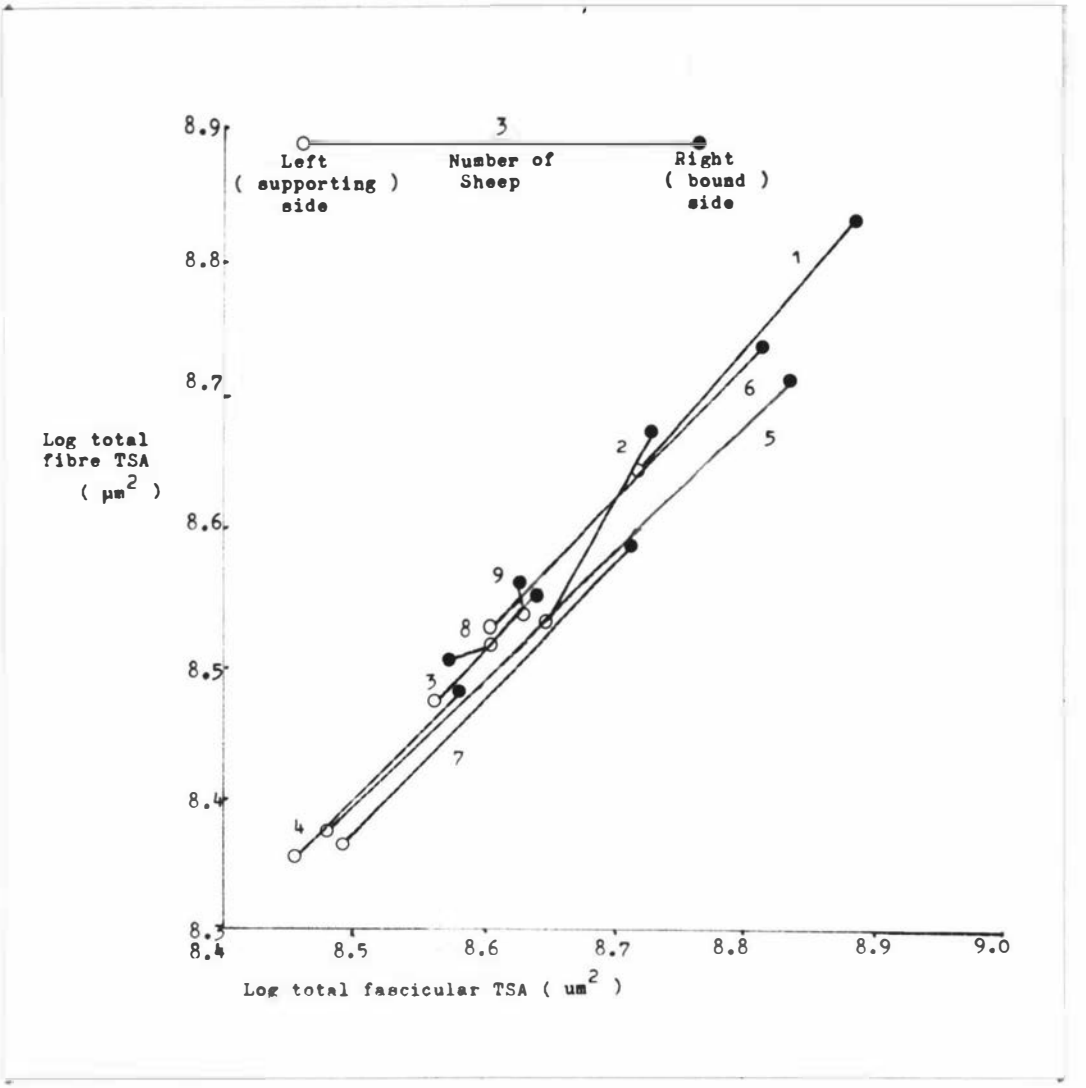


Figure 28: Changes in connective tissue content of the semitendinosus muscle of the sheep due to chronic stretching : Endomysium.

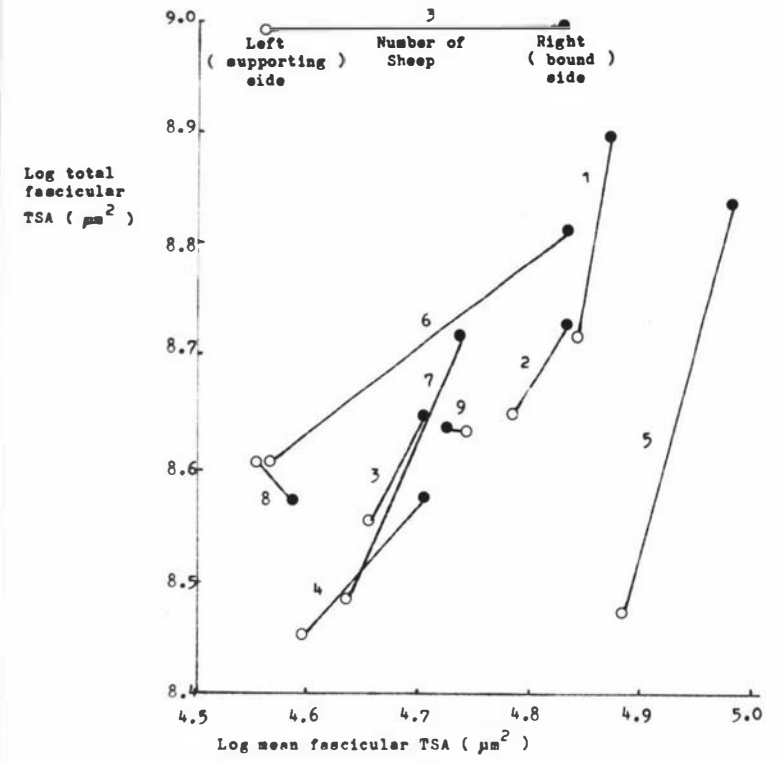


Figure 29: Changes in fascicle number of the semitendinosus muscle of the sheep due to chronic stretching.

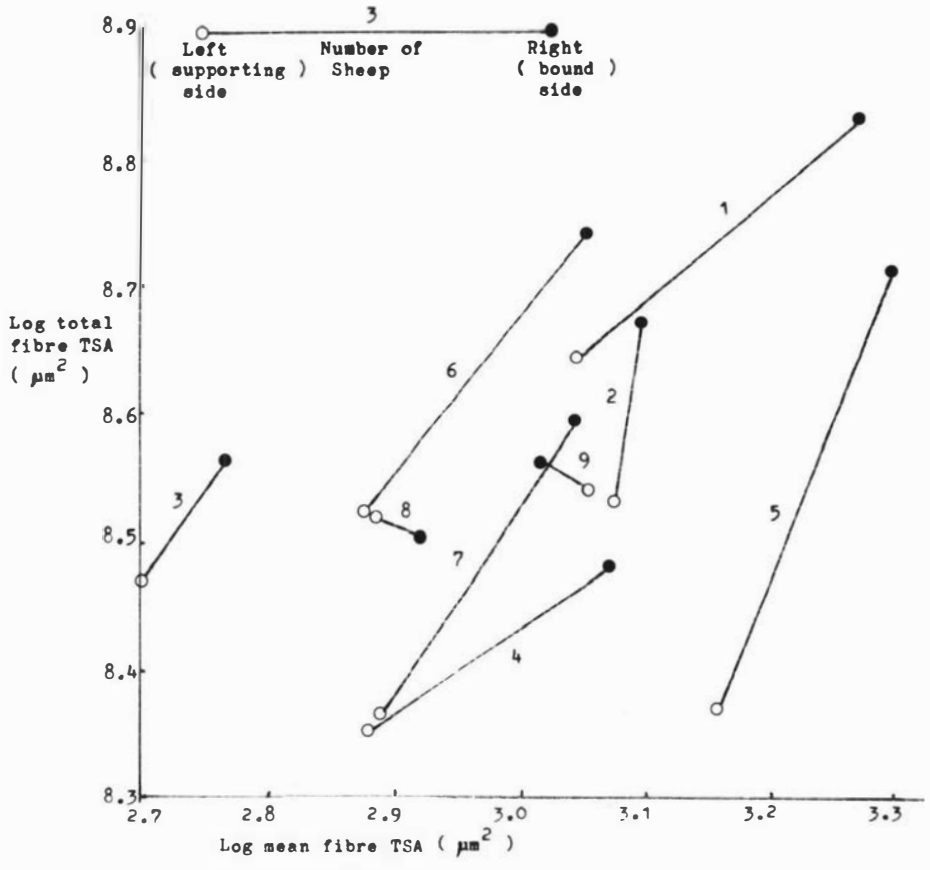


Figure 30: Changes in fibre number of the semitendinosus muscle of the sheep due to chronic stretching.

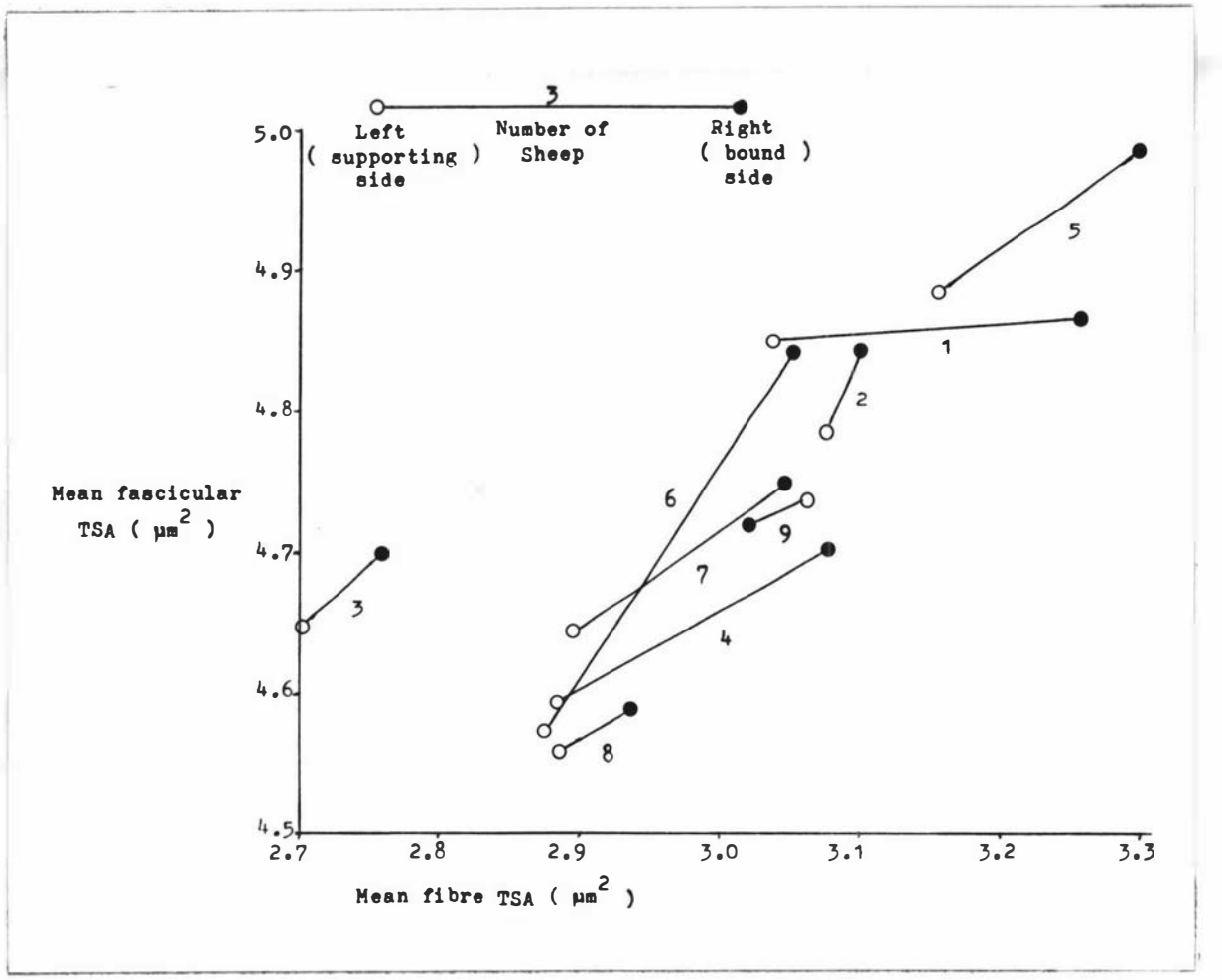


Figure 31: Changes in fibre number within individual fascicles of the semitendinosus muscle of the sheep due to chronic stretching.

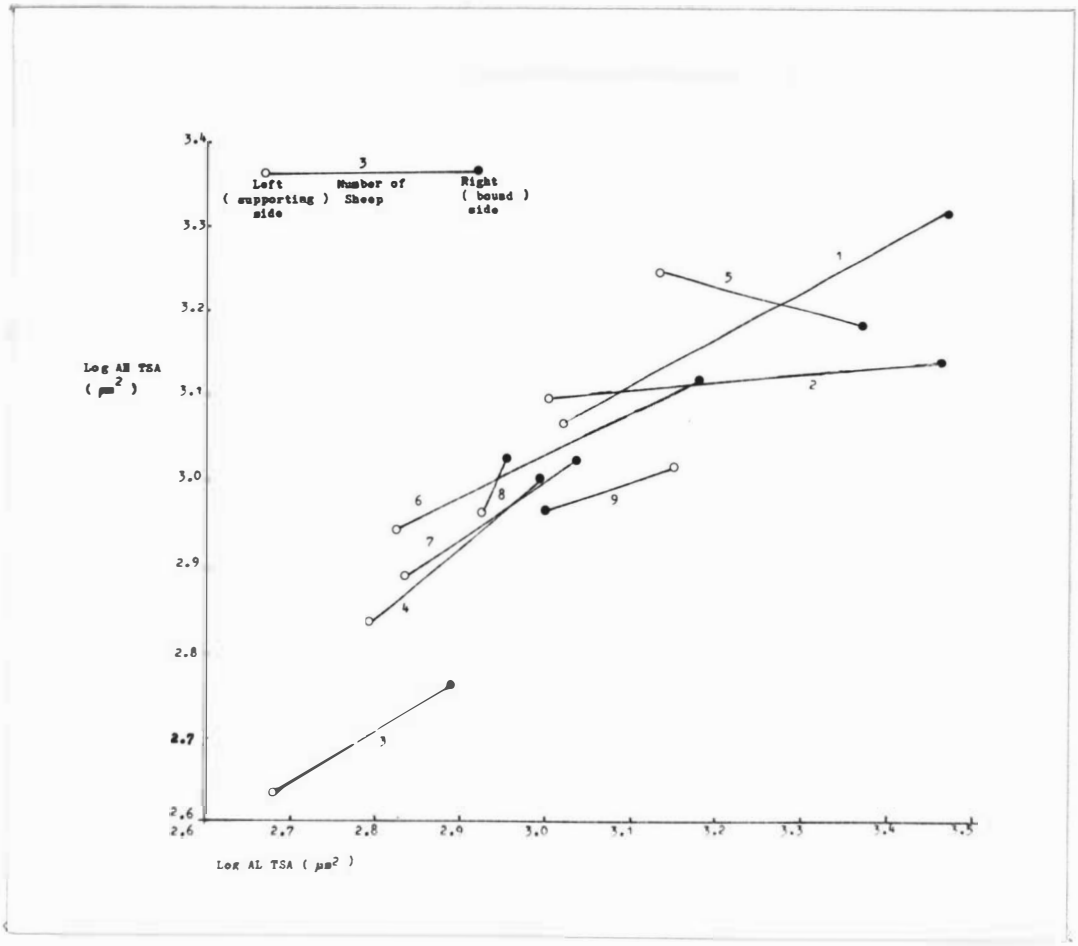


Figure 32: Changes in fibre size proportions in the AL sparse area of the semitendinosus muscle of the sheep due to chronic stretching.

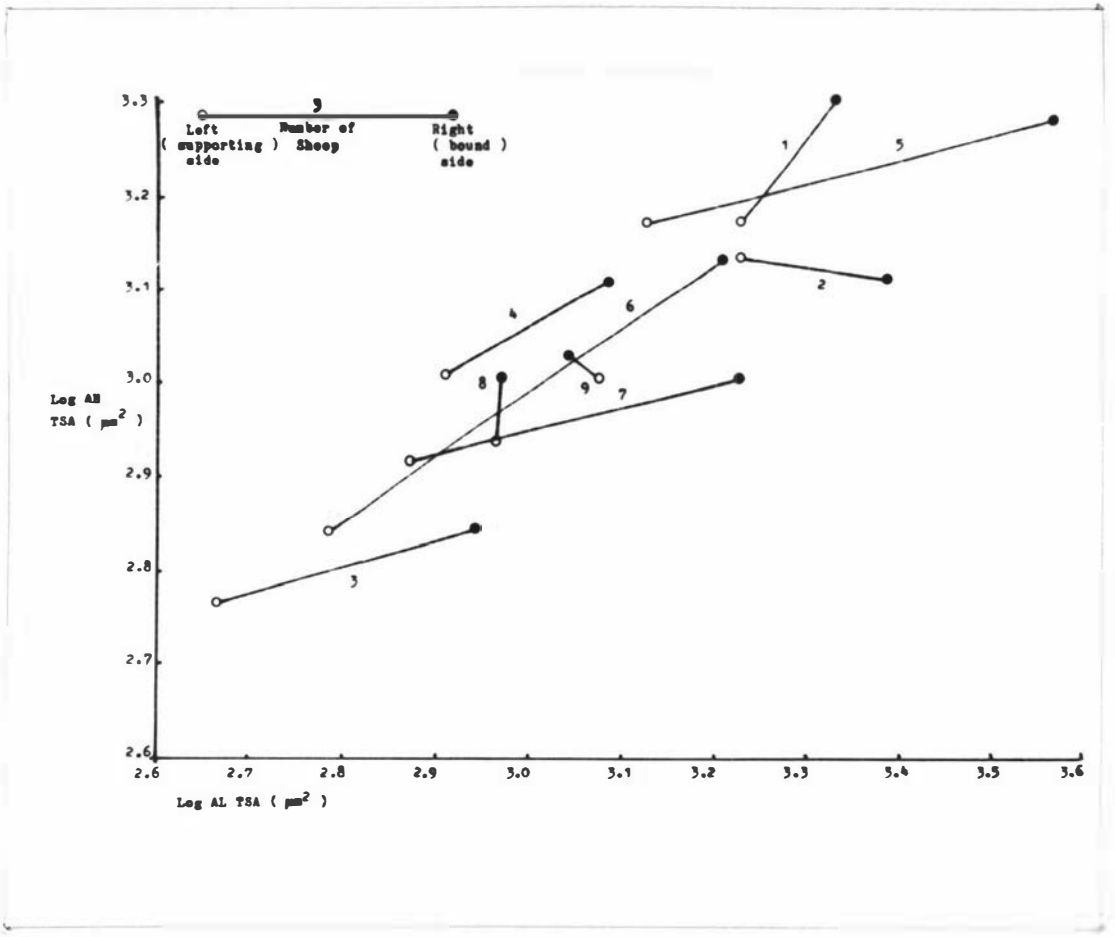


Figure 33: Changes in fibre size proportions in the AL dense area of the semitendinosus muscle of the sheep due to chronic stretching.

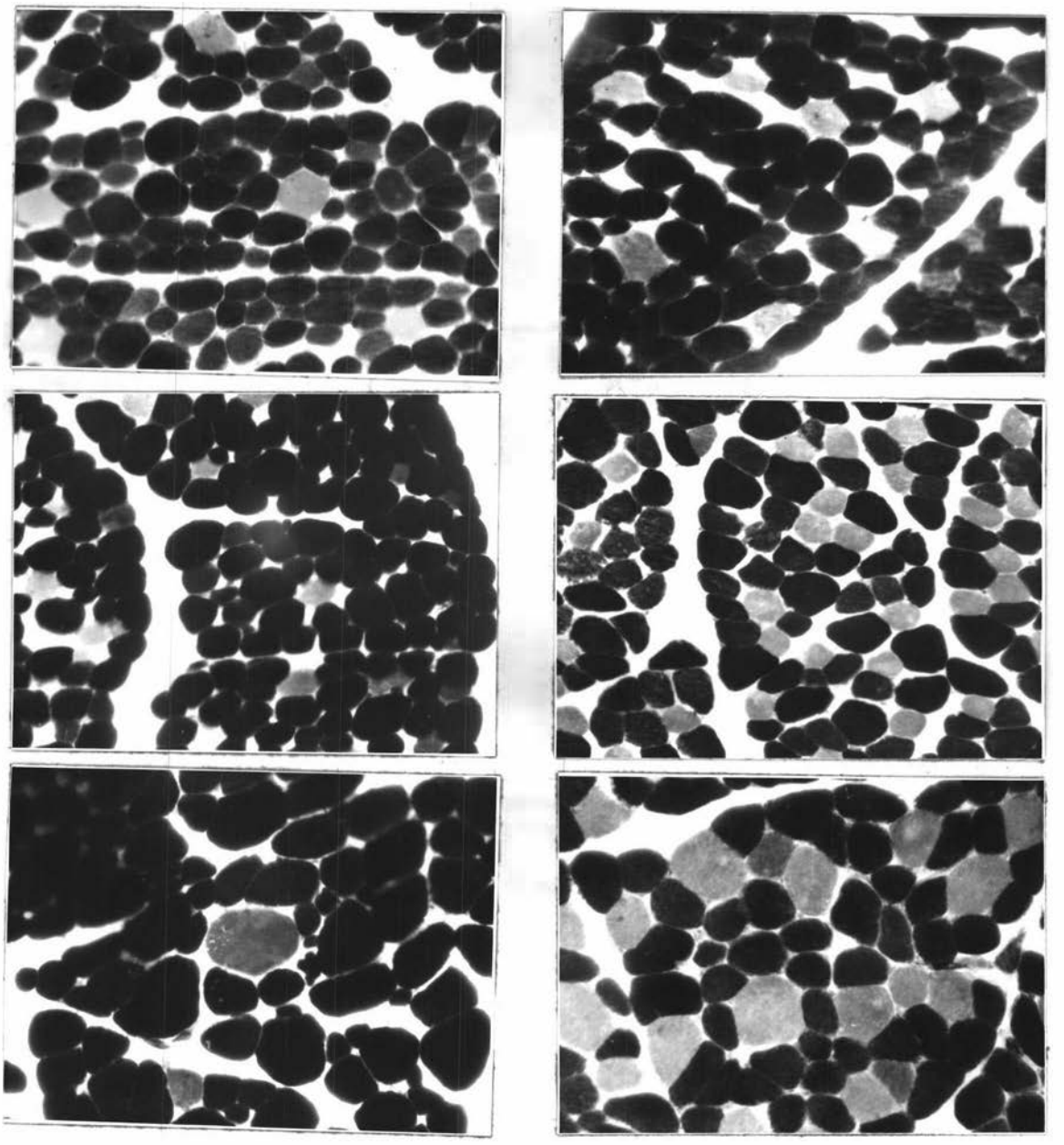


Figure 34: Frozen transverse sections of the semitendinosus muscle stained for alkali-stabile myosin ATPase. x 160.

Top row, control; middle row, supporting limb; and bottom row, bound limb. Left side, AL sparse area; right side, AL dense area.

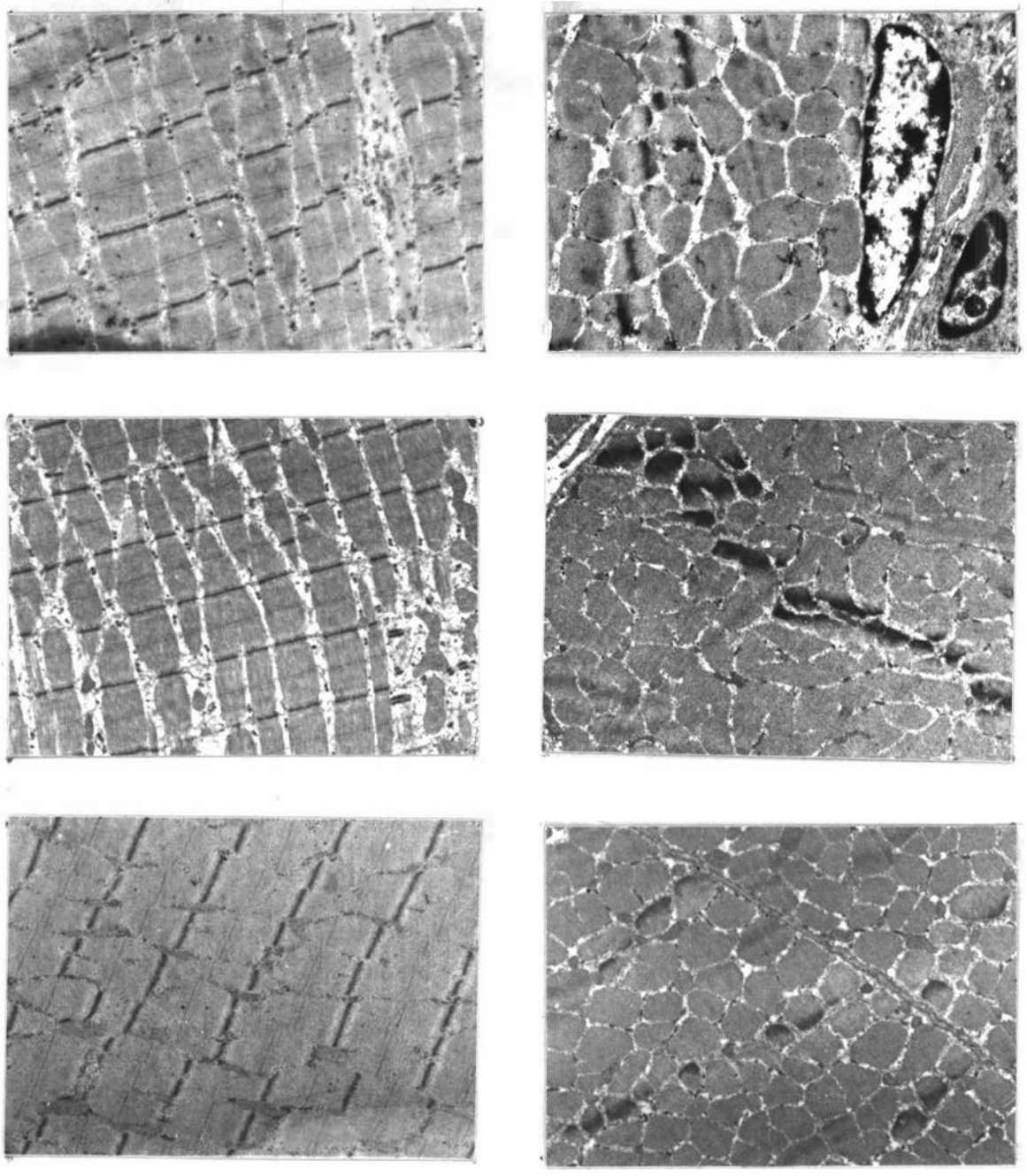


Figure 35: Low power electron micrographs of fibres from the semitendinosus muscle of the sheep. x 6400.

Top row, control; middle row, supporting limb; and bottom row, bound limb. Left side, longitudinal section; right side, transverse section.

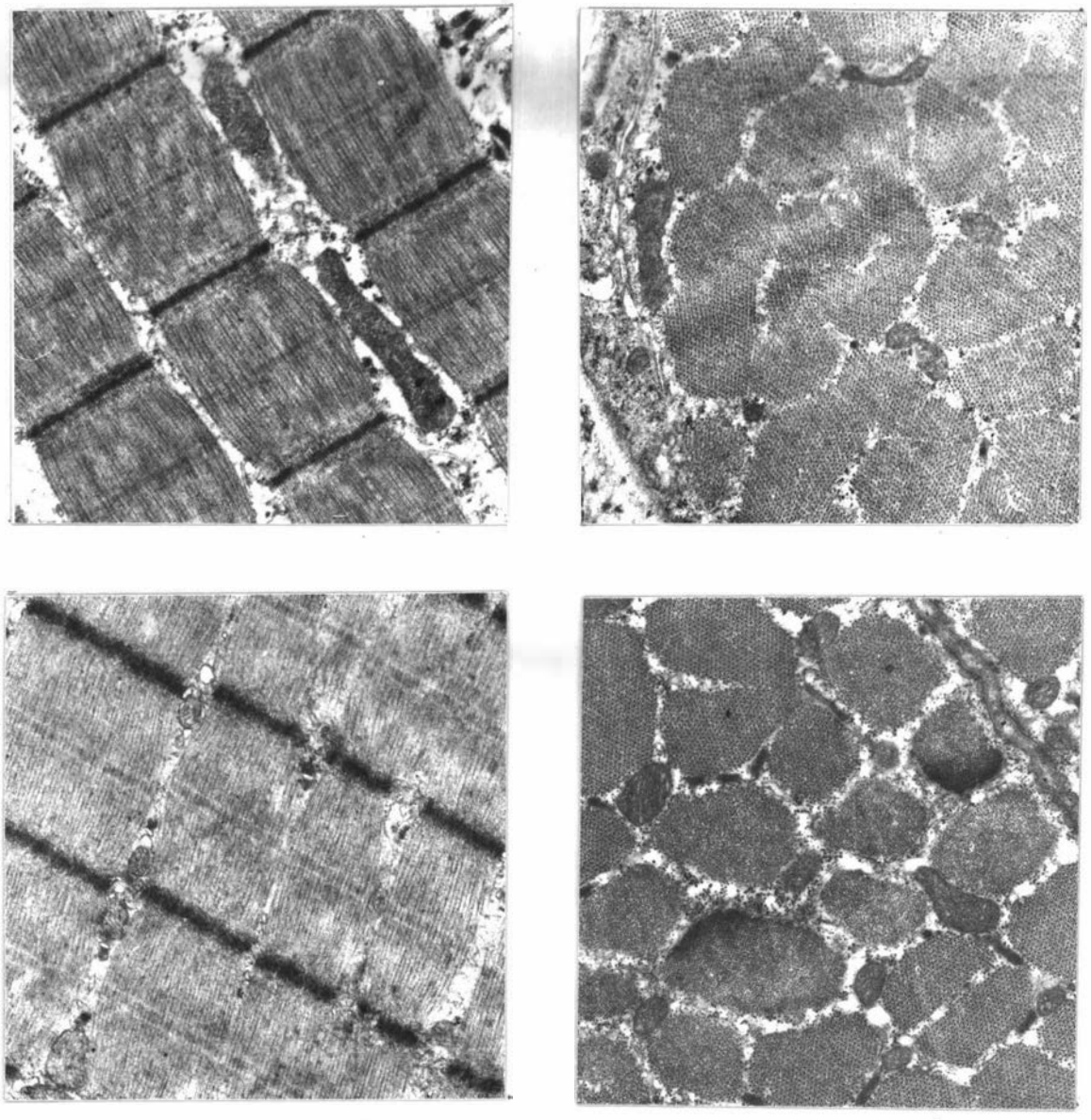


Figure 36: High power electron micrographs of fibres from the semitendinosus muscle of the sheep. x 17500

Top row, supporting side; bottom row, bound side.

Left side, longitudinal section; right side, transverse section.

Appendix 1.

Alkali-stabile Myosin ATPase Technique

Substrate

1.0 M Tris- (hydroxymethyl) - aminomethane (M.W. 121.14)		8 ml
0.18 M Calcium Chloride $CaCl_2 . 6H_2O$		4 ml
Adenosine triphosphate (disodium dihydrogen salt)		60 mg
Distilled water	up to	30 ml

Adjust pH to 9.5 with 0.1 M HCl. Add distilled water to make up a final volume of 40 ml. Incubate for 20 minutes.

Method

(1) Cut fresh frozen sections at 10-15 µm thickness

(2) Mount on slides (and leave in refrigerator for at least 30 min. This prevents the section peeling off from the slide later).

(3) Fix in 4% Formaldehyde buffered with cacodylate (see below) pH 6.8 for 2 minutes (exactly).

(4) Wash in two changes of distilled water.

(5) Incubate for 20 minutes in the above substrate

(6) Wash in distilled water

(7) Treat with 2% Cobalt Chloride solution for 2-3 minutes

(8) Develop in dilute (1%) yellow Ammonium Sulphide solution for 30 seconds.

(9) Wash, mount in aqueous mountant.

(Note: Concentrations of calcium chloride seems critical in getting good reaction).

Cacodylate buffered Formaldehyde

2.14 g Sodium cacodylate $.3H_2O$ in 50 ml distilled water

50 ml of above solution + 6.3 ml 0.2M HCl

+ 20 ml 40% W/V Formaldehyde

Make up to 200 ml with distilled water. Final pH 6.8.

Appendix 2

Succinate Dehydrogenase Histochemical Technique

Special reagents required

(1) Substrate

0.1 M Phosphate buffer pH 7.6 (see below)	7.5 ml
0.2 M Sodium succinate (M.W. 270.15)	7.5 ml
Nitro blue tetrazolium (1mg/ml)	15 ml
(needs to be made fresh each time)	

(2) Phosphate buffer (pH 7.6)

Potassium dihydrogen orthophosphate KH_2PO_4	(1.36g/100 ml)	10 ml
Disodium hydrogen orthophosphate Na_2HPO_4	(1.78g/100 ml)	90 ml
Distilled water		to 500 ml

(3) 10% Formol (Stock solution)

Method

1. Fresh frozen sections cut at 10-15 µm are mounted on coverslips.
2. Incubate at 37°C in the substrate (1) for 20 minutes.
3. Wash in two changes of distilled water.
4. Dry the sections to eliminate gas bubbles.
5. Fix in 10% formalin for 10 minutes.
6. Wash and mount in glycerine jelly.

Appendix 3

Electron microscopy technique

a) Fixatives and embedding media

1) Phosphate buffer (0.1 M, pH 7.2)

36 ml of 0.2 M $Na_2HPO_4 . 12H_2O$ (71.64 g dissolved in distilled water to make 1 ℓ).

14 ml of 0.2 M $NaH_2PO_4 . 2H_2O$ (31.21 g dissolved in distilled water to make 1 ℓ).

Made up to 100 ml with distilled water.

2) Phosphate buffered sucrose (pH 7.2-7.4)

Dissolve 35.0 g of sucrose in 300 ml of 0.1 M phosphate buffer.

3) Modified Karnovsky's fixative

2% Formaldehyde

3% Glutaraldehyde

in 0.1 M phosphate buffer pH 7.2

To make 100 ml:

i) Heat 2 g of paraformaldehyde in 80 ml water to 60-70°C

ii) Slowly add 1 N NaOH dropwise until milky solution clears. Leave for 5 minutes at room temperature.

iii) Add buffer salts 2.51 g $Na_2HPO_4 . 12H_2O$
0.41 g KH_2PO_4

iv) Add 6 ml of 50% Glutaraldehyde solution

v) Make up to 100 ml. Store in refrigerator.

4) Osmium tetroxide fixative

1% Osmium tetroxide (1 g of OsO_4 in 0.1 M phosphate buffer to make 100 ml). (Work in a fume cupboard; score the OsO_4 ampoule about its circumference with a file; place the vial in a strong brown glass-stoppered bottle and shake it to break the vial; introduce correct volume of 0.1 M phosphate buffer).

5) Durcupan ACM epoxy-resin

To make 20 ml:

10 ml Component A (10.94 g) - Epoxy resin

10 ml Component B (9.58 g) - Hardener (Anhydride of a dicarboxylic acid aliphatic side chain).

0.4 ml Component C - Accelerator (Phenol derivative with amino group)

0.2 ml Component D - Plasticizer (Di-n-butyl phthalate)

(Use 30 ml disposable glass vial; measure components A & B by weight using a top-loading balance; mix components by inversion of the vial; warm with hot air blower or at 60°C in an over and mix again; add components C & D using disposable plastic 1 ml syringes).

b) Staining solutions

1) Uranyl acetate stain

Saturated Uranyl acetate in 50% Ethanol

(Add uranyl acetate to 50% ethanol until it will no longer dissolve; centrifuge and store supernatant in a brown glass bottle).

2) Lead citrate stain

0.025 g lead citrate

10 ml distilled water

0.1 ml 10 N NaOH

Shake vigorously until dissolved.

A-14

MORPHOGENESIS OF ISOENZYMES OF MYOSIN

M.N. Sivachelvan

Department of Physiology and Anatomy, Massey University, Palmerston North, New Zealand

The myosin adenosine triphosphatase (myosin ATPase) histochemical technique was employed in a quantitative study on antenatal and postnatal development of the semitendinosus muscle of the sheep. This enabled the pattern of development of the fibres and their sequence of differentiation into functionally distinct fibre types to be determined, inferring the morphogenesis of specific isoenzymes of myosin.

Two distinct generations of fibres develop early in foetal development. The primary generation of cells is recognisable at forty days gestation while the secondary generation of cells begin to appear at sixty days. Up to seventy days of gestation, both generations of cells stain dark with myosin ATPase stain. At seventy days gestation, the primary generation of cells are larger in size (mean transverse sectional area (TSA) of 150 μm^2)with pale central cores containing nuclei and occupying a more central position in the newly forming perimysial enclosed fascicles. The secondary generation of cells shows a homogeneous dark appearance with the stain, and have a mean TSA of 100 μm^2. The fascicles at this stage have a mean TSA of 3,500 μm^2.

From eighty days of gestation onwards, two distinct myosin isoenzyme fibre types are observed histochemically in the muscle. The primary generation of cells presumably has lost its dark staining characteristic to become pale staining ATPase low (AL) fibres whereas the secondary generation of cells continues to retain its dark staining ability and form the ATPase high (AH) fibre population. The distribution of the fibre types is heterogeneous from this period of adulthood. Regions of highest and lowest AL fibre density are recognisable in all these stages and are referred to as the AL dense and the AL sparse areas of the muscle. The percentage of AL fibres in the AL dense area increases from 10 percent at eighty days to 30 percent in the adult, while no appreciable change occurs in the percentage of AL fibres (4 percent) in the AL sparse area. The mean fibre TSAs of the AL and AH fibres in both AL dense and AL sparse areas do not differ significantly in the adult, with a value of 3,500 μm^2. The mean fascicular TSA ultimately attains a value of 175,000 μm^2.

These findings indicate that the specificity of the myosin isoenzymes is not revealed during early foetal development. By eighty days foetal stage the fibres begin to show specificity, shown by others to be due to loss of an initial dimorphic isoenzyme activity. The presence of an ATPase dense and an ATPase sparse area in the muscle is related to the postural and the propulsive functional requirements of the postnatal semitendinosus muscle, as shown in electromyographic studies. The antenatal and postnatal increase in AL fibre percentage in part of the muscle with a predominantly postural function reflects both antenatal anticipation of, and postnatal adaptation for, postural demand on the muscle during growth.

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ANTENATAL ANTICIPATION OF MUSCLE POSTNATAL FUNCTION

M.N. SIVACHELVAN and A.S. DAVIES

Massey University

The semitendinosus of the sheep was used as an experimental model to test the antenatal genetic anticipation of muscle to its postnatal functional demands. The alkaline myosin ATPase histochemical technique was used to identify and quantify postural, ATPase low (AL), and propulsive, ATPase high (AH), fibres. Areas of highest and lowest AL fibre density were recognisable within the muscle in all stages of development from 80 days gestation. The number of AL fibres, as a percentage of total fibres in the AL dense area, increased from 10% at 80 days gestation up to 30% in the adult, whereas in the AL sparse area the AL density remained at about 4% throughout this growth period.

Electromyographic recordings made simultaneously in the AL dense area and the AL sparse area of the semitendinosus muscle in adult sheep proved that the AL dense area is active posturally and AL sparse area is active in propulsion.

The presence of postural and propulsive parts within the muscle and the increase in percentage of AL fibres along with increasing body weight during postnatal growth suggests a functional adaptatory change. In the antenatal period, however, the fetus is an amniotic aquanaut supported by forces of buoyancy, and does not experience adaptatory influence. Thus the presence of postural and propulsive regions even during antenatal growth and an accompanying increase in AL fibre density within the postural region indicates an antenatal genetic anticipation of muscle to its postnatal functional demands.

Department of Physiology and Anatomy

M.N. Sivachelvan

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