# UNIVERSITY OF SOUTHAMPTON
# FACULTY OF MEDICINE

## Department of
## Human Nutrition

## Doctor of Philosophy

## Nutrition, Infection and
## Skeletal Muscle Function.

## By Louis Bernard Levy

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To move things is all mankind can do,
   and for such the sole executant is muscle,
whether in whispering a syllable
   or in felling a forest.


Sir Charles Sherrington.

## ACKNOWLEDGEMENTS

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ABSTRACT

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The influence of nutrition on skeletal muscle function is not clearly undertood. In particular the changes that may occur within the muscle have not been addressed independently of those occurring via central or cardiovascular mechanisms. In addition the manner in which concurrent infection may further influence muscle function is unknown.

The influence of food deprivation, food restriction, consumption of low protein diets and endotoxin administration on the in situ function of skeletal muscle was investigated in male wistar rats using novel computer–aided techniques. Specifically, three muscles were chosen for study to reflect the different muscle fiber types. In addition, the resting metabolite profile was examined.

Twitch, tetanic and fatigue characteristics were preserved following fasting, food restriction and endotoxin administration. Deficits in function were only observed in food restricted animals compared to those animals allowed to grow normally (age–matched, heavier animals).

Following consumption of low protein diets animals were stunted and wasted. These animals exhibited prolongation of the soleus muscle twitch time, shifts in the force–frequency relationship and a tendency for increased fatigue. Preservation of force generation was noted despite loss of muscle tissue.

Endotoxin administration to animals consuming low protein diets did not further alter the twitch characteristics, but all three muscle types studied exhibited shifts in the force–frequency relationship and further increases in fatigue susceptibility.

No changes in muscle metabolite concentrations was discernible in any animals.

The relevance of these results in relation to the lower physical working capacity reported in malnourished labourers is discussed.

# CHAPTER 1.

## INTRODUCTION

Physical activity is an integral part of normal life. Activities including work, sport and exercise programmes are pursued for survival and also to promote physical, social and mental well being of the individual. Physical activity is important in promoting optimal growth and development of children and there is a minimum level of habitual physical activity which is consistent with maintaining good health.

It is generally accepted that malnutrition may be associated with reduced physical activity resulting in decreased fitness and increased fatigue leading to further reductions in physical activity. Yet the influence of nutrition on the capacity of skeletal muscle to function normally is not fully understood. The extent to which physical working activity may be maintained in the presence of malnutrition is believed to be dependent upon the extent of muscle wasting. Muscle mass is not the sole determinant of skeletal muscle function. It has been suggested that changes in muscle function may become apparent in the presence of undernutrition prior to significant changes in muscle mass. The nature of these changes remains unclear. Infection may be present against a background of malnutrition. It is unclear whether infection in the presence of malnutrition further impairs physical activity.

Adaptations to reduced food intake may involve changes in the central, cardiovascular of peripheral (i.e. within muscle) systems. Changes taking place within muscle have not been addressed independently of those occurring via central or cardiovascular mechanisms. In addition the manner in which infection may specifically influence muscle function following undernutrition requires further investigation.

The first part of this introduction will describe the structure and energy metabolism of normal skeletal muscle. In addition, the manner in which skeletal muscle structure and energy metabolism may alter in various states of food intake and infection will be considered. The final section will discuss our present understanding of the extent to which changes in metabolism and muscle mass associated with alterations in food intake and infection influence the functional characteristics of skeletal muscle.

## 1.1 <u>STRUCTURE, FUNCTION AND ENERGY METABOLISM OF NORMAL SKELETAL MUSCLE.</u>

Human skeletal muscle function can be described in many ways. These include measurements of the force generated by a maximal voluntary contraction (MVC), the ability to maintain a given work load or the degree of loss of force with time during a contraction. Assessment of muscle function using these principles relies upon the motivational state of the subject and the ease with which the subject can use the measuring equipment. Electrical stimulation of peripheral nerves can be used to elicit contraction of the muscle in order to overcome the limitations imposed by motivation when measuring voluntary contractions. This would enable muscle function to be assessed without the subject's motivation influencing the measurement. Using this approach in animal studies the functional capacity of the muscle can be directly related to its structure and metabolic state.

The contractile characteristics of mammalian skeletal muscle have been of interest to physiologists since the early work of Ranvier (see Close 1972). In the late 19th century muscles had been classified as "red" and "white" based on their appearance. Subsequent studies of Ranvier (see Close 1972), Kronecher & Stirling (1878) and Cooper & Eccles (1930) demonstrated that certain "red" muscles of rabbits and cats contracted more slowly than "white" muscles, although the colour of the muscle did not always correlate with a particular speed of contraction. Histological studies (see Close 1972 for references) revealed that muscles were not the homogeneous entities originally described being made up of at least two kinds of fiber. The heterogeneity of muscle fibers is discussed in greater detail in Section 1.1.1.

In order to understand the manner in which muscle functions, a knowledge of muscle structure and metabolism is important.

### 1.1.1 <u>The structure of skeletal muscle.</u>

Skeletal muscles are usually distinct, relatively large structures. A whole muscle is composed of fibers containing multinucleate myofilaments. Figure 1.1 represents a diagramatic view of the composition of a generalised muscle.

As noted earlier, muscle fibers had been subdivided into "red" and "white" by early investigators (see Close 1972) but this distinction remained unsatisfactory. The development of histochemical techniques resulted in further classification of fibers into three broad types whose morphological features are listed in Table 1.1. It should be noted that Table 1.1 represents only a guide to these features rather than a complete list.
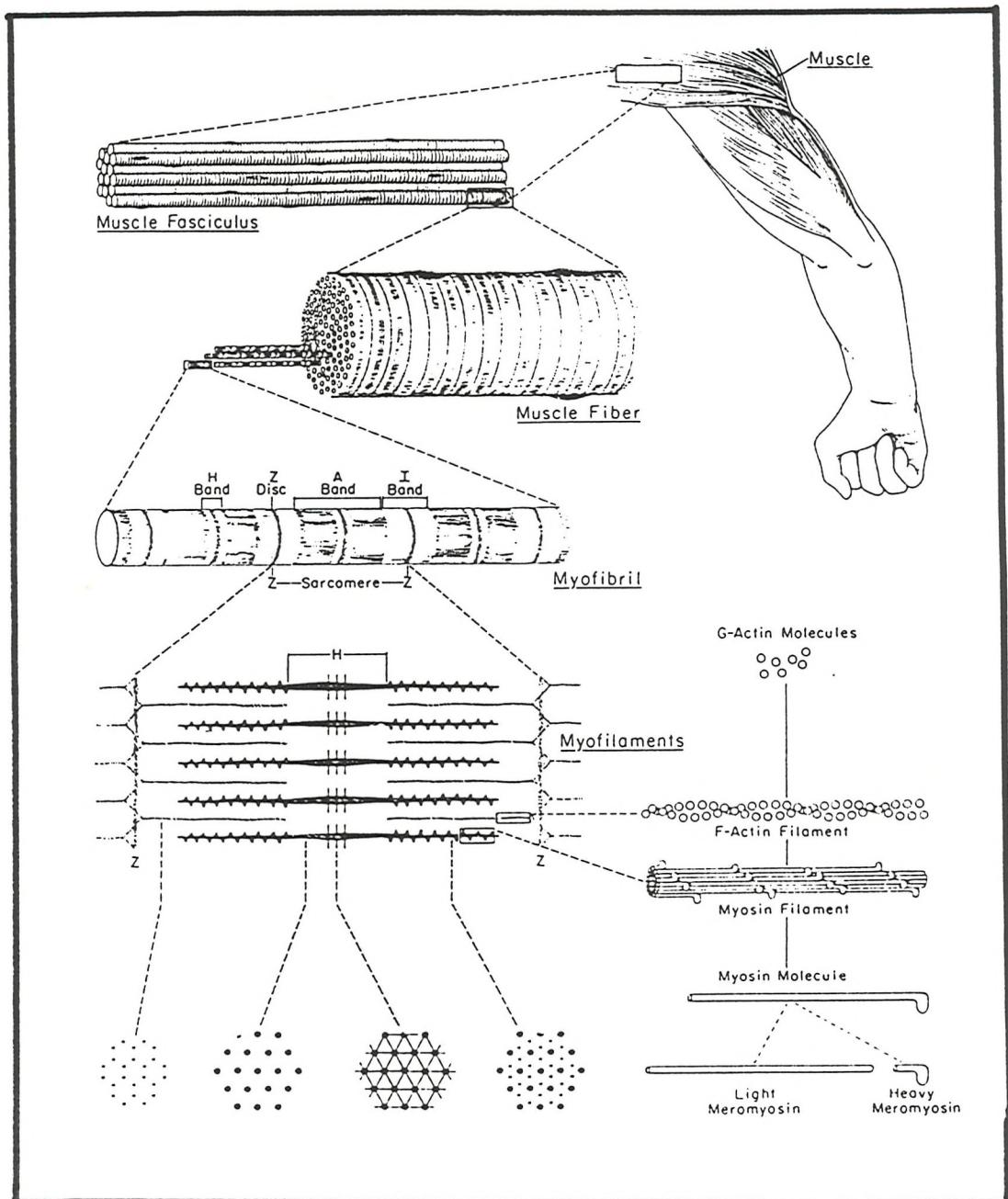
Figure 1.1 Diagram to show the organisation of vertebrate skeletal muscle from gross structure to ultrastructure (adapted from Bloom & Fawcett eds 1968: A textbook of Histology, 9th Edition. Saunders. Philadelphia).

Table 1.1. Morphological and histochemical types of fiber in a mammalian skeletal muscle (adapted from Close 1972).

CLASSSIFICATIONS:

| | | | |
|---|---|---|---|
| Ogata & Mori (1958) | white | medium | red |
| Stein & Padykula (1962) | A | B | C |
| Engel (1962;1970) | II | I | II |
| Romanul (1964) | I | III | II |
| Padykula & Gauthier (1967) | white | intermediate | red |
| Kugelberg & Edstrom (1968) | A | C | B |
| Barnard et al (1971) | fast twitch white | slow twitch intermediate | fast twitch red |
| Brooke & Kaiser (1970) | IIB | I | IIA(c?) |

PROPERTIES:

| | | | |
|---|---|---|---|
| Mitochondrial content | small | large | intermediate |
| Z line | narrow | intermediate | broad |
| Fiber diameter | large | small | intermediate |
| Neuromuscular Junction | large & complex | intermediate | small & simple |
| Distribution of Succinic Dehydrogenase | even network | even network | subsarcolemal |
| Oxidative enzyme activity | low | high | intermediate/ high |
| Mitochondrial ATPase | low | intermediate | high |
| Glycolytic activities | high | low/ variable | intermediate/ low |
| Myoglobin content | low | high | high |
| Glycogen content | high | low | high |
| Myofibrilar ATPase (at pH 9.4) | high | low | high |
| pH sensitivity of ATPase | acid labile | acid stable | acid labile |
| | alkali stable | alkali labile | alkali stable |
| Fatigue | fast | resistant | slow |

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In 1925 Sherrington developed the concept of the motor unit to describe a motorneurone and the muscle fibers which the motorneurone innervated. It has since become clear that motor units are composed of single types of muscle fibers but that the number innervated varies (Hennerman & Olsen 1965). Since a motor unit is homogenous, it follows that stimulation of motorneurones would define the contractile characteristics of individual motor units and thus allow determination of the properties of muscle fibers. From the work of many investiagtors including Wuerker, McPhedron & Hennerman (1965), Edstrom & Kugelberg (1968) and Burke (Burke 1967; Burke, Levine, Tsaris & Zajac 1973) it appears that the morphological and metabolic characteristics of the fibers are associated with specific functional characteristics as shown in Figure 1.2.

The classification of fiber types used in this thesis will be:

a) Slow twitch type I fibers with relatively long contraction times, low ATPase activity, poorly developed glycolytic enzyme patterns, high mitochondrial content, showing little or no fatigue;

b) Fast twitch type IIA fibers with relatively short contraction times, high ATPase activity, moderate glycolytic capacity, high oxidative capacity and relative resistance to fatigue;

c) Fast twitch type IIB fibers with relatively short contraction times, high ATPase activity, well developed glycolytic enzyme systems, low oxidative capacity and which fatigue rapidly.

From this classification it can be seen that the properties of each fiber indicate its probable function. Thus, type IIB fibers would perform as fast units for short term powerful phasic activity whereas type IIA fibers are better adapted for sustained physical activity. Type I fibers are low speed ideally suited for tonic activity. Muscles are not however, composed of one type of fiber being heterogenous and tending to contain all three fiber types (Table 1.2) whose activity is integrated to perform suitable tasks.

1.1.1.1 Ultrastructure of skeletal muscle fibers.

Since muscle structure appears to be conserved between the different types of muscle fiber the differences in function must reflect differences between fast and slow fibers at a more detailed level. Muscle contraction occurs following an impulse by excitation of the muscle membrane. This impulse is transmitted via the transverse tubule system to the depths of the muscle resulting in depolarisation of all the muscle fibers at approximately the same instant (Figure 1.3) The actual mechanism by which excitation−contraction coupling is achieved remains a matter of debate (see Caswell & Brandt 1989) but the ultimate outcome is the release of calcium into the interstitium from the terminal cisternae of the sarcoplasmic reticulum.
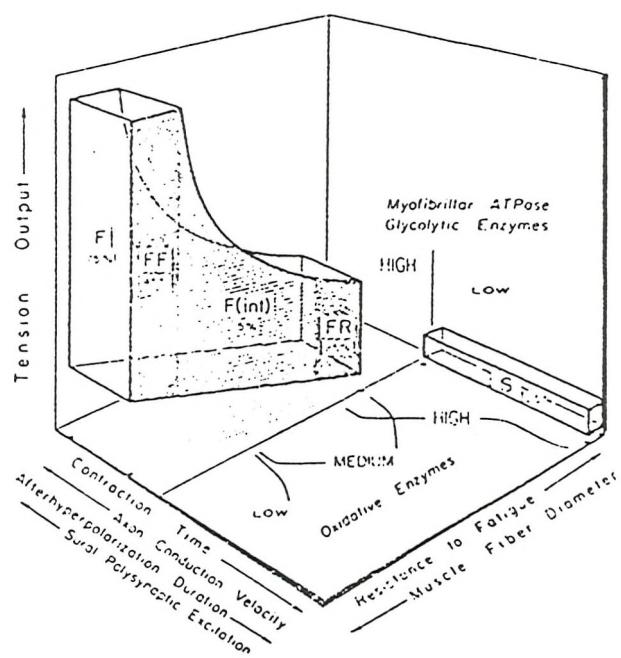
Figure 1.2. A three dimentional diagram summarising the contractile, morphological and biochemical characteristics of cat skeletal muscle motor units (taken from Burke 1980).

Table 1.2: Composition of different muscles often used in physiological studies (adapted from Close 1972)

| ANIMAL AND MUSCLE | % TOTAL FIBER NUMBER | | | REFERENCE |
|---|---|---|---|---|
| | I | IIA | IIB | |
| CAT | | | | |
| Flexor Digitorum Longus | 28.0 | 30.4 | 41.6 | Olson & Swett 1966 |
| Flexor Hallucis Longus | 14.0 | 35.8 | 50.2 | Olson & Swett 1966 |
| Medial Gastrocnemius | 21.3 | 27.8 | 50.9 | Hennerman & Swett 1966 |
| Soleus | 95-100 | | | Guth & Samaha 1969 |
| | | | | Hennerman & Olson 1965 |
| | | | | Karparti & Engel 1967 |
| | | | | Wuerker et al 1965 |
| | | | | |
| RAT | | | | |
| Diaphragm | 20.0 | 60.4 | 20.6 | Gauthier 1969 |
| Red Semitendinosus | 40.0 | 52.4 | 9 | Gauthier 1969 |
| White Semitendinosus | 14.0 | 4 | 82 | Gauthier 1969 |
| Tibialis Anterior | 18 | 41 | 41 | Kugelberg & Edstrom 1968 |
| | 11 | 42 | 47 | Guth & Samaha 1969 |
| | | | | Samaha et al 1970 |
| | 3 | 19 | 78 | Larsson & Edstrom 1986 |
| Extensor Digitorum Longus | | | 60 | Edgerton & Simpson 1969 |
| | 3.8 | 45 | | Edgerton & Simpson 1971 |
| | 3 | 23 | 76 | Larsson & Edstrom 1986 |
| | 3 | 19 | 78 | Edstrom & Larsson 1987 |
| Soleus | 80 | 20 | | Edgerton & Simpson 1969 |
| | 85-90 | 10-15 | | Karpati & Engel 1967 |
| | 91 | 7 | | Edstrom & Larsson 1987 |
| | 88 | 10 | | Larsson & Edstrom 1986 |
| | | | | |
| GUINEA PIG | | | | |
| Flexor Digitorum Longus | 10.7 | 35.5 | 53.8 | Barnard et al 1971 |
| Flexor Hallucis Longus | 17.7 | 9.7 | 72.8 | Barnard et al 1971 |
| Medial Gastrocnemius | 11.7 | 50.2 | 38.6 | Barnard et al 1971 |
| Red vastus lateralis | 4.1 | 78.2 | 17.7 | Barnard et al 1971 |
| Soleus | 100 | | | Barnard et al 1971 |
| | | | | Brooke & Kaiser 1969 |
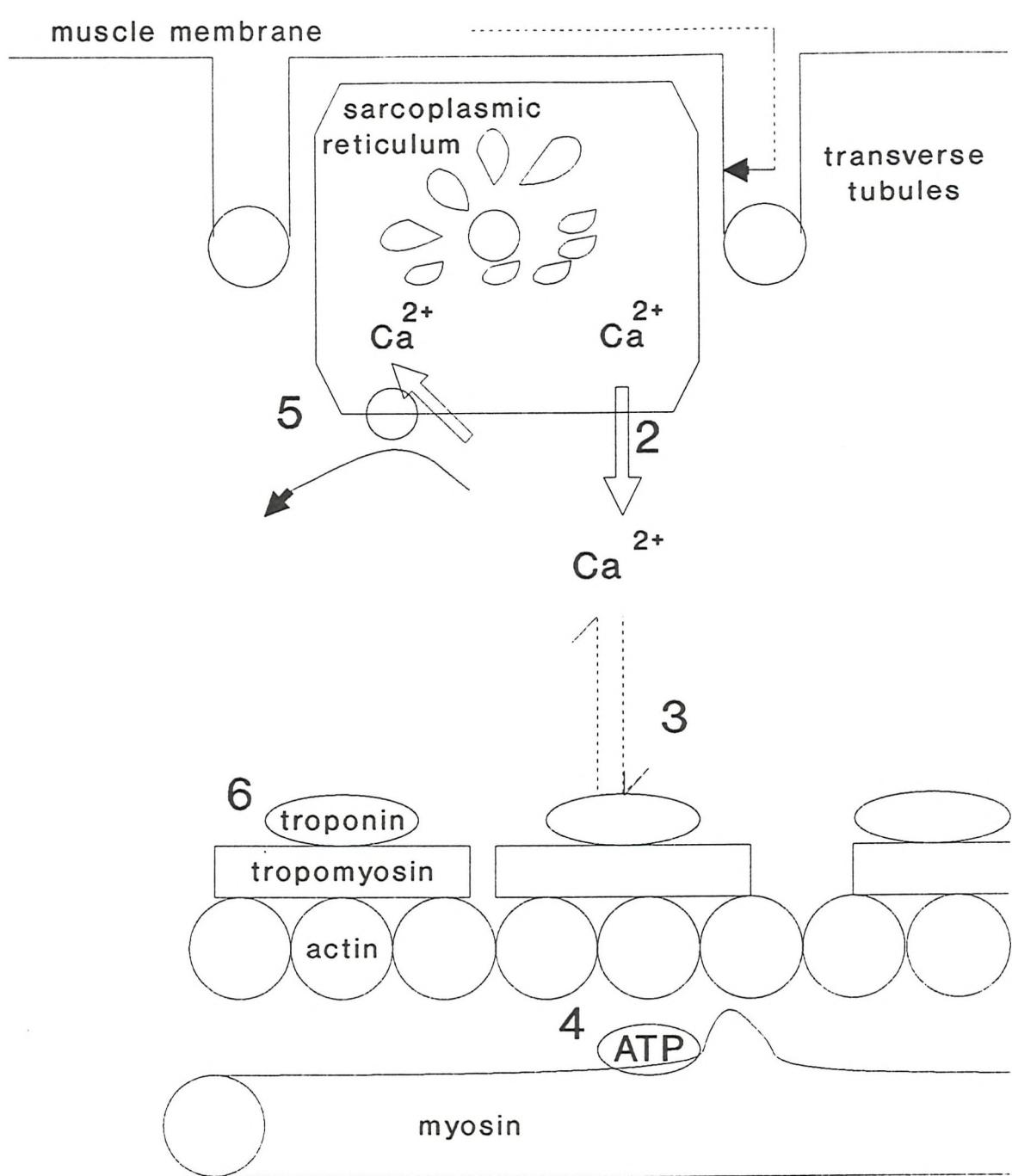
Figure 1.3. A Schematic diagram to illustrate the excitation–contraction coupling initiated by depolarisation of the muscle membrane (1) which is transmitted via the transverse tubules (2) to the interior of the muscle. Calcium is released across the sarcoplasmic reticulum (3) and interacts with troponin disinhibiting the block on tropomyosin allowing interaction of actin and activated myosin (4). This results in crossbridge attachment. Hydrolysis of ATP results in detachment and allows sliding of filaments over each other as the myosin head attaches further along the actin filament. Sequestration of calcium by the sarcoplasmic reticulum (5) lowers the interstitial concentration of calcium resulting in the reimposition of the troponin block (6) hence relaxation occurs.

Calcium binds to troponin causing a conformational alteration occurs resulting in the uncovering of a binding site on the actin filament. Energised myosin cross bridges on the thick filament bind to actin which triggers the release of energy stored within the myosin producing an angular movement of the cross bridge and the cleavage of ATP. ATP binds to myosin, breaking the link to actin thereby causing cross bridge dissolution. The ATP is split transfering energy to the myosin cross bridge and thus prepares it for another cycle of attachment. As cross bridge formation and breakage occurs in a cyclical fashion movement of thin filaments over thick filaments occurs and is repeated for the duration of the binding of troponin and calcium. Removal of calcium by sequestration over the sarcoplasmic reticulum restores the block on the binding sites and muscle tension declines. This sequence of events is summarised in Figure 1.3 and for references and greater detail the reader is referred to Huxley & Hanson (1954), Huxley & Niedegerke (1954) and Sandow (1965, 1970). It should be noted however, that muscle contains other proteins whose exact function remains unclear at present.

For a more detailed description of skeletal muscle structure the reader is referred to general physiological texts and the work of Peachey (1968) and Huxley (1969).

### 1.1.2 Skeletal muscle energy metabolism.

Energy is required within skeletal muscle to maintain a variety of metabolic processes within the cell including membrane transport of ions and substrate and for the turnover of carbohydrates, fats and proteins essential for the maintenance of the cell. Marked increases in the rate of energy utilization will be required for many of the stages of muscle contraction and relaxation. Energy released from the hydrolysis of ATP is directly coupled to the movement of crossbridges, ATP binding to myosin is necessary for the breakage of crossbridge attachment and the energy released from hydrolysis of ATP at the sarcoplasmic reticulum provides energy for the sequestration of calcium thus initiating relaxation. The concentration of ATP within the muscle is sufficient to enable few twitches to be elicited, yet muscle contraction does not necessarily result in reductions of muscle ATP concentration. Maintenance of cellular homeostasis and function demand that the rate of ATP supply satisfies the rate of ATP utilization within the muscle cell.

The resynthesis of ATP within skeletal muscle occurs via three mechanisms. Firstly, phosphagenolysis either from the myokinase reaction (2ADP = ATP + AMP) or from creatine phosphate hydrolysis. Creatine phosphate is a rapid means by which ATP can be resynthesised:

$$\text{Creatine Phosphate + ADP} \rightleftharpoons \text{ATP + Creatine}$$
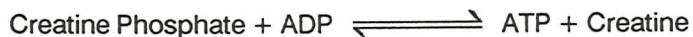
The equilibrium of this reaction lies to the right, thus as the concentration of ATP falls during a contraction it is immediately resynthesised from creatine phosphate. Creatine phosphate itself is synthesised by the reversal of this reaction, thus when ATP concentrations rise above that required for maintenance of skeletal muscle function and metabolism, the formation of creatine phosphate occurs. Creatine phosphate, however, does not form an inexhaustable source of energy via ATP.

An additional consideration apart from the relative proportion of total creatine that is available in the phosphorylated form, is the size of the total creatine pool. Whilst creatine may be derived from the diet, the principle source of creatine is from *de novo* synthesis (Beesman & Geiger 1981). As the rate of creatine degradation to creatinine appears constant, the primary determinant of creatine availability appears to be the rate of creatine synthesis and incorporation within the muscle cell (Beesman & Geiger 1981). Similarly, the size of the adenine pool must also be maintained by achieving a balance between the rates of adenine synthesis, degradation and salvage (Flanagan, Holmes, Sabina & Swain 1986). Any factor that limits the relative activity of each of these processes will in turn limit the capacity for ATP resynthesis from phosphagenolysis within skeletal muscle.

Energy provision is ultimately derived from oxidative phosphorylation and glycolysis. At moderate intensities of muscular activity much of the required energy is provided by the oxidation of fatty acids and glucosyl units from intramuscular glycogen stores and blood borne glucose. Skeletal muscles are composed of different types of muscle fiber, some with greater oxidative capacities than others. The slow−twitch muscle fibres (type I) are better able to maintain the rates of energy provision via oxidative phosphorylation than the more glycolytic fast−twitch fibers (type IIB). With increasing rates of ATP utilization, the demand for ATP can only be satisfied by greater dependency upon glycolysis initially through oxidative metabolism and ultimately through anaerobic glycogenolysis (McGilvery 1975). Other substrates may be used to maintain the rates of ATP resynthesis including amino acid and ketone body oxidation.

Optimal delivery of ATP via these metabolic processes and ATP utilization can only be maintained with functional enzyme systems and mitochondria, an intact cardiovascular system, an intact sarcoplasmic reticulum/enzyme system to compartmentalise appropriate concentrations of calcium, a system capable of buffering against ionic (pH) alterations and the substrate stores which can be metabolised. If any of these systems fail to operate appropriately, the capacity of the muscle to generate force and hence its functional capacity would be limited.

### 1.1.3 Determinants of skeletal muscle function.

Stimulation, representing a change in environment, is required to initiate muscle contraction. These stimuli tend to be specific and may be electrical, mechanical, electromagnetic, chemical, temperature or osmotic in nature. The use of electrical stimulation in physiological studies has several advantages in that they are reproducible, controlable, the responses occur rapidly and the tissue normally recovers without injury.

The contraction induced by a single, brief electrical stimulus which activates all the muscle fibers almost simultaneously is referred to as a twitch. A typical twitch response is shown in Figure 1.4. It can be described by three phases: the latent period, the contraction phase and the relaxation phase. The latent period is the time between the stimulus and the initiation of force generation. During this period chemical changes occur as the result of the stimulus and represents excitation—contraction coupling. The contraction phase occurs due to the sliding of actin filaments over myosin within the myofibrils which comprise the muscle (see Section 1.1.1). The relaxation phase is that time during which the muscle tension declines. During normal activity, however, muscular contractions are not merely twitches lasting for short periods of a second but are sustained contractions for longer periods during which compound or tetanic contractions are generated. Thus, when a muscle receives a stimulus it undergoes a single contraction. When a second stimulus is applied to muscle still in its relaxation phase further force generation results. This response is known as summation resulting in the production of clonus (Figure 1.5).

Application of several stimuli in rapid succession but with little interval between successive stimuli results in contractions which fuse together to maximal contraction. This is a complete tetanus. By measuring the forces generated at frequencies of stimulation resulting in incomplete and complete tetany a force—frequency curve may be constructed to show the relative force generating capabilities of the muscle compared to the maximal force generation (Fmax, Figure 1.5).

The proteins and structures which form skeletal muscle are highly conserved between fast and slow twitch muscle fibers. Thus it is assumed that interaction between myosin and actin molecules follow the same cyclical pattern in both slow and fast muscles (Huxley 1985; Close
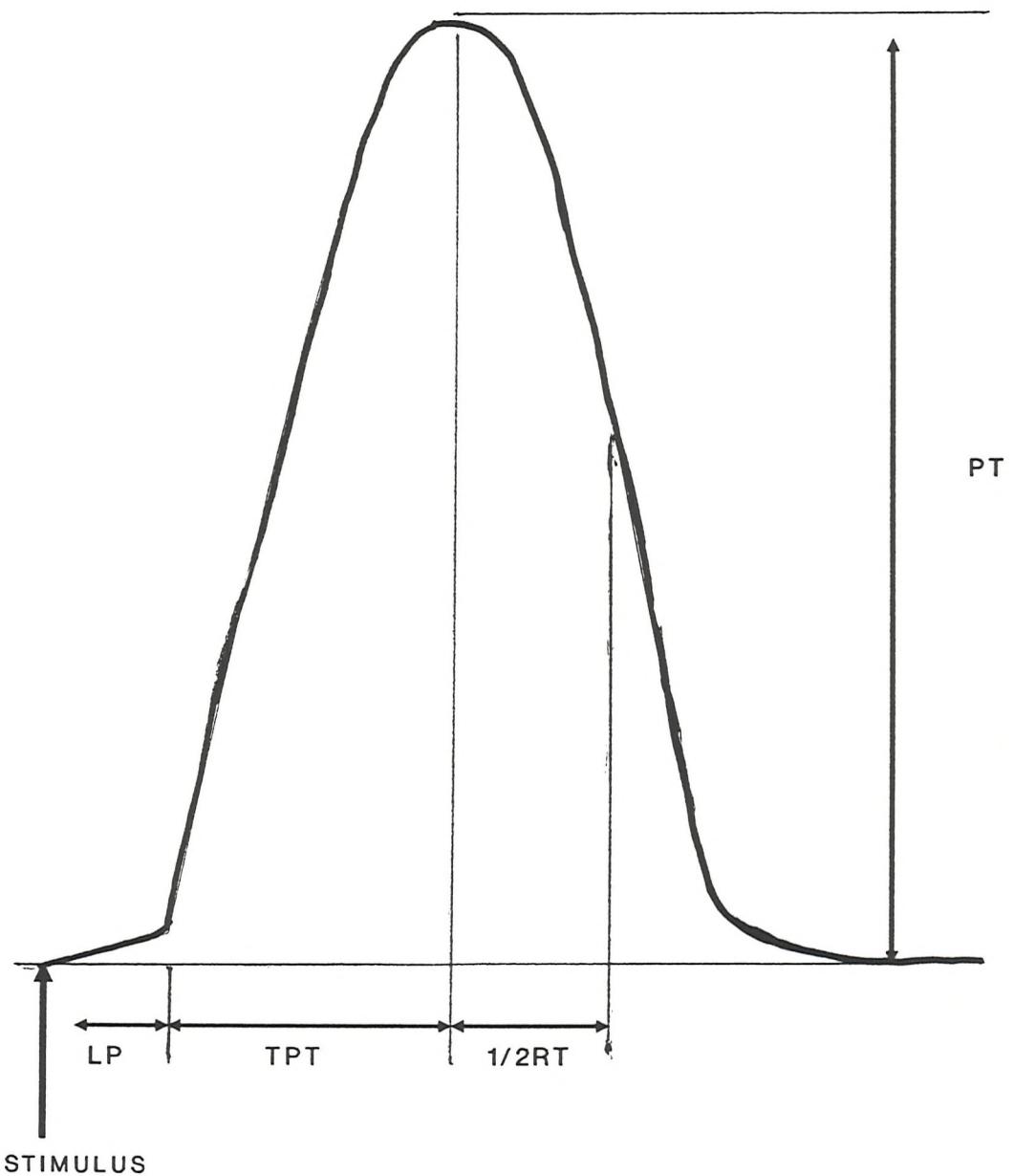
Figure 1.4. A twitch response following a single, brief electrical stimulus resulting in the production of force during the contraction phase after a short latent period (LP) and terminating in relaxation with the force generated returning to baseline tension during the relaxation phase. From these traces measurements of peak twitch tension (PT), time to peak tension (TPT) and half relaxation time (½RT) can be made.
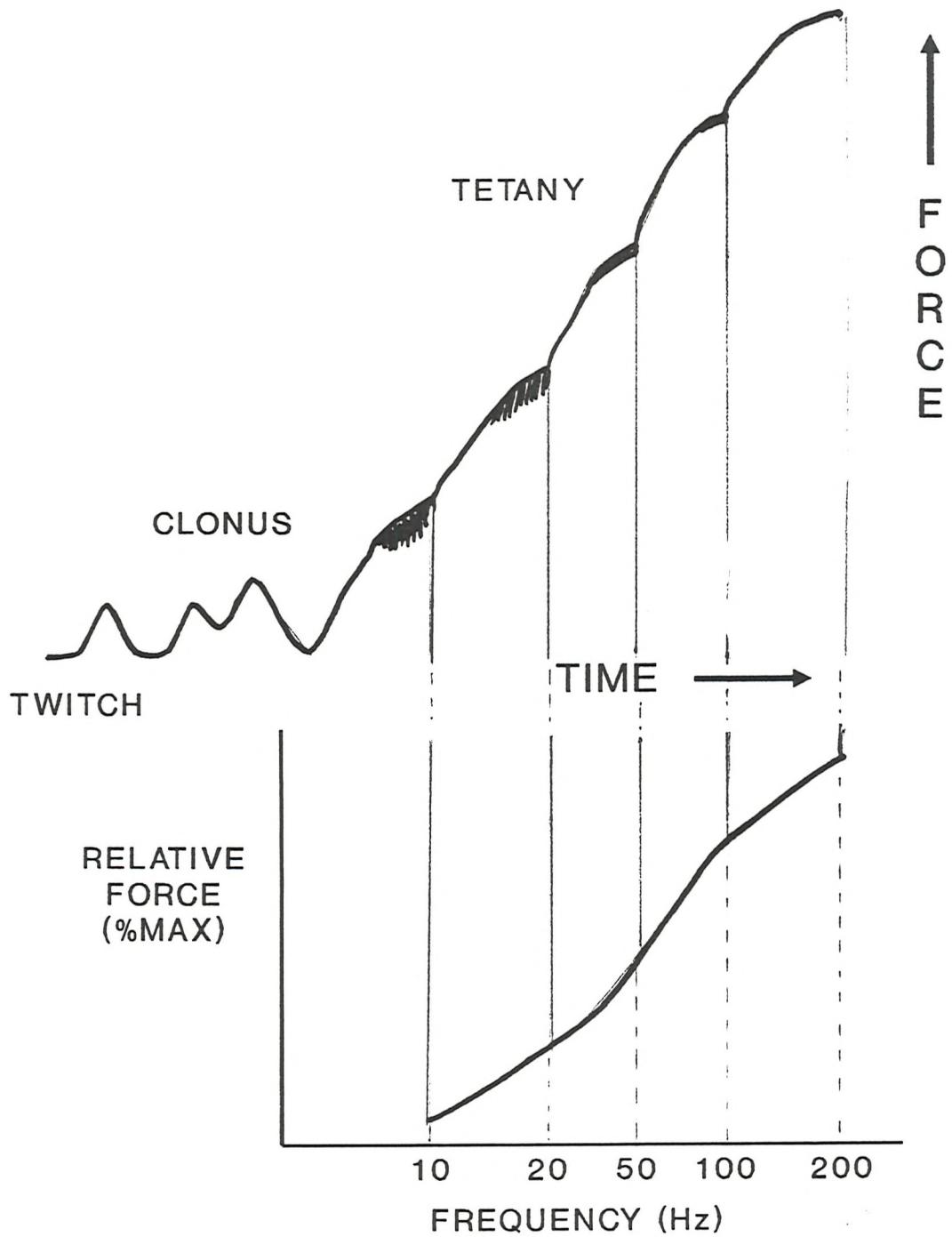
Figure 1.5. The production of force within a muscle increases with higher frequencies due to the contraction phase a twitch being elicited when the muscle is still in the relaxation phase from the previous stimulation. This results initially in summation, then clonus and eventually tetany— at which point the maximal tetanic force (Fmax) is elicited. By plotting the force generated at each frequency relative to this maximal tetanic force against the appropriate frequency of stimulation a force—frequency curve can be constructed.

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1972). It seems likely therefore that an individual cross bridge will be capable of the same force generation in all muscles. Total isometric twitch tension will therefore be proportional to the number of cross bridges formed during contraction. The tension development within a half sarcomere of the myofibril is reliant upon the number of cross bridges formed whose individual tensions will add up in parallel.

Over each half of an A band these forces are additive in series since they form in opposite directions. This may suggest that the peak twitch tension is determined by the number of myosin molecules in each half A band and the number of filaments per unit area of muscle. The lateral spacing of the A bands however, appears to be uniform between different muscles (Huxley 1953) and the length of the A band is fairly constant (Huxley 1985). Hence one might expect the peak twitch tension of different muscles to be similar. Yet muscles differ in total size thus larger muscles are capable of greater force generation due to the greater number of muscle fibers and hence greater number of crossbridges formed.

### 1.1.3.1 What determines the speed of contraction?

The speed of contraction can be assessed by measurement of the time to peak twitch tension (TPT) during a single twitch (Figure 1.3). This is the time taken for the muscle to attain peak tension following the latent period. The rate of ATP hydrolysis differs between fast and slow muscles despite conservation of muscle structures (Close 1965; Barany 1967). Barany and associates measured the myosin−ATPase activity and speed of contraction of the flexor hallucis longus, soleus and crureas muscles of the cat and sloath (Barany, Barany, Reckard & Volpe 1965; Barany 1967). Contraction of the flexor was 2.6 and 2.8 times that of the crureas and soleus muscles respectively in the cat, correlating with a 2.3 & 3.2 times greater ATPase activity. This suggested that ATPase activity determines the speed of contraction (Barany *et al* 1965). Comparison of the sloath extensor digitorum longus (EDL) and gastrocnemius muscles with those of the cat however, reveals that contraction is 6.7 & 4.7 times slower in the sloath and the ATPase activity lower by 5.6 & 3.7 times (Barany 1967). Thus it seems likely that the ATPase activity does determine the speed of contraction. There also appears to be a neural influence, however, since cross−innervation studies have indicated that both slow and fast muscles come to exhibit the contraction times and intrinsic speeds of shortening similar to muscle which the nerve originally innervated (Close 1969).

As motorneurones supplying different types of muscle discharge at different rates, the alterations in muscle contraction time observed in these cross−innervation studies implies that it is the frequency of stimulation which is important in influencing the contractile characteristics. The site at which this influence or interaction occurs remains unclear.

Another consideration is that of muscle components other than the contractile elements. Thus the connective tissue, nervous tissue and blood vessels within the muscle will offer a degree of resistance and elasticity to the muscle. The precise contribution of such resistance or elasticity has not been adequately determined, but never the less this would dampen the rate of force development.

### 1.1.3.2 What determines the speed of relaxation?

Relaxation of muscle appears to be associated with the removal of calcium from troponin (see Figure 1.3). The amount of calcium which must be sequestered for complete relaxation has been estimated *in vivo* to be of the order of $0.1 - 0.2$ $\mu$moles/g muscle (Close 1972). The differences in the rate of relaxation of fast and slow muscles is likely therefore to reflect the uptake of calcium over the sarcoplasmic reticulum. Kinetic studies on fragmented sarcoplasmic reticulum in vitro have indicated that maximal rates of calcium uptake by fast muscle fibers is $4-11$ times greater than slow muscle sarcoplasmic fragments at room temperature (Fiehn & Peters 1971). There also appears to be twice as much sarcoplasmic reticulum in fast compared to slow muscles per unit weight of muscle (Fiehn & Peters 1971), and by volume of reticulum as assessed by electron microscopy (Luff & Atwood 1971). In terms of actual activity, Lucas–Heron and colleagues observed that the soleus muscle of rats exhibited 10 times less $Ca++Mg++-ATPase$ activity than the EDL muscle and that upon denervation the activity in the EDL declined to that seen in the soleus (Lucas–Heron, Loirat, Ollivier & Leoty 1986).

Components of the muscle other than the contractile elements would also influence the relaxation time by dampening the rate at which relaxation occurs. Thus relaxation would be expected to last for a longer period than that which could be accounted for by the myofibers alone. The magnitude of this effect cannot be determined.

### 1.1.3.3 What determines the force–frequency relationship and the generation of maximal tetanic force?

The normal functioning of muscle does not occur via the generation of single twitches, but is the result of repetitive stimuli giving rise to the production of complete or incomplete tetani. The force development in a muscle under these conditions is described by the number of fibers contracting and the tension produced in each contracting fiber. The number of fibers contracting is dependent upon the recruitment of muscle fibers associated with a single motor unit (Burke 1980). The number of fibers associated with each motor unit varies considerably between muscles. Muscles involved in small, delicate movements are associated with motor units whose individual size is small. Hennerman & Olsen (1965) have indicated that small motor units tend to develop small forces and the range of force development per motor unit is dependent upon the type of

fiber innervated. If a muscle is composed of small motor units the total tension of the muscle can be increased in small increments by the recruitment of additional motor units. If motor units are large, much greater increments in force development occur as each additional motor unit is recruited. Thus, fine control of muscle tension is achieved with small motor units.

The motor neurones to a given muscle fire asynchronously. Thus, some motor units may be active while other units are inactive. In muscles which are active for long periods of time, such as the postural muscles, this asynchronous activity helps prevent fatigue that might otherwise result from prolonged activity. Asynchronous activity is able to maintain a nearly constant tension in a muscle. The tension developed by a contracting fiber is also dependent upon the frequency at which the muscle is being stimulated ( i.e. whether or not summation or tetany has occurred), the muscle fiber length (which determines the degree of overlap of the filaments) and the duration of activity (i.e. fatigue). Thus for an increasing frequency of stimulation, motor unit recruitment and the summation properties of the muscle result in graded increases in tension development. At maximum tetanic tension (Fmax) all the muscle fibers are contracting and developing their maximal force. A force−frequency curve can be constructed by expressing the force generated at each stimulation frequency as a percentage of maximal force development (see Figure 1.5 earlier).

### 1.1.3.4 What determines the extent to which muscles fatigue?

Muscle fatigue has been extensively studied, but there is no general agreement as to its definition. Muscle fatigue has been used to mean many different things and has been defined as an inability to sustain the required or expected force (Edwards 1975) and also as a loss of force−generating capacity (Bigland−Ritchie & Woods 1984). Fatigue can be quantitatively measured by the execution of maximal voluntary contractions over a period of time or by electrical stimulation at a tetanic frequency. Generally, following repetitive stimuli muscle exhibits impaired contractile performance, characterised by reductions in the maximal contraction velocity, peak twitch and tetanic tensions, rate of ATP hydrolysis and slower relaxation rates (Maher, Goodman, Francesceoni, Bowers, Hartley & Angelakos 1972; Edwards, Hill & Jones 1975; Dawson, Gadian & Wilkie 1978,1980; Petrofsky, Guard & Phillips 1980; Edman & Mattiozzi 1981; Fitts, Coutright, Kim & Witzman 1982). Whether the site of fatigue is the nervous system or the muscle itself is of great debate (see Edwards 1975). Several possible mechanisms have been proposed, including excitation−contraction coupling (Edwards, Hill, Jones & Merton 1977), changes in membrane function (Jones 1975), reduction of high energy phosphates (Sahlin 1978; Spande & Schottelius 1970) and accumulation of hydrogen ions (Sahlin, Edstrom, Sjoholm & Hultman 1981; Sahlin, Edstrom & Sjoholm 1983; Hermansen 1981; Hultman, Del Canale & Sojoholm 1985).

A number of studies indicate that fatigue of the quadraceps is confined to the muscle since maximal voluntary force and maximal tetanic force produced by direct stimulation of the muscle are reduced in parallel (Merton 1954). However, reduced nervous system activity may be responsible for fatigue in the soleus and diaphragm (Bigland–Ritchie, Furbush & Woods 1986). Fatigue could be produced at all sites involved in the transmission and contraction mechanisms within the muscle (see Figure 1.3).

During ischemic exercise mitochondrial respiration is limited by a lack of oxygen supply and cells resort to anaerobic metabolism for maintenance of ATP supply. As ATP demand increases during prolonged exercise, demand may exceed the capacity of the citric acid cycle to support mitochondrial oxidation. Under both these conditions a raised muscle lactate concentration is likely to arise. Muscle pH declines due to raised levels of hydrogen ion production and creatine phosphate concentration may reach near total depletion (Sahlin, Harris & Hultman 1975; Dawson *et al* 1978). Lowering of muscle ATP concentration was only apparent following a 60% reduction in force generation in frog muscles when measured by phosphorus nuclear magnetic resonance (Dawson *et al* 1978). Similarly, ATP concentration of human *musculus quadraceps femoris* fell from 25.8 mmol/kg dry weight to 18.5 mmol/kg dry weight following knee extension exercises (Sahlin *et al* 1975). Breakdown of creatine phosphate and ATP yield raised levels of ADP and inorganic phosphate (Sahlin *et al* 1975; Dawson *et al* 1986). The muscle potassium concentration may also be raised (Sjogaard, Adams & Saltin 1985).

Depletion of glycogen stores may reduce the rate of ATP production but this may not be related to fatigue since recovery of maximal strength occurs rapidly (Edwards, Hill & Jones 1977). When creatine phosphate is low ATP turnover may be reduced hence limiting further contraction, but again this has been observed without changes in force production (Hultman & Sjoholm 1983; Edwards *et al* 1975). This is possibly due to slower cross bridge turnover. *In vitro* studies of skeletal muscle fibers however, have demonstrated that reduced creatine phosphate and glycogen do not depress maximal tetanic force generation (Godt & Nosek 1986; Fitts *et al* 1982).

Accumulation of hydrogen ions has been reported to reduce isometric tension (Edman & Mattizoni 1981; Curtin & Rawlingson 1984; Donaldson & Hermansen 1978; Fabiato & Fabiato 1978; Cooke, Franks, Luciani & Pate 1988). This may be the result of reduced supply of ATP from glycolysis due to reduced activity of phosphofructokinase (Hermansen 1981). However, raised hydrogen ion concentrations have also been reported to compete with Ca++ ions for the calcium binding site on troponin C (Fuch, Reddy & Briggs 1970), reduce myosin–ATPase activity (Cooke *et al* 1988), enhance calcium binding to sarcoplasmic reticulum (Nakamura & Schultz 1970), influence membrane depolarisation (Hagberg 1985) and influence the excitation–contraction coupling (Katz & Hechi 1969). Thus, hydrogen ions will effect both the provision and utilization of ATP.

Table 1.3. Normal values for mammalian skeletal muscle function taken from the literature.

|  | TPT (ms) | ½RT (ms) | PT | FMAX | REF |
|---|---|---|---|---|---|
|  |  |  | (units defined below) | | |
| RAT |  |  |  |  |  |
| SOLEUS | 10.75 | 9.25 | 0.5 | 2.7 | 1 |
|  | 52 | 30 | 0.7 | 3.1 | 2 |
|  | 35 | 40 | -- | -- | 3 |
|  | 24 | 46 | 0.63 | 2.99 | 4 |
|  | 31 | 35 | -- | -- | 5 |
|  | 34 | 49 | 30.5 | 184 | 6 |
|  | 36 | 48 | -- | 156 | 7 |
| EDL | 28 | 36 | 0.6 | 2.6 | 1 |
|  | 17 | 20 | -- | -- | 3 |
|  | 12.6 | 8 | 64.6 | 317 | 6 |
|  | 12.5 | 8 | -- | 224 | 7 |
| TA | 15 | 18 | 3.9 | 13 | 2 |
|  | 17 | 14 | 3.3 | 13.5 | 4 |
|  | 17 | 10 | -- | -- | 5 |
| HAMSTER |  |  |  |  |  |
| PLANTARIS | 16.4 | 11.4 | 262 | 4.6 | 8 |
| SOLEUS | 27.5 | 29.3 | 271 | 3.4 | 8 |
| EDL | 20.8 | 16.0 | 529 | 8.0 | 8 |

KEY: TPT - Time to Peak Tension; ½RT - Half Relaxation Time; PT - Peak Twitch Tension; F - Maximal Tetanic Force.

REFERENCES: 1 - Close & Hoh 1968 (forces given as $kg/cm^2$); 2 - Larsson *et al* 1988 (forces in N); 3 - Kossler & Kuchler 1987 (forces in N); 4 - Larsson & Edstrom 1986 (forces in N); 5 - Edstrom & Larsson 1987; 6 - Close 1969 (forces in g); 7 - Close 1965 (forces in g); 8 - Drew *et al* 1988 (forces in $g/cm^2$).

Conduction block may occur across the transverse tubules due to potassium accumulation (Bigland–Ritchie, Jones & Woods 1979; Jones 1979) but this may only occur under exhaustive conditions (Vollestadt & Sejersted 1988). Alteration in potassium and sodium concentration over the muscle membrane may alter the membrane potential and result in the gradual reduction in calcium release per depolarisation (Edwards 1975). This evidence taken together tends to suggest that the major contributor to fatigue is ATP availability, although this does not explain all the conditions under which fatigue is noted.

### 1.1.3.5 "Normal" skeletal muscle function.

Normality is difficult to define as most physiological investigations are conducted with the muscle under different conditions. Table 1.3 lists some functional characteristics of mammalian skeletal muscle available in the literature, and is only included as a representative guide. From this table, however, it is clear that different muscles exhibit distinct characteristics. The soleus muscle is relatively slow in comparison to the EDL and TA, yet even within a given muscle of a named species the characteristics may vary with the nature of the conditions of measurement. Despite the problem associated with taking data from the literature, the soleus muscle of different animals is generally slower to contract and relax than the EDL of that animal. Similarly, the force generated by the soleus muscle tends to be lower than that generated by the faster–twitch muscles and may partially be attributed to an effect of size. The other major problem with summarising the literature is the nature of the units of measurement. Thus the force quoted in Table 1.3 range form Newtons to kg or g per square centimetre. Since it is not always possible to calculate these figures from the information available in the literature this does not provide a satisfactory basis for comparison.

### 1.2 HOW UNDERNUTRITION AND INFECTION INFLUENCE SKELETAL MUSCLE STRUCTURE AND ENERGY METABOLISM.

From the foregoing section it is evident that normal function is dependent upon those factors determining skeletal muscle contraction and relaxation being optimal. Before describing the experiments of this study which have assessed skeletal muscle function following limitation in quantity or quality of food intake and infection it is necessary to describe how the factors which determine skeletal muscle function become altered under these conditions.

### 1.2.1 How is skeletal muscle mass maintained?

A major determinant of skeletal muscle strength is the size or mass of the muscle. Skeletal muscle mass is composed of intracellular contractile and non–contractile proteins (15%), extracellular proteins (3.4%), glycogen (0.5%) and triglyceride stores (1.6%), the remainder of muscle mass is

water (Layman, Merdian−Bender, Hegarty & Swan 1981). The protein content of muscle is determined by the balance between the rates of protein synthesis and degradation. In growing muscle the rates of both protein synthesis and degradation may be high, and decreases with age (Millward & Waterlow 1978). In order to lay down lean tissue, however, the rate of protein synthesis must exceed the rate of degradation. Growth of skeletal muscles involves both hypertrophy and hyperplasia (Reeds, Jackson, Picou & Poulter 1978) thus the provision of the components of intra− and extra−cellular proteins, carbohydrates and triglycerides is required.

Following limitation in food intake and infection muscle mass may be reduced (Montgomery 1962; Heymsfield, Raymond, Kutner & Nixon 1979; Layman *et al* 1981; Lopes,Russell, Whitwell & Jeejeebhoy 1982).

Reductions in muscle mass may arise in several ways. A reduction in the synthetic rate could be associated with raised degradation leading to net protein breakdown. Similar magnitudes of net degradation could be achieved by reduced synthetic rates with no alteration in the rate of protein degradation or no change in the protein synthetic rate with an increased rate of protein degradation. Alternatively, the rate of synthesis may be increased whilst greater increases in the rate of degradation occur. Before discussing the situations and predominance of each of these variations in the rates of protein synthesis and degradation it is necessary to look at the manner in which protein balance is regulated.

One of the major regulators of protein balance is the endocrine system. Hormonal regulation of protein deposition is not clearly understood (Millward 1990). However, Millward (1990) has proposed a scheme in which the high concentrations of insulin in response to a meal effect activation of the initiation of translation. Insulin action is dependent upon the levels of circulating counter−regulatory hormones, since high glucocorticoid concentrations exert a great catabolic action and the rapid fall in circulating glucocorticoids following refeeding allows maximal insulin stimulation of protein synthesis (Odedra, Bates & Millward 1983). Insulin would appear to be aided in its activity by tri−iodothyronine (T3), insulin−like growth factor−1 (IGF−1) and growth factors (eg. somatostatin). Thyroxine−deiodinase is an insulin−dependent enzyme (Sato & Robbins 1981; Gavin & Moeller 1983) thus circulating levels of insulin dictate the serum T3 concentration. The low levels of IGF−1 in diabetic rats is also believed to reflect low insulin concentrations (Phillips, Fusco & Unterman 1985).

The scheme proposed by Millward (1990) therefore requires sustained dietary intake at a level at which insulin concentrations are maintained at elevated levels for periods prolonged sufficiently to initiate elevated concentrations of circulating T3 and IGF−1, thus stimulating growth.

The regulation of protein breakdown is likely to be the reverse of synthesis thus requiring low concentrations of circulating insulin and raised concentrations of the counter−regulatory hormones. This may involve lysosomal (Baracos, Ruderman, Dinerello & Goldberg 1983), calcium activated proteases (Lowell, Ruderman & Goodman 1986), prostaglandin mediated (Baracos *et al* 1983) or gelsolin−like compounds (D′Hasse, Rutschmann, Dahlmann & Hinssen 1987), Although the mechanism of protein loss from skeletal muscle remains unclear each of the processes may contribute to the net loss of muscle tissue under different conditions.

Under conditions of severe wasting the intracellular proteins become degraded in order to provide both carbon for energy provision and nitrogen for utilization at other organ sites. This may firstly arise by degradation of intracellular non−contractile proteins although some contractile protein may be lost as indicated by 3−methylhistidine excretion (Cahill 1976). Extracellular proteins (e.g. collagen) would appear to be more resistant to degradation during skeletal muscle wasting (see later). This loss of muscle protein may not however, be solely satisfying energy requirements. It is believed that the body may regulate lean body mass to a level at which the energy and protein consumed is appropriate. Thus a lower lean body mass represents a lower energy expenditure requiring a lower energy and protein intake. Thus this may form part of an adaptive process.

A number of possible conditions could underlie the need to degrade protein. The provision of carbon for gluconeogenesis with wastage of nitrogen or provision of nitrogen associated with acid base balance with the wastage of carbon may occur. There may be specific demands for a component of the protein with both carbon and nitrogen being wasted. A specific demand for individual amino acids may arise, for example for detoxification. Amino acids of particular quality and quantity may be required for example to maintain the acute phase protein response. Demands for adrenaline, nucleotides and other metabolites derived from proteins or amino acids may increase. There may be demands for specific metabolites from skeletal muscle, such as taurine. Alternatively, negative nitrogen balance may simply be a whole body response with the need for a loss of appetite, in that feeding itself produces an undesireable metabolic state. These options are not mutually exclusive and may occur as part of a well organised response.

Changes in the various components of skeletal muscle when muscle mass diminishes do not occur simultaneously. Glycogen and triglyceride stores are likely to become reduced, as they are utilised to provide cellular energy, before detection of large amounts of protein loss from the muscle. As glycogen within skeletal muscle becomes depleted there may be an apparent increase in water content of the muscle due to alterations in serum albumin concentration allowing leakage of water out of the circulation and the high binding affinity of collagen (Layman *et al* 1981).

Since water comprises the greatest component of muscle mass, profound alterations in components other than water would not greatly influence muscle mass, yet may cause subtle alterations in the functional capacity of the muscle.

What happens to these processes under conditions of limitations in food intake or infection?

### 1.2.2. The influence of reductions in food intake on skeletal muscle structure and energy metabolism.

Undernutrition may result from a variety of causes. The simplest to consider is the situation of a complete reduction in food intake (e.g. starvation) or the partial reduction of food intake (e.g dieting). Maldigestion and malabsorption as the cause or consequence of disease processes may also result in undernourishment and may be responsible for specific nutrient deficiencies (e.g. fat soluble vitamin deficiencies associated with cystic fibrosis). Undernutrition may also result from the consumption of a diet in which there is a limiting nutrient. In today's society it is rare to see such cases of specific nutrient deficiency resulting from dietary intake due to the large variety of foods which constitute the diet. However, where the nutrient source is limited such that the major energy source is from a particular food or foods such specific nutrient deficiencies may arise.

While each of these areas of undernourishment warrants further study this thesis is particularly concerned with undernourishment as a result of limitations in the amount and composition of the food consumed. Hence, the subsequent sections will concentrate on these areas.

The mammalian body responds to the ingestion of food in a specific manner such that ingestion of food results in the secretion of hormones responsible for the stimulation of metabolism and storage of the carbohydrate, fat and protein. Two states of food ingestion have been described: namely the absorptive state during which the ingested nutrients are entering the blood from the gastrointestinal tract (GIT) and the post−absorptive state during which the GIT is empty and energy must be supplied from the body's endogenous reserves. The absorptive state can be summarised as follows. During absorption of a meal glucose provides the major energy source with a small proportion of absorbed amino acids and fats being utilised for energy. Part of the amino acids and fats is used in the resynthesis of degraded proteins and fats from the previous postabsorptive state. Most of the amino acids and fats, as well as the carbohydrates not oxidised for energy, are metabolised into adipose tissue fat or become part of the triglyceride pool stored in skeletal muscle.

In the postabsorptive state carbohydrate is derived from gluconeogenesis but the utilization of glucose for energy is reduced. Oxidation of endogenous fats provides most of the body basal energy requirements and fat and protein synthesis is reduced leading to a net breakdown of these substances.

The endocrine responses during the absorptive and post—absorptive periods have been well documented and are described below. Clearly, lowered food intake will attenuate the pattern of hormone release with consequent alterations in the manner in which substrates may be metabolised by the body. Since energy provision is important in the maintenance of skeletal muscle function, the contractile characteristics of skeletal muscle may become impaired.

### 1.2.2.1 Response to acute food deprivation.

Few investigators have measured the absolute amount of high energy compounds within skeletal muscles of animals following food deprivation. Russell and co—workers reported that following both 2 and 5 days fasting the concentration of ATP in the rat gastrocnemius muscle remained unaltered (Russell, Atwood, Whittaker, Itakura, Walker, Mickle & Jeejeebhoy 1984). There was evidence of a 15% lower creatine phosphate concentration but this difference was not reported to be statistically significant (Russell *et al* 1984). The muscle biopsies were taken following a period of sustained contraction however, and may reflect the utilization of ATP in the absence of sufficient blood supply to replenish high energy compounds. Creatine turnover in muscle of 5 day fasted rats was increased two fold (Waterlow, Neale, Rowe & Palin 1972), probably reflecting an increased utilization of creatine phosphate to maintain the muscle ATP concentration. It therefore remains unclear whether acute starvation results in changes in the concentration of high energy compounds within skeletal muscle.

Since there is a limited capacity for the resynthesis of ATP from creatine phosphate it is important to also know the effects of starvation on the processes of glycolysis and oxidative phosphorylation.

Glycolysis is generally believed to be suppressed following acute starvation (Young & Scrimshaw 1971; Cahill 1976). The maximal activity of pyruvate kinase (PK) in skeletal muscle homogenates, a rate limiting enzyme in glycolysis, has been reported to be lower following fasting in rats (Layman *et al* 1981; Russell *et al* 1984). The maximal activity of succinyl dehydrogenase (SDH), a rate limiting enzyme of the tricarboxylic acid cycle, in skeletal muscle homogenates of fasted rats was also reduced (Layman *et al* 1983) or did not change (Russell *et al* 1984). Such changes may be indicative of an overall reduction in the glycolytic and oxidative capacities of skeletal muscle. Oxygen uptake by homogenates of rat skeletal muscle following 4 days starvation was 25—30% lower than that of control muscles, indicative of a reduced oxidative capacity — but was increased

in the heart and soleus muscle (Layman *et al* 1981). Thus it appears that the fiber composition may play an important role in determining the metabolic capacity of skeletal muscle in response to acute changes in food intake.

The relative contribution made by the oxidation of fatty acids to energy provision is increased as fatty acids are mobilised in response to low circulating insulin concentration and raised glucagon concentration resulting in reduced adipocyte lipoprotein lipase activity suppressing the storage of fatty acids as triacylglycerol (Young & Scrimshaw 1971; Cahill 1976). It is generally accepted that following acute starvation the rates of both protein synthesis and degradation are reduced (Henshaw, Hirsh, Morton & Hiatt 1971; Goldberg & Goldspink 1975; Millward, Garlick, James, Nnanyelugo & Ryatt 1978; Odedra *et al* 1983; Rennie 1985). The reduced rate of protein synthesis is associated with low concentrations of insulin. It remains unclear, however, as to the degradative mechanism(s) which may be influenced by starvation. Circulating fatty acids themselves may play a role, directly attenuating degradation of myofibrilar proteins – treatment of rats with tetradecylglycidate (an inhibitor of long chain fatty acid oxidation) reduced the elevated degradation of non–myofibrilar protein (Lowell & Goodman 1987). The response of other degradative processes for example prostaglandin–mediated proteolysis, lysosomal degradation and calcium–mediated mechanisms is unclear. Russell and colleagues have reported raised intracellular calcium concentrations in the gastrocnemius muscle of 5 day fasted rats. Thus it would seem unlikely that calcium–mediated mechanisms are involved in determining the rate of protein degradation in skeletal muscle following starvation (at least in the rat). Similarly, gelsolin concentration is reported to be raised following starvation (D'Hasse *et al* 1987) and is also unlikely to play a role in this reduced rate of protein degradation.

The net outcome of the reductions in the rates of both protein synthesis and degradation is that little skeletal muscle mass is lost following starvation (Layman *et al* 1981; Heymsfield, Stevens, Noel, McManus, Smith & Nixon 1982). Despite a 50% reduction in the glycogen content of the rat psoas muscle no change in muscle size could be detected (Heymsfield *et al* 1982). However, Heymsfield and associates calculate that a loss of glycogen of this magnitude should incur a loss of water equivalent to a reduction in size of 3.6%, this was not seen (Heymsfield *et al* 1982). Other components of skeletal muscle did not change at this time (Heymsfield *et al* 1982).

It remains unclear whether the skeletal muscles following acute food deprivation are energy deficient. It is generally accepted that the rate of glycolysis may be reduced, but oxidative capacities may vary allowing some muscles particularly those with high content of slow type I

fibers (see Section 1.1) to maintain energy provision. Since skeletal muscle mass changes little in this acute situation it is assumed that the processes of oxidative phosphorylation, glycolysis and ATP resynthesis from creatine phosphate are sufficient to satisfy the energy requirements of the muscle.

### 1.2.2.2 Response to chronic food restriction.

Skeletal muscle appears to be robust in the face of acute changes in food intake. Changes in food intake are likely to occur for long periods of time and it is therefore possible that the ability of muscle to preserve its function and metabolism is impaired following more chronic limitations of food intake.

The concentration of ATP within rat gastrocnemius muscle following 21 days of food restriction at a level of 25% that consumed by control animals was not different to that of control animals (Russell *et al* 1984). Muscle creatine phosphate concentration was reduced by 50% in the food restricted animals (Russell *et al* 1984). This suggests that the rate of regeneration of ATP cannot be maintained from creatine phosphate alone. The maximal activity of both PK and SDH in rat skeletal muscle homogenates were reduced following both 9 days partial food restriction (Layman *et al* 1981) and following 21 days food restriction (Russell *et al* 1984). This indicates a reduced glycolytic flux which was greater than that observed following acute starvation (Layman *et al* 1981; Russell *et al* 1984).

Oxygen uptake of muscle homogenates was 50% lower in the gastrocnemius, biceps branchii and psoas muscles of 9 day partially food restricted rats but was increased in the heart and soleus muscles (Layman *et al* 1981). The reduction in aerobic capacity would be in agreement with studies of Goldspink and Waterson (1979) where raised binding of nitroblue tetrazonium to skeletal muscle sections were taken to indicate a reduced mitochondrial number which has also been reported by Hansen–Smith and colleagues (Hansen–Smith, Van Horn & Maksud 1978). Following 9 days partial restriction of food intake in rats, however, histological examination revealed mitochondria to be enlarged and that internal cisternae were disrupted suggestive of a reduced capacity to mediate oxidative metabolism. Since no change in the mitochondrial number or distribution was reported following 4 days starvation in the rat (Layman *et al* 1981) it is tempting to suggest that this is a specific response to the prolongation of a reduction in food intake. This data therefore agrees with the generally held view that chronic food restriction results in reductions in both glycolysis and oxidative phosphorylation (Young & Scrimshaw 1971; Cahill 1976). Such responses may reflect the hormonal regulation of substrate metabolism. Thus under conditions of prolonged restriction of food intake little mobilisation of muscle and liver glycogen would occur, fatty acid mobilisation may be reduced, the concentration of circulating ketone bodies may be raised and protein degradation may be increased (Cahill 1976). This may result

form the reduced insulin, T3, IGF−1 and growth hormone concentrations and the increased concentration of circulating counter−regulatory hormones (Young & Scrimshaw 1971; Cahill 1976; Goldgerg, Tischler, De Martino & Griffin 1980).

It is generally accepted that net degradation of protein under conditions of chronic food restriction results from reduced rates of protein synthesis and raised rates of protein degradation (see Rennie 1985). Raised intracellular calcium concentration following 21 days food restriction in the rat (Russell *et al* 1984) may indicate the involvement of calcium−mediated mechanisms. The increased concentrations of gelsolin seen with food restriction (D′Hasse *et al* 1987) may also play an important role although there is no direct evidence to support this.

The net outcome of this increased protein degradation is the loss of skeletal muscle tissue. Muscle size is reported to be lower in both humans (Heymsfield *et al* 1982; Russell, Walker, Leiter, Sima, Tanner, Mickle, Whitwell & Jeejeebhoy 1983) and in the rat (Heymsfield *et al* 1982; Russell *et al* 1984) following prolonged food restriction. The animal studies however, indicate that muscles composed of slow twitch fibers may not be effected to the same extent as those of fast−twitch fibers. Thus while the gastrocnemius, EDL, biceps branchii and psoas muscles lost 24−32% of their weight following 9 days partial food restriction, the soleus muscle lost only 9% (Layman *et al* 1981). All components of muscle mass were reduced, the greatest effect being seen in the water and glycogen content. The relative water content of the muscle was increased (Layman *et al* 1981). Myofibril diameter was smaller and there appeared to be an increase in the relative amount of connective tissue content, associated with intrafibrilar oedema. The apparent increase in the connective tissue content may reflect the slower turnover rates of these proteins in comparison to the soluble intracellular proteins.

The apparent raised water content of skeletal muscle is believed to reflect a lower serum albumin concentration which allows leakage of water out of the circulation and the relative increase in connective tissue which is believed to have a high affinity to bind water (see Layman *et al* 1981).

Thus, following periods of prolonged limitation of food intake the energy available within the skeletal muscle appears to be reduced due to a lower capacity of all three ATP resynthesising mechanisms. The components of muscle are greatly changed with large reductions in glycogen and water resulting in lower skeletal muscle mass. Some muscles, particularly those with a high content of slow−twitch type I fibers, may be effected to a lesser extent than others. These limitations in energy provision and size of the skeletal muscle could therefore limit the functional capacity of skeletal muscles and is discussed further below.

Table 1.4 summarises the discussion of those factors which may influence skeletal muscle function. These factors may be of importance during states which deviate from the "normal". Clearly, alteration in these factors may occur through endocrine, neural of other routes and would therefore be expected to effect skeletal muscle function.

### 1.2.2.3 Response to the consumption of low protein diets.

Energy availability is believed to be reduced in the clinical condition of protein energy malnutrition associated with the consumption of low protein diets. No biochemical studies have been completed to determine the absolute muscle ATP concentrations. Reeds and colleagues have noted that creatine turnover in malnourished Jamaican infants was unaffected following recovery from malnutrition (Reeds *et al* 1978), in agreement with data presented by Waterlow and colleagues (Waterlow *et al* 1972). It would seem likely that there was a general inability to maintain energy production within the muscle which may be associated with changes in substrate turnover, ion pumping and membrane transport mechanisms. Specific details of such changes in protein energy malnutrition are not clearly defined.

Pyruvate kinase activity, as a measure of glycolytic flux, has been reported to be lower in skeletal muscles of protein calorie malnourished individuals (Metcoff 1967) but no studies are available to confirm this observation. The oxidative capacity of skeletal muscles under conditions of protein deficiency are not known.

The hormonal profile is not necessarily that expected from the consideration of lowered food intake which is commonly associated with the consumption of low protein diets. While the circulating insulin concentration may be either normal or reduced in the protein deficient individual, fasted individuals exhibit reduced serum insulin concentrations (Heard, Frangi, Wright & McCartney 1977). Serum T3 concentration is reported to be reduced in fasted individuals and in kwashiorkor while the T3 concentration may be normal in the marasmic individual (see Becker 1983). Circulating glucagon is normal in the protein−deplete host while cortisol, growth hormone and catecholeamines (though not dopamine) are raised (Becker 1983). The hormonal profile of marasmus and kwashiorkor are presented in Table 1.5, for the readers reference the hormonal profile observed following starvation has been included. For greater detail the reader is referred to the review of Becker (1983). Thus the hormonal profile in the protein deficient host is likely to stimulate gluconeogenesis and mobilise fat stores. However, with prolongation of the consumption of a low protein diet it is likely that adipocyte fat stores will become near depleted and degradation of protein will occur to provide essential amino acid supplies to maintain nitrogen and energy for body requirements. Net degradation of muscle associated with the consumption of low protein diets may represent a reduction in the rate of protein synthesis and an increased rate of degradation. The rate of protein synthesis per DNA unit is reduced in

Table 1.4. Factors which may influence skeletal muscle function. (Adapted from Vollestadt & Sjoholm 1988)

| FACTOR | TWITCH RESPONSE | | | TETANIC RESPONSE | REFS |
|---|---|---|---|---|---|
| | PT | TPT | $\frac{1}{2}$RT | FMAX | |
| MUSCLE MASS DECREASE | R | N | N | R | 1 |
| pH DECREASE | R | ? | R | R | 2-8 |
| CP DECREASE | ? | ? | R | ? | 9 |
| ATP DECREASE | ? | R | ? | R | 10-11 |
| ADP INCREASE | ? | ? | R | I | 5,7,10 |
| Pi INCREASE | R | ? | R | R | 7,12-15 |
| LACTATE INCREASE | R | R | ? | R | 7 |
| Na/K INCREASE | R | ? | R | R | 16,17,24 |
| CALCIUM INCREASE | R | I | ? | R | 18-20 |
| | I | I | ? | ? | 21 |
| TEMPERATURE INCREASE | I | R | I | I | 22-23 |

KEY: I - INCREASED; R- REDUCED; ?- EFFECT UNKNOWN OR UNREPORTED;N- NORMAL; FOR TIME TO PEAK TENSION (TPT) AND HALF RELAXATION TIME ($\frac{1}{2}$RT) - REFLECTS SLOWING OF RESPONSES; PT- PEAK TWITCH TENSION;FMAX- MAXIMAL TETANIC FORCE; MRR MAXIMAL RELAXATION RATE. CP - CREATINE PHOSPHATE; Pi - INORGANIC PHOSPHATE

REFERENCES: 1-Drew 1988; 2-Trivedi 1966; 3-Donaldson 1978; 4-Nakamura 1972; 5-Sahlin 1975; 6-Godt 1986; 7-Cooke 1985; 8-Fabiato 1978; 9-Edwards 1975; 10-Dawson 1978; 11-Wiles 1982; 12-Kentish 1986; 13-Herzig 1981; 14-Chance 1965; 15-Kawai 1987; 16-Glyn 1975; 17-Jones 1981; 18-Chance 1965; 19-Rossi 1963; 20-Julian 1986; 21-Skomedal 1987; 22-Close 1968; 23-Kossler 1987; 24-Vergara 1978

young rats consuming protein free diets (Millward *et al* 1973), being 14% that of control muscles at 30 days following the commencement of consumption of the diet. Muscle concentration of RNA is reduced in protein deficient rats resulting in a lower rate of protein synthesis as a result of reduced translation (Young & Alexis 1968). The reduction in the rates of protein synthesis and degradation with age reported in well-nourished rats has been demonstrated in malnourished animals although the extent of this reduction is limited (Millward 1990).

Muscle wastage in malnourished individuals (Montgomery 1962; Reeds *et al* 1978) has been frequently reported in animal models (e.g. McCance & Widdowson 1956; Anthony & Edozein 1975; Lowrey, Pond, Barnes & Loosli 1962). The nature of this atrophy is unclear. McCance & Widdowson were unable to detect alterations in the muscle structure (McCance & Widdowson 1956), but Lowrey reported partial or complete loss of striations within the muscles (Lowrey *et al* 1962). The components of skeletal muscle which are lost have not been completely investigated. Montgomery noted that there was a reduced cross sectional area, reduced area of muscle bundles in comparison to the area of the whole muscle and a reduced fiber size. This was associated with an increase in extracellular space, connective tissue and possibly water content (Montgomery 1962). This may reflect the increase in total water content observed by Smith (1960), Garrow, Fletcher & Haliday (1965) and Waterlow & Mendes (1957). However, no change in the muscle water content was observed in rats consuming a low protein-casava based diet (Widdowson & McCance 1957).

The lowered body weight in protein deficient animals will also reflect the reductions in liver weight which has been recorded in many animal studies (McCance & Widdowson 1956; Anthony & Edozein 1975; Lowrey, Pond, Barnes & Loosli 1962). This weight probably reflects the expected size of the liver that is occupied by weight matched controls (McCance & Widdowson 1956). Development of fatty liver in some animals (e.g. Lowrey *et al* 1962; Anthony & Edozein 1975; Ausman, Gallina & Heested 1989) reflects the kwashiorkor type of malnutrition. The changes which may occur with respect of the other processes involved in the regulation of muscle function, such as intercellular concentrations of calcium or hydrogen ions for example remain to be elucidated.

1.2.2.4 <u>Response to infection.</u>

Infection may arise through numerous mechanisms. Common to many bacterial infections is the endotoxemia resulting from the effects of components of bacterial cell walls (Biesel 1977). Many of the effects of endotoxin are thought to be mediated via interleukin-1 (IL-1) and tumour necrosis factor (TNF). Elevated interleukin-1 concentrations have been demonstrated in the blood and tissues of infected men and animals (Powanda, Cockerell, Moe, Abeles, Pekarek & Canconico 1975). Septic and infected patients exhibit a raised urinary excretion of

Table 1.5. Summary table of the influence of consumption of low protein diets and fasting on circulating hormonal profile. For further details see text.

|  | STARVATION | MARASMUS/KWASHIORKOR |
|---|---|---|
| Growth Hormone | R | I |
| Somatomedin | R | R |
|  |  |  |
| Thyroid Hormones |  |  |
| TSH | R/N | I(N/R in marasmus) |
| T3 | R | R(N in marasmus) |
| T4 | I | I(N/R in marasmus) |
|  |  |  |
| Leutenising Hormone | R | N/R |
| FSH | R | N/R |
| Testostertone | R | R |
| Oestrogen | R | R |
| Prolactin | variable | R |
|  |  |  |
| Cortisol | I | I |
| Aldosterone | ? | I |
| Nor/Adrenaline | R | I |
| Dopamine | ? | R |
| Antidiuretic Hormone | ? | I(N in marasmus) |
|  |  |  |
| Insulin | R | R/N |
| Glucagon | R | N |

KEY: N - normal, I - increased, R - reduced, ? - unknown or not quantified.

methylhistidine (Long, Birkhahn, Geiger, Betts, Schiller & Blakemore 1982) implicating muscle degradation in this response. Similarly, the septic rat model exhibited muscle wastage in response to pneumonia infection (Ruff & Secrist 1984). Studies showing alterations in skeletal muscle protein turnover in rats following administration of endotoxin and IL−1 would suggest that these effects are due to endotoxin and IL−1 (Baracos *et al* 1983; Jepson, Pell, Bates & Millward 1988; Jepson, Bates & Millward 1988) and are likely to involve reduced protein synthesis (Jepson, Cox, Bates, Rothwell,Stock, Cady & Millward 1986).

The increased demand for energy to support the phagocytic, proliferative and secretory events of the inflammatory response is initially provided by body glucose (see Beisel 1977). Plasma creatine concentration increased at 4.5 hours following administration of endotoxin in sheep (Southorn & Thompson 1986). Increased creatine turnover has also been reported in rats with lung infections (Waterlow *et al* 1972). Reduced creatine phosphate concentration has also been reported in the muscle biopsies of severely ill patients resulting in lowered muscle ATP concentrations, the magnitude of difference becoming greater as the illness progressed (Bergstrom, Bostrom, Furst, Hultman & Vinnars 1976). This suggests that demands upon phosphagenolysis to resynthesis ATP cannot be maintained.

In the study of sheep by Southorn & Thompson, plasma glucose concentration had fallen 3 hours following endotoxin administration and remained reduced for 52 hours (Southorn & Thompson 1986). Similar observations were made by Ward and colleagues in ponies with plasma glucose falling 50% by 24 hours with endotoxin injections at 0, 6, 12 and 18 hours (Ward, Fessler, Bottom & Turek 1987). No alteration in plasma glucose concentration were noted following repeated endotoxin administration to rats over 30 hours (Jepson *et al* 1986). In the study of Southorn & Thompson plasma insulin concentration appears not to reflect the observed alterations in plasma glucose: being reduced at 1.5 hours (before the fall in plasma glucose) and then becoming raised from 4 to 13 hours (Southorn & Thompson 1986).

As the circulating glucose concentrations fall glycogen degradation increases, but glycogen stores may rapidly become depleted. Protein degradation becomes a source of gluconeogenic substrates. Unlike the situation during prolonged starvation, during infection hepatic ketogenesis is impaired − thus reducing the concentration of ketone bodies in the circulation (Neufield, Pace & White 1976). Changes in oxidative metabolism have not been studied during endotoxemia, but inhibition of energy dependent calcium transport in the mitochondria indicates that the capacity for oxidative phosphorylation would be reduced (Nicholas, Mela & Miller 1974). This may result in raised intracellular calcium concentrations within skeletal muscle which may initiate increased protein degradation via calcium−mediated proteases, a reduced sequestration of calcium by the sarcoplasmic reticulum and may further inhibit mitochondrial function. As such peripheral tissues rely to greater amounts on protein degradation for the maintenance of energy

supply. Lysozyme secreted by neutrophils has been suggested to be responsible for part of this proteolysis (Baracos *et al* 1983) and IL−1 has been shown to be a potent stimulator of lysosome activity via PGE2 production.

The extent of muscle protein degradation may be compounded due to endotoxin−induced anorexia. Administration of endotoxin resulted in reduced food intake of 33% in sheep (Southorn & Thompson 1986), 25% in rats (Wan 1987) and 39% in rats undergoing continuous infusion of endotoxin for 24 hours (Fish, Lang & Spitzer 1986). Such a reduction in food intake may be responsible for the reduced body weight that is observed in rats (Fish et al 1986; Wan 1987). However, this reduced body mass is also a reflection of the reduced weight of muscle (13% of control) and skin (11% of control) (Wan, Grimble & Gore 1984; Wan & Grimble 1986) and the increased mass of liver (27% of control) (Fish et al 1986; Wan & Grimble 1986). These changes in liver and muscle weights are reflected in changes in the protein content and fractional synthesis rates of these organs (Wan *et al* 1984; Wan & Grimble 1986).

Sepsis is associated with reduced protein synthesis (Powanda *et al* 1972; Stein & Padykula 1962). However small doses of IL−1 stimulate protein synthesis in rat muscle while similar doses of IL−1 in conjunction with tumour necrosis factor result in net protein degradation (Flores, Bistrian, Pomposelli, Dinerello, Blackburn & Istfan 1989). There may also be a species difference with an efflux of nitrogen form skeletal muscles of patients treated with TNF (Starnes, Warren, Jeevanandum, Gabrilove, Larchian, Ottgen & Brennan 1988) but no degradation in rats to either *in vitro* (Goldberg, Kettlekut, Furuno, Fageu & Baracos 1988) or *in vivo* (Kettelkut & Goldberg 1988) administration.

The rate of protein degradation isolated rat muscle preparations increased when incubated with a peptide, thought to be IL−1, isolated from septic patients (Clowes, George, Villee & Savaris 1983). They also reported an increased release of amino acids from the limb muscles of septic patients. Negative nitrogen balance appears to result from many types of insult and has been demonstrated to be due to an inhibition of synthesis and stimulation of degradation in sepsis in man (Long *et al* 1977), trauma (Birkhahn, Long, Fitkin, Jeevanandam & Blackmore 1981) and in live typhoid−cholera vaccination (Garlick, McNurlan, Fern, Tomkins & Waterlow 1980). These changes in protein turnover are likely to reflect the endocrine profile during endotoxemia with reduced concentrations of insulin and either raised or lowered concentrations of glucagon (Powanda 1977; Neufeld, Pace, Kaminski, Jahring, Wannemacher & Beisel 1980). Insulin insensitivity may develop during sepsis resulting in a lower rate of protein synthesis becoming apparent (Rocha, Sansteusa, Fabina & Unger 1973; Zenser, DeRubertis, George & Rayfield 1974).

Circulating T3 concentration during infection is reported to be lowered, thus limiting protein synthesis. This may represent a protective measure, since T3 injections given to prevent the normal lowering of circulating thyroid hormones observed during bacterial infection in rats raised mortality (Little 1985). Plasma total and free T3 concentrations did not, however, appear to be related to muscle proteolysis following *E.coli* lipopolysaccharide (LPS) treatment of rats (Jepson *et al* 1986). Tri−iodothyronine also plays an important role in the regulation of Na+,K+−ATPase resulting in reduced ion pumping activity over membranes (e.g. myocardium Tulp, Krupp, Danforth & Horton 1979), it is unclear if this is also apparent in skeletal muscle.

The ultimate end point for this increased demand for energy over prolonged periods may be the degradation of skeletal muscle. Endotoxin has been reported to result in reduction in skeletal muscle mass as reported above, however endotoxemia also has other implications. Coincident with these alterations in mass, organ blood flow is altered with an increased flow to the liver (68%) and heart (65%) 30 hours after the commencement of continuous infusion of endotoxin (Fish et al 1986; Burnier, Waeber, Aubert, Nussberger & Brunner 1988). Blood flow to other organs were observed to be reduced 30 hours following administration of endotoxin being 64%, 40% and 3% lower to the lungs, pancreas and skin of rats respectively (Fish *et al* 1986; Burnier *et al* 1988). While blood flow to muscle was reduced in both studies this only attained statistical significance in the study of Fish and colleagues (Fish *et al* 1986). Muscle blood flow in rats receiving repeated challenge with *E.coli* LPS was 57% lower in the back muscles and 64% lower in the hindlimb compared to control animals (Jepson *et al* 1986). Burnier and associates also noted depressed blood flow to other organs including the kidney, stomach, brain and intestine however these differences did not attain statistical significance (Burnier *et al* 1988).

Since blood flow to muscle may not be significantly impaired it is unlikely to influence the oxygen supply, although it should be noted that where reductions in blood flow were reported (Jepson *et al* 1986) oxygen uptake was reduced in hindlimb muscle of *E.coli* LPS−treated rats suggesting a reduced aerobic capacity. Plasma lactate concentration was reported to be increased following injections of endotoxin in ponies and may explain the lack of change in muscle lactate concentrations (Ward *et al* 1987). Reduced muscle pH also supports the suggestion of lactate production during endotoxemia (Guenter, Fiorca & Hinshaw 1969).

While a considerable amount is known about some of the processes which may influence ATP availability for example within skeletal muscle during endotoxemia further work is required to determine the nature of changes in these processes and their functional implications.

### 1.2.2.5 Response to infection following consumption of protein deficient diets.

Malnourishment is commonly associated with infection (Waterlow 1986). Thus the capacity of the body to supply energy for the protective functions of the immune response is of prime importance in these patients. In 1937, Vint (see Alleyne, Ray & Picou 1977) noted that children suffering from kwashiorkor showed thymic atrophy on post mortum study. Since T cells are involved in the response to infection, atrophy of the thymus during malnutrition obviously effects the hosts' immune response. Thymus atrophy has also been reported by Smythe and colleagues who noted that the size of the tonsils of malnourished children during infection were smaller than in normal children (Smythe, Schonland, Brenton–Stiles, Coovadia, Grace, Loening, Mofayne, Parent & Vox 1971). These malnourished children also exhibited delayed and reduced hypersensitivity, impaired lymphocyte transformation in response to phytohaemaglutinin and on post mortum exhibited reduced spleen weight. Impaired antibody production during infection in malnourished individuals has been reported by numerous workers (see Scrimshaw 1975).

Young and colleagues were unable to observe infection related changes in protein synthesis in rats fed low protein diets (Young, Chen & Newbere 1968). Recent studies suggest that whole body protein turnover is increased during infection in malnourished children but to a lesser extent than that of well nourished children (Tomkins, Garlick, Schofied & Waterlow 1983). In the study of Tomkins, enhanced excretion of methylhistidine was also noted in agreement with that of Long and associates where methylhistidine excretion reached 227% of that of normals during the febrile response to sepsis (Long *et al* 1982).

The acute phase response to infection may not be evident for example in the protein deplete guinea–pigs investigated by Drabik and co–workers (Drabik, Schnure, Mok, Moldawer, Dinarello, Blackburn & Bistrian 1987). In this study, however, the degree of malnutrition was severe, causing dramatic body weight loss indicating that the lack of an acute phase response may be due to an insufficient amino acid supply. From these studies it would appear that the production of acute phase proteins is dependent upon nutritional status, with some production of proteins in moderate protein malnutrition but none in severe cases.

The changes in body and electrolyte concentrations which occur during infection reported earlier did not develop in the protein deplete guinea pig administered endotoxin (Moldawer, Hamawy, Bistrian, George, Drabik, Dinarello & Blackburn 1985) although these electrolyte changes could be observed when the animals received exogenous IL–1. The magnitude of the change in the protein depleted animals was similar to that in control animals, suggesting that the lack of response represents a reduced *in vivo* capacity of IL–1 production. Peritoneal macrophages from these protein malnourished guinea pigs were also unable to secrete IL–1 *in vitro* confirming this hypothesis.

The maintained availability of energy in infected malnourished hosts has not been fully investigated. This provision of energy would have importance in the maintenance of skeletal muscle function which also requires further investigation.

## 1.3 HOW UNDERNUTRITION AND INFECTION EFFECT SKELETAL MUSCLE FUNCTION.

As noted earlier, malnutrition may result from either a partial or complete reduction of food intake, an alteration in food composition or through maldigestion and malabsorption. Our knowledge of the manner in which skeletal muscle functions as a result of these changes in food intake is limited and represents the main interest of this thesis.

### 1.3.1 Skeletal muscle function following limitation of food intake.

Human muscle function can be assessed using either maximal voluntary contractions (MVC) or via stimulation of a muscle via a peripheral nerve. While the MVC contains a motivational component which may limit force generation, this can be overcome by electrical stimulation of the peripheral nerve.

Despite the assumption that skeletal muscle performance is impaired in the malnourished state little conclusive evidence exists. A lower physical working capacity, defined as the ability to maintain productivity, has been reported for Guatamalan farm labourers (Viteri 1971), Columbian sugar cane cutters (Maksud, Spurr & Barac−Nieto 1976; Spurr, Barac−Nieto & Maksud 1977; Spurr, Barac−Nieto, Reina & Ramirez 1984) and Kenyan road diggers (Wolgemuth, Latham, Hall, Cheshire & Crompton 1981) compared to well nourished workers. These reports have been taken to indicate an impairment of the skeletal muscle, yet activity patterns in these workers may be altered such that a greater proportion of the day is spent completing the work task and less energy expended outside the work environment (See Ferro−Luzzi 1989; 1990). Supplementation of the diets of undernourished individuals has been reported to redress the imbalance in activity patterns (Viteri 1971; Prentice, Whitehead, Roberts & Paul 1984), but it is unclear whether this represents a change in central (i.e. motivational) or peripheral (i.e. within the skeletal muscle) mechanisms.

The report of a 21% lower grip strength in surgical patients, undernourished due to a variety of gastrointestinal disorders, when compared to gender, age and height matched control subjects (Windsor & Hill 1988) supports the contention that limitation of food intake impairs skeletal muscle function . This difference may also be due to the lower motivational state of the undernourished patients. Further evidence for the impairment of skeletal muscle function following limitation of food intake has come from studies of electrical stimulation of skeletal muscle. Thus, Jeejeebhoy and co−workers have reported a lower force generation in the

stimulated adductor pollicis muscle of obese subjects on maintenance diets (Russell, Leiter, Whitwell, Marliss & Jeejeebhoy 1983) and in patients with anorexia nervosa (Russell, Prendergast, Darby, Garfinkel, Whitwell & Jeejeebhoy 1983). This has also been reported in surgical patients less than 90% ideal body weight (Bruce, Newton & Woledge 1989). This lower force generation appears to be a rapid response to nutritional status apparent following 48 hours of starvation in human volunteers (Wootton, Sutton & Dague 1987). In these studies only that of Bruce and co–workers indicated a reduction in the size of the adductor pollicis (Bruce *et al* 1989).

Maximal relaxation rates of the human adductor pollicis following tetanic contractions were reported to be slower (Russell *et al* 1983; Wootton *et al* 1987; Bruce *et al* 1989). Slowing of the relaxation rate following limitation of food intake has been used to explain the greater relative force generation at low frequencies of stimulation noted in obese subjects on maintenance diets (Russell *et al* 1983a), anorectic individuals (Russell *et al* 1983b) and following 48 hours starvation (Wootton *et al* 1987).

The adductor pollicis of undernourished individuals has also been reported to fatigue more easily (Russell *et al* 1983b; Bruce *et al* 1989). The muscle may become ischemic resulting in reduced ATP resynthesis and a build up of metabolic by–products limiting the functional capacity of the muscle (see Section 1.1.2). Russell and colleagues, however, were unable to discern significant alterations in either substrate or energy status of the gastrocnemius muscle of obsess subjects after the consumption of maintenance diets.

The study of skeletal muscle function in human subjects is complicated by the central and cardiovascular factors which cannot be controlled or accurately assessed, and by the nature and uncertainty of the cause of the malnutrition. The development of animal models of undernutrition in which stimulated muscle function could be assessed independently of such factors may help to clarify this area.

Motor performance in previously undernourished and rehabilitated animals assessed by inherent activity (Gutherie 1968), ability to balance on rods (Galler & Turerwitz 1977) and maintaining position on a revolving drum (Smart & Bedi 1982) was not impaired compared to control animals. How this relates to physical working capacity in humans however, is unclear.

In 1978, McCarter and co–workers reported that a 60% reduction in food intake in early life did not impair *in vitro* function of the rat lateral omohyoides muscle in later life (McCarter, Yu & Radicke 1978). Similarly, undernutrition resulting from large litter size did not impair *in vitro* function of the rat soleus or EDL muscles later in life (Wareham, Mahon, Bedi & Smart 1982). In fact, the force generation was greater per unit muscle in the animals of large litters when

compared to those in litters of normal size (Wareham *et al* 1982). These reports are in contrast to those of Russell and colleagues who noted stimulated force generation of the rat gastrocnemius muscle following 5 days fasting was 22–26% lower than age matched controls, while the force production following 21 days food restriction was 37% lower (Russell *et al* 1984). Twitch force generation in the muscles of these fasted and restricted animals was unaltered compared to control animals (Russell *et al* 1984). Maximal force development in the soleus, plantaris and diaphragm muscles of rats consuming 50% their normal intake was 35–40% lower than control animals, commensuate with a similar reduction in body mass (Drew, Farkas, Pearson & Rochester 1988). Thus there would appear to be a difference in the ability to preserve skeletal muscle function depending upon animal maturity at the time of undernutrition. There may also be an effect of size since reduction of force generation was only observed when large well–nourished animals were compared to smaller animals. This begs the question as to whether these studies are in fact looking at two different areas, firstly a lack of development or secondly an atrophy of tissue.

Twitch contraction and relaxation times appear to be resistant to change following limitation of food intake. No change in the twitch contraction and relaxation times of skeletal muscles has been reported either following early life undernutrition (Wareham *et al* 1982), following 5 days fasting and 21 days food restriction (Russell *et al* 1984) or following food restriction at 50% normal intake (Drew *et al* 1988). Yet maximal relaxation rate following sustained tetanic contraction was 35–40% slower following 5 days fasting and 60% slower following 21 days food restriction (Russell *et al* 1984). The twitch response therefore appears to be preferentailly preserved in comparison to the tetanic responses. It may be this slowing of maximal relaxation rate which influences the force–frequency relationship. Russell and associates observed a shift in the force–frequency curve of the rat gastrocnemius muscle resulting in greater relative force generation at low frequencies of stimulation following both 5 days fasting and 21 days food restriction (Russell *et al* 1984). In animals consuming 50% their normal intake, a shift in the force–frequency relationship of the diaphragm was observed, but not of the soleus and plantaris muscles (Drew *et al* 1988). If the maximal relaxation rate is important in determining the force–frequency relationship there would appear to be a distinct muscle effect, which may be due to differences in muscle fiber composition.

Fatigue of the rat gastrocnemius muscle was greater in animals fasted for 5 days and following food restriction for 21 days than in control muscles (Russell *et al* 1984). Fatigue of the diaphragm muscle of rats restricted to 33% their normal intake was not greater, in fact fatigue resistance became enhanced (Sieck, Lewis & Blanco 1989). To some extent this difference may be the result of fiber atrophy patterns. While the cross sectional area of both type I and type II fibers become reduced following reductions in food intake, the magnitude of this atrophy is greater in the type II fibers (Sieck *et al* 1989). However, it is difficult to envisage significant loss of

one fiber type to account for the changes in fatigue susceptibility. It is unclear whether this difference in fatigue susceptibility reflects differences in the distinct muscles, methodoloical differences or may simply be the result of species differences.

The fatigue response, however, could reflect the muscle metabolite concentration. Russell and co-workers observed reduced concentration of creatine phosphate and increased concentrations of ADP in the gastrocnemius muscle of fasted and food restricted rats (Russell *et al* 1984). These alterations in muscle metabolites were believed to be indicative of impaired energy provision (see Section 1.1.2). Yet, resting muscle metabolite concentrations of the lateral omohyoides muscle of undernourished rats was not different to control muscles (McCarter *et al* 1978). It is possible that the altered metabolite profile in the study of Russell and colleagues could be the result of the sustained contractions comprising the function trial prior to biopsy.

Muscle membrane potential may prove to be another important factor limiting skeletal muscle function. No change in the membrane potential following 2 days fasting and the gastrocnemius muscle following 21 days food restriction was observed, however, depolarisation of soleus muscle membrane was apparent (Pichard & Jeejeebhoy 1988). Since the impairment of skeletal muscle function reported by this group previously was similar following fasting and food restriction the significance, if any, of this observation is unclear.

1.3.2 Skeletal muscle function following alterations of food composition.

This discussion will be limited to the changes in function associated with changes in the protein and energy content of the diet since this represents the interest of this thesis. There is little information on skeletal muscle function in conditions of reduced protein and energy intake. Human data is particularly sparse and where available functional assessment is difficult to discern from other alterations at the central and cardiovascular levels. No studies have been conducted using electrical stimulation of muscles via peripheral nerves to determine the underlying contractile capabilities of skeletal muscle in protein energy malnourished individuals.

Reduced physical working capacity in malnourished individuals has been described by several workers in developing countries (Viteri 1971; Maksud *et al* 1976; Spurr *et al* 1977; Spurr *et al* 1984). The findings of these studies have previously been discussed under limitations in the amount of food consumed since it is unclear what the dietary intake of the individuals was prior to the studies. It is likely that the diets consumed were largely deficient in protein, but energy intake would also be reduced. It is unclear whether protein intake becomes limiting prior to energy intake (Jackson & Golden 1987; Jackson 1990). No functional data is available as to the strength of skeletal muscles in malnourished individuals although it would appear that the malnourished workers fatigue more readily during manual work than well nourished individuals

and thus require a greater number of rest periods during the working day (see Ferro–Luzzi 1989, 1990).

Clearly, an animal model of protein deficiency would clarify some of these areas, yet few models have been developed which characterise the clinical conditions of protein energy malnutrition (see Golden 1985). In fact, no study has investigated the strength of muscles in animal models of protein deficiency.

Another line of evidence indicating a possible impairment of skeletal muscle function arose from studies of animals depleted of creatine phosphate by feeding diets containing $\beta$–guanidinoproprionic acid (GPA), an inhibitor of creatine transport into muscle. No alteration in the soleus muscle time to peak tension or half relaxation time of animals fed GPA–containing diets was observed but both peak twitch tension and maximal tension were lower than that observed in well nourished animals (Petrofsky & Fitch 1980). The force–frequency relationship of the soleus muscle of GPA fed animals was shifted to the left i.e. greater forces were generated at low frequencies of stimulation (Petrofsky & Fitch 1980), which has been taken to indicate an impairment of muscle function (Russell *et al* 1984). Since GPA ultimately depletes the muscle of creatine phosphate it would be expected that enhanced fatigue susceptibility would be observed, but this was not assessed in the study of Petrofsky & Fitch.

In summary, therefore, the investigations of skeletal muscle during/following protein deficiency do not allow conclusions to be drawn regarding the ability of muscle to perform mechanical work. Further investigations of protein/energy deficient states are required to determine what impairments occur at the peripheral level (i.e. within muscle) and the implications of these impairments on muscle function for the host.

### 1.3.3 Skeletal muscle function following infection.

Little is known about the changes in skeletal muscle contractile characteristics during the course of infective insults. Brough and co–workers reported that severe sepsis (Stoner index above 10) in patients who were eating normally before sepsis occurred was associated with an elevated force generation of the electrically stimulated adductor pollicis muscle at 10 Hz relative to that at 50 Hz when compared with control subjects, but with normal maximal relaxation rates (Brough, Horne, Irving & Jeejeebhoy 1986). Trauma without sepsis, however, was reported to be associated with a slower relaxation rate on assessment in the emergency room, but with no alteration in force–frequency characteristics when assessed the following day (Brough *et al* 1986). This may indicate that skeletal muscle function is impaired in infected individuals.

To obtain more precise knowledge of the changes in skeletal muscle function in infection, Drew and colleagues developed a model of infection in the hamster produced by *Leishmania donovi*, a parasitic infection of the reticuloendothelial system (Drew *et al* 1988). This study assessed *in vitro* contractile characteristics of the soleus, plantaris and diaphragm muscles following 12 weeks of infection resulting in up to 30–40% loss of body weight. The contraction and relaxation characteristics of the muscles were preserved except in the case of the diaphragm where half relaxation time was 16% slower than control or calorie restricted animals. Twitch force generation was increased in the diaphragm muscle (1.3 fold) but unaltered in the soleus and plantaris muscles. Maximal force generation was lower in the plantaris muscle (55%) but unaltered in the soleus and diaphragm muscles. In all three muscles alterations in force generation with higher frequencies of stimulation were noted with lower force generation at frequencies below 35 Hz in the soleus and plantaris (but an increase in relative terms) while the plantaris muscle of infected animals exhibiting a greater reduction in force generation at high frequencies (>50 Hz) than that seen in the calorie restricted group. Thus once again distinct muscles are responding differently to the insult and may be due to the differences in fiber type composition.

Much has been reported as to skeletal muscle wasting and stimulation of acute phase proteins during endotoxemia (see Section 1.2.2.4). To date, no investigation of the effects of endotoxemia on skeletal muscle function have been conducted. However, the anorexia associated with endotoxemia (e.g. Southorn & Thompson 1986; Fish *et al* 1986; Wan *et al* 1984; Wan & Grimble 1986 Wan 1987) may initiate changes in skeletal muscle function by mechanisms described above (see Section 1.3.1). The reported changes in organ blood flow (Fish *et al* 1986; Burnier *et al* 1988; see Section 1.3.1) may also be responsible for changes in functional capacity due to a reduced supply of nutrients and oxygen. Reported lactate accumulation (Guenter *et al* 1969) associated with alterations in muscle pH may influence contractile characteristics including those associated with translocation of calcium or may influence mitochondrial function (Nicholas *et al* 1974). It should also be remembered that muscle membrane potential may be reduced (Cunningham, Carter, Rector & Sheldin 1971; Trunkey, Illner, Wagner & Shires 1979).

Although no studies have investigated skeletal muscle contractile characteristics using endotoxin as a model for infection, Wakabayshi and colleagues have indicated that the contraction of helical strips of thoracic aorta of endotoxin–injected rats are diminished prior to an observed drop in blood pressure (Wakabayshi, Hatake, Kakishita & Nagai 1987). This appears to be the result of a disorder of calcium utilization. Direct addition of endotoxin to the incubation medium resulted in decreased calcium uptake in cardiac sarcoplasmic reticulum (Hess & Briggs 1971) and decreased Na+,K+–ATPase activity (Hulsman, Lamers, Stam & Breeman 1981). Sayeed & Maitra (1987) measured cytosolic concentrations of calcium and the exchangeable calcium in isolated hepatocytes from endotoxin injected calcium animals which

were higher in the endotoxin treated animals.

Thus it appears that calcium disturbances are occurring in endotoxic rats which would affect those variables of the contractile responses which are calcium dependent, namely contraction, relaxation and force generation.

### 1.3.4 Summary.

The evidence for changes in the contractile characteristics of skeletal muscle following partial or complete reductions in food intake, alteration in food composition and infection remain equivocal. Studies conducted in malnourished humans indicate that impairment of function may occur although the mechanisms which may be involved are ill defined due to the difficulties of this type of investigation in human subjects. Findings from animal studies are inconclusive due to the different muscles studied, wide range of techniques used in the studies and differences in temperature at which measurements have been made. In general, it would appear that skeletal muscles become slower to relax, generate lower forces and exhibit raised relative forces at low frequencies of stimulation following changes in nutritional status and possibly following infection. The mechanisms by which these impairments in function arise are unresolved and may be related to the skeletal muscle wasting which has been noted for each condition. To date no data regarding skeletal muscle function following the combined insult of malnutrition and infection is available. The extent of our current knowledge of the contractile characteristics of skeletal muscle following limitations in the amount or composition of food consumed, endotoxin administration and the combined insults of malnourishment and endotoxin administration is summarised in Table 1.6.

Table 1.6. Muscle characteristics under various nutritional states and in infection.

|                  | A     | B     | C   | D     | E   |
|------------------|-------|-------|-----|-------|-----|
| CONTRACTION      | N     | N     | ?   | ?(R)  | ?   |
| RELAXATION       | R     | R     | ?   | ?(R)  | ?   |
| FATIGUE          | I     | I     | I   | I     | ?   |
| FORCE GENERATION | R     | R     | R   | ?(R)  | ?   |
| WORK CAPACITY    | ?(R)  | ?(R)  | R   | ?(R)  | ?   |

KEY:

A - Food Restriction; B - Starvation; C - Low Protein Diet; D - Endotoxin; E - Low Protein Diet + Endotoxin; R - Reduced; N- Normal; I - Increased; ? - unknown; ( ) - inferred.

## 1.4 <u>AIMS</u>

The introduction has described the processes underlying normal muscle function and the various factors which may influence them. The most profound characteristic associated with malnutrition is that of skeletal muscle wasting. Yet little is known as to the nature of the changes which occur within skeletal muscle in response to malnourishment. One of the principle difficulties associated with attempting to investigate the changes in functional capacity at the level of the skeletal muscle is the interaction with changes at the level of the central nervous and cardiovascular systems. It is therefore important to investigate each level independently.

Investigations of this type can be most readily achieved by the use of animal models of undernutrition. Undernourishment may be due to limitations in the amount of food available, limitations in the composition of the food available or due to malabsorption and maldigestion as the cause or consequence of disease processes. In addition, many malnourished individuals exhibit associated infections which may be primarily or secondarily related to the malnutrition.

This project therefore investigated the contractile characteristics of rat skeletal muscles following limitation in the amount or composition of food available and following an infectious insult. This was achieved by developing rat models of acute food deprivation for 5 days, chronic food restriction for 21 days at 25% the intake of paired control animals on the previous day, the consumption of low protein diets and the development of a model of infection using endotoxin administration. Since infection is commonly associated with malnutrition the combined response to endotoxin administration in animals consuming low protein diets was also studied. These studies specifically examined the effect of these nutritional and infectious measures on skeletal muscles selected to reflect the different muscle fiber types, namely soleus (type I), extensor digitorum longus (EDL, type IIB) and tibialis anterior (TA, type IIA/B).

# CHAPTER 2
# METHODS

This chapter is subdivided into several sections. Firstly, the maintenance of animals throughout the studies will be described in relation to the food consumption and duration of the experiments. Secondly, the muscle function measurements conducted will be discussed and an assessment of the reliability of the techniques will be made. Finally, the collection and preparation of the body and muscle samples will be described in connection with the biochemical analysis conducted.

## 2.1 ANIMAL MAINTENANCE.

Male Wistar rats were housed individually in a room maintained at 23°C with a 12 hour light–dark cycle. Prior to the studies animals received CRM(X) pellets ( Labsure; see Table 2.1) *ad libatum* and were allowed free access to water. Body weight and food intake was monitored during the studies at the same time during each light period.

### 2.1.1 Animal Groups.

Animals were allocated to groups as follows:

#### 2.1.1.1) acute food deprivation.

| | |
|---|---|
| A) CONTROL : | fasted overnight with skeletal muscle assessment on day zero (12–14 hours); |
| B) FASTED 5 DAYS : | fasted 5 days with free access to water. |

#### 2.1.1.2) chronic food restriction.

| | |
|---|---|
| A) CONTROL : | fasted overnight (12–14 hours) at the commencement of the study period; |
| B) CONTROL FED : | receiving CRM(X) pellets *ad libatum* for 21 days, followed by an overnight fast (12–14 hours) prior to muscle function assessment; |
| C) FOOD RESTRICTED : | receiving a quantity of food (CRM(X)) equivalent to 25% of that consumed by paired CONTROL FED animals in the previous 24 hours. |

Table 2.1: The composition of CRM(X) rat diet.*

| COMPOSITION BY % | | ADDED MINERALS (PPM) | |
| --- | --- | --- | --- |
| Crude oil | 2.9 | Manganese | 25.0 |
| Crude protein | 18.3 | Copper | 7.0 |
| Crude fibre | 3.5 | Cobolt | 0.4 |
| Calcium (as Ca) | 0.8 | Iron | 30.0 |
| Phosphorus (as P) | 0.6 | Iodine | 1.3 |
| Salt | 0.7 | Magnesium | 102.0 |
| Carbohydrate | 56.3 | | |

| AMINO ACIDS BY % | | VITAMINS (ADDED PER Kg) | |
| --- | --- | --- | --- |
| Threonine | 0.6 | Vitamin A | 8000iu |
| Glycine | 0.9 | Vitamin D3 | 1000iu |
| Valine | 0.8 | Vitamin B2 | 8mg |
| Cystine | 0.2 | Nicotinic acid | 50mg |
| Methionine | 0.3 | Pantothenic acid | 12mg |
| Isoleucine | 0.7 | Vitamin B12 | 12$\mu$g |
| Leucine | 1.4 | Vitamin E | 60iu |
| Tyrosine | 0.6 | Vitamin K | 10mg |
| Phenylalanine | 0.8 | Folic acid | 10mg |
| Lysine | 1.0 | Choline chloride | 200mg |
| Histidine | 0.4 | Vitamin B1 | 4mg |
| Argenine | 1.2 | Vitamin B6 | 6mg |
| Tryptophan | 0.2 | | |

*
  - As given by the manufacturer.

2.1.1.3) protein deficiency.

A) CONTROL                :animals weighing 97 ± 4.7 g at time of study;

B) FED                    :animals consuming a normal diet containing 15% protein, 580 mg sodium/ g diet and 2930 mg potassium/kg diet *ad libatum* for 21 days;

C) Low Protein, Low Electrolyte (LPLE):

animals consuming a diet containing 0.5% protein, 580 mg sodium/kg diet and 2930 mg potassium/kg diet for 21 days.

D) Low Protein, High Electrolyte (LPHE):

animals consuming a diet containing 0.5% protein, 4900 mg sodium/kg diet and 4900 mg potassium/kg diet for 21 days;

The exact composition of the diet for the animals in this study is shown in Table 2.2.

2.1.1.4) endotoxin.

A) ACUTE CONTROL  :      receiving intraperitoneal (ip) saline injection at 0 hours with assessment of muscle contractile characteristics at 8 hours;

B) ACUTE ENDOTOXIN :      receiving *E.coli* endotoxin (Sigma Chemical Co. Ltd, Dorset, UK; 1.2 mg/kg body weight) ip at 0 hours with assessment of muscle function at 8 hours.

C) CHRONIC CONTROL :      receiving saline injections ip at 0 and 24 hours with muscle function assessment at 32 hours;

D) CHRONIC ENDOTOXIN:

receiving *E.coli* endotoxin (1.2mg/kg body weight) ip at 0 and 24 hours with muscle function assessment occurring at 32 hours;

Table 2.2: The composition of diets in the protein deficiency study.

| COMPOSITION BY % | CONTROL | LPHE | LPLE |
|---|---|---|---|
| Casein | 15 | 0.5 | 0.5 |
| Dextrin | 44.5 | 53.1 | 54.2 |
| Sucrose | 22.3 | 25.6 | 27.1 |
| Cellulose | 5.0 | 5.0 | 5.0 |
| Corn Oil | 10.0 | 10.0 | 10.0 |
| Choline | 0.2 | 0.2 | 0.2 |
| Mineral Mix [!] | 2.0 | 2.0 | 2.0 |
| Vitamin Mix [$] | 0.5 | 0.5 | 0.5 |
| Lecithin | 0.5 | 0.5 | 0.5 |

FINAL SODIUM AND POTASSIUM CONCENTRATION (mg/Kg) [*]

| | CONTROL | LPHE | LPLE |
|---|---|---|---|
| Sodium | 580.0 | 4900.0 | 580.0 |
| Potassium | 2930.0 | 4900.0 | 2930.0 |

! - AIN-76 Mineral Mix, SDS Products Ltd. $ - AIN-76 Vitamin Mix, SDS Products Ltd. * - final concentration of sodium and potassium by addition of NaCl and KCl.

LPHE- Low Protein, High Electrolyte Diet; LPLE -Low Protein, Low Electrolyte Diet.

E) PAIR FED CONTROL      :animals were pair fed the quantity of food consumed by animals in the CHRONIC ENDOTOXIN group on the previous day. Saline injections were administered ip at 0 and 24 hours. Assessment of skeletal muscle function occurred at 32 hours.

### 2.1.1.5) protein deficiency and endotoxin.

Animals consuming the low protein diets additionally received endotoxin administration. Thus, the following groups were studied:

A) FED + ENDOTOXIN :      animals consuming the 15% protein diet for 21 days following which endotoxin was administered ip (0.5 mg/kg body weight) with the assessment of skeletal muscle function at 8 hours.

B) LOW PROTEIN, LOW ELECTROLYTE + ENDOTOXIN: animals consuming a diet containing 0.5% protein, 580 mg sodium/ kg diet and 2930 mg potassium/kg diet for 21 days. Assessment of skeletal muscle function was made 8 hours following ip administration of endotoxin (0.5 mg/kg body weight).

C) LOW PROTEIN, HIGH ELECTROLYTE + ENDOTOXIN: animals consuming a diet containing 0.5% protein, 4900 mg sodium/kg diet and 4900 mg potassium/kg diet for 21 days. Assessment of skeletal muscle function was made 8 hours following ip administration of endotoxin (0.5 mg/kg body weight).

### 2.1.2 Measurement of body temperature.

Rectal temperature was measured by inserting a plastic coated probe into the rectum at a depth of 6 cm. Temperatures were displayed on an electronic thermometer (Bright Laboratories, Brighton, England).

### 2.1.3 Anaesthesia.

On the day of study animals were anaesthetised by ip injection of 0.44ml Sagatal/kg body weight (May & Baker Ltd, LONDON, England) with additional doses administered to maintain the level of anaesthesia during the experiments.

### 2.1.4 Surgical procedures.

The skin of the right hind limb was shaved and the underlying muscles exposed with the use of a cautary in order to reduce blood loss. The soleus, extensor digitorum longus (EDL) and tibialis anterior (TA) muscles were carefully separated by ablation at their lower insertion (see Photo 1) and isolated in a manner designed to preserve the blood supply to these muscles.

The proximal sciatic nerve was isolated at two sites. Firstly, the branch of the sciatic nerve was isolated at the site of emergence from the gastrocnemius−plantaris group for stimulation of the soleus muscle. Secondly, the main trunk of the sciatic nerve was isolated proximally for the stimulation of the EDL and TA (see Photo 1). The right hindlimb was immobilised in a specially designed rig by clamping of the femur and foot in order to prevent the measurement of muscle activity other than that associated with the stimulation of the muscle under investigation (see Photo 2). Throughout the experiment, the animals body temperature, monitored via a rectal probe, was maintained at 37 °C using a heated blanket. The tendons were tied using silk thread for attachment to the force transducer (Harvard Bioscience) and its orientation adjusted by means of a racking system in order to ensure that the line of force development was optimal for the measurement of force by the transducer. The contractile characteristics of each muscle was studied independently and although initial pilot studies indicated no influence of the order of assessment of the measurements (data not shown), the muscles were always assessed in the following order: soleus, EDL and then TA. The assessment of contractile characteristics in each animal lasted approximately 2 hours, including acclimatization and rest periods. Muscle temperature was maintained at $37 \pm 0.2°C$ with the aid of a small diameter spot lamp and a saline drip, the muscle temperature being continually monitored using a small temperature probe. This was particularly important as the literature would suggest that changes in muscle temperature will alter the skeletal muscle contractile characteristics (see later, section 2.1.6). In order to ascertain the influence of skeletal muscle temperature on functional characteristics validation experiments were conducted and are described below (2.1.6i).

Photo 1. The diagram on the following page illustrates the dissected soleus (SOL), extensor digitorum longus (EDL) and tibialis anterior (TA) muscles of a rat. The muscles are displaced from their normalorientation for ease of vision. SNs is the branch of the sciatic nerve innervating the soleus muscle, SNe is the branch innervating both the EDL and TA muscles.
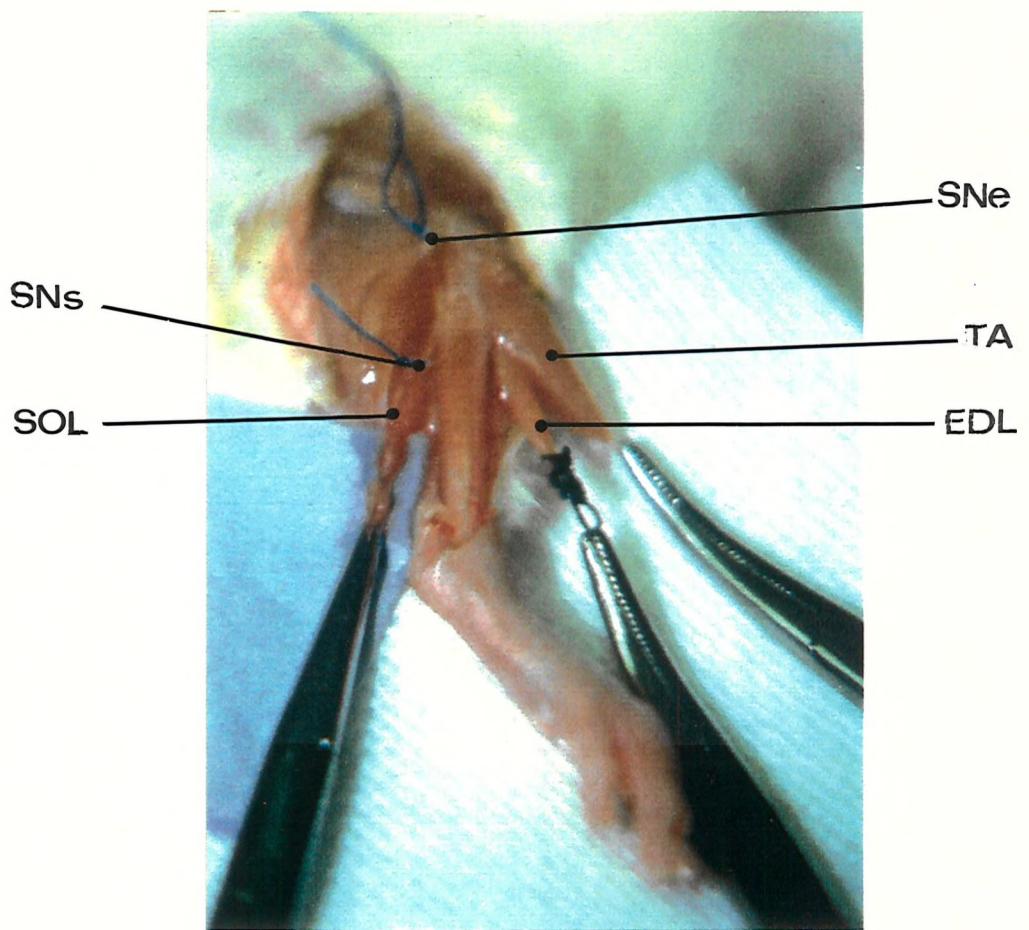
Photo 2. The diagram on the following page illustrates an anaesthetised animal immobilised in the assessment rig by clamping of the femur (clamp CL) and foot (not visible) in order to prevent the measurement of muscle activity other than that associated with the stimulation of the msucle under investigation. Body temperature, monitored via a rectal probe (A) was maintained at 37 °C using a heated blanket (B). Muscle temperature was maintained at 37 ± 0.2 °C with the aid of a small diameter spot lamp (C) and a saline drip (D), the muscle temperature being continuously monitored using a small temperature probe (E). Force development in response to electrical stimulation via an electrode (F) under the branch of the sciatic nerve was measured using a force transducer (G) connected to a computer.
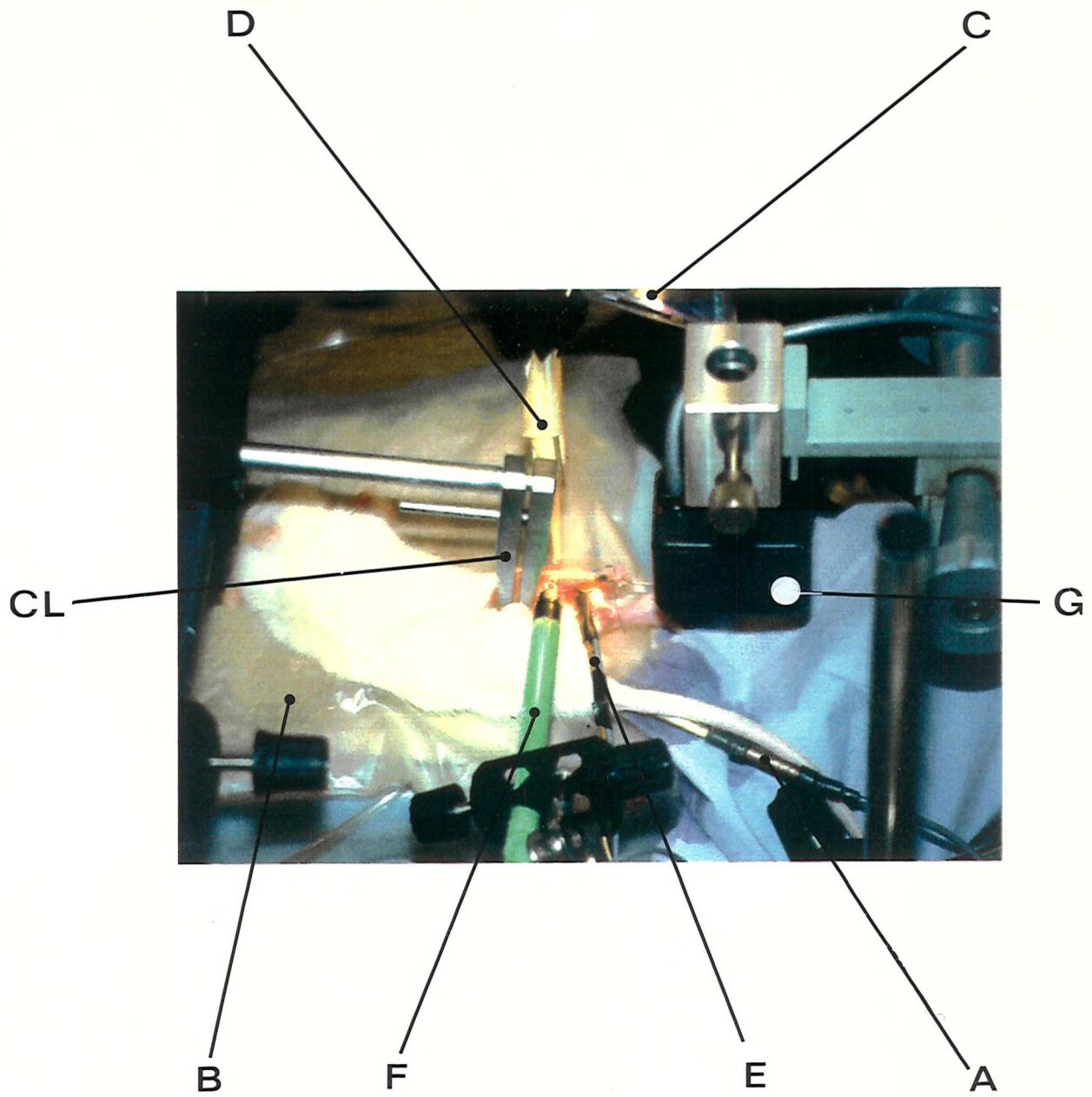
2.2 <u>ASSESSMENT OF CONTRACTILE CHARACTERISTICS.</u>

This section will describe the methods employed in the *in situ* assessment of skeletal muscle contractile characteristics of the rat. This will include a discussion of the unique processes used in the collection and analysis of the muscle contractile responses as the result of electrical stimulation. In the absence of well defined protocols for assessment of *in situ* skeletal muscle function, computer software was written to initiate stimuli and record force generation via an IBM computer. This ensured that the stimulation and recording procedure were reproducible in all cases.

2.2.1 <u>Collection and analysis of data.</u>

Computer software, written in Turbo Pascal, allowed the option of setting the stimulation frequency, duration, interval between sequences and the subsequent repeat patterns. Twitch sequences (frequency 1 Hz), force−frequency sequences (frequency range 10 − 200 Hz) and fatigue trials (Burke's Paradigm) could be defined and saved. For further details of the computer system the reader is referred to the users guide which I have written for this software (Appendix I.1−I.6). Collection of the force generated was achieved using a 16 channel A/D 12 bit collector (Amplicon Liveline, Brighton, England) which enabled a sampling rate of 4000 per second resulting in the distinction of time intervals of 0.25 msec. At this sampling frequency the smallest measurable force generation was $6 \times 10^{-5}$ N with the transducer set over the 0−500 g range and $7 \times 10^{-6}$ over a 0−50 g range..

Data analysis was completed using custom written, Turbo Basic software. This was capable of analysing the data to the specifications of collection. The analysis program plotted the force development onto the screen and was written to enable the choice of one of four data smoothing options, the ability to adjust the baseline force, the start of the contraction phase of the twitch and maximal force production. For further details of the analysis programme the reader is referred to the users manual which I have written for the software (Appendix I.7). From this data the half relaxation time could be calculated and displayed. From the series of frequency measurements the computer program enabled direct display of force generation in Newtons using a calibration system such that with the force transducer set over a range of 0−50 g 128000 units was equivalent to 1 N.

During analysis of the force−frequency relationship the force generated at each frequency was plotted and the option of 4 data smoothing levels and the option to adjust the baseline (see Appendix I.7). Maximal force was set by the software package and could be adjusted either by calculation of the average of troughs and peaks or by manual override. Spurious data points could be excluded from this procedure by indicating the start and end positions of maximal force

averaging. From the displayed force profiles, force generation could be calculated with 1 N being equivalent to 14400 units over the 0−500 g range of the transducer. The force generation as a percentage of the maximal tetanic force could be calculated and displayed.

### 2.2.1.1 Twitches.

A period of acclimatisation was allowed before adjustment of the muscle to its optimal length by determination of the maximal twitch force elicited to supramaximal stimuli. Contractile characteristics of the muscles were measured by application of supramaximal square wave stimuli of 0.25 ms duration at 1.0 Hz.

Peak twitch tension (PT, Figure 1.4), time to peak twitch tension (TPT, see Figure 1.4) and half relaxation time (½RT, see Figure 1.4) were derived from 5 twitches and a mean value recorded. The coefficient of variation of the time and force measurements over five twitches was less than 1%, averaging over larger number of observations did not further reduce the variance.

### 2.2.1.2 Force−frequency curve.

The force−frequency relationship was examined by measuring the maximal force produced by repetitive stimuli for 3 seconds at 10, 20, 50, 100 and 200 Hz allowing 30 seconds between the start of each sequence of stimuli. Construction of the force−frequency curve was achieved by plotting the maximal force noted at each stimulation frequency relative to the average maximal force evoked (corresponding to the maximal tetanic force, Fmax, see Figure 1.5) against the frequency of stimulation.

### 2.2.1.3 Fatigue − Burke's Paradigm.

Following assessment of contractile characteristics tetanus was elicited to a train of stimuli consisting of 13 pulses at 40Hz lasting 330 ms once a second for 5 minutes (Burke *et al* 1973). The tetanus produced was observed using a pen recorder. From the traces obtained the fatigue index was calculated as the force generated at 2 minutes of stimulation compared to the initial force (see Burke 1973). Thus muscles which do not fatigue during 2 minutes of stimulation will have a fatigue index of 1.0, while those exhibiting total fatigue will have a fatigue index of 0.

## 2.2.2 Assessment of reliability of contractile measurements.

Before describing the methodology further it is necessary to assess the reliability of these measurements obtained using this unique, computer-aided system.

In order to assess the reliability of the measurements obtained several procedures were conducted:

    i) assessment of the influence of muscle temperature on skeletal muscle contractile characteristics;

    ii) calculation of the within animal (intraanimal) variance was assessed by measuring the difference between the muscles on the right and left limb of a single control animal;

    iii) calculation of the between animal (interanimal) variance was measured to assess the inherent biological variation of the animal population.

## 2.2.2.1 Effect of muscle temperature on skeletal muscle contractile characteristics.

Muscle function was assessed in soleus muscle of 4 male Wistar rats using the techniques described above. Muscle temperature was maintained at temperatures between 35 and 39°C and the assessment of contractile characteristics carried out with increasing and decreasing temperature, allowing time for acclimatisation to the new temperature at each step. This range of temperatures was chosen as it lies around the temperature at which body temperature was set. Additionally, the maintenance of constant temperature could not be reliably maintained outside this range of temperatures. The average of the measurements was taken as the contractile characteristics of the soleus muscle at that temperature. The effects of temperature changes have been assessed by assuming that normal muscle temperature is 37°C and comparing responses at other temperatures to this. The effect of changes in temperature on the time to peak tension (TPT), half relaxation time (½RT) and peak twitch tension (PT) are shown in Table 2.3.

Both increasing and decreasing the soleus muscle temperature by up to 2°C resulted in slight, non-significant rise in time to peak tension (slower by 1ms maximal $p>0.05$). Half relaxation time was faster at temperatures below 37°C (1ms, $p>0.05$) and prolonged at temperatures above 37°C (1.8 ms, $p>0.05$). Peak twitch tension was lower at 35°C and greater above 37°C but this difference did not attain statistical significance ($p>0.05$). The effect of this temperature

Table 2.3. The effect of temperature on the contractile
characteristics of the rat soleus muscle (mean ± sem, n=4).

| TEMPERATURE | TPT | $\frac{1}{2}$RT | PT |
|---|---|---|---|
| (˚C) | (ms) | (ms) | (mN/g muscle) |
| 35.0 | 43.8 ± 0.3 | 24.1 ± 0.3 | 31.5 ± 3.0 |
| 36.0 | 43.5 ± 0.4 | 24.1 ± 0.3 | 33.0 ± 3.2 |
| 37.0 | 42.9 ± 0.3 | 25.1 ± 0.4 | 34.2 ± 3.3 |
| 38.0 | 43.5 ± 0.4 | 26.1 ± 1.7 | 35.1 ± 1.3 |
| 39.0 | 43.8 ± 0.9 | 26.8 ± 0.9 | 35.1 ± 3.4 |

TPT - Time to Peak Tension; $\frac{1}{2}$RT - Half Relaxation Time; PT - Peak
Twitch Tension.

range on the force–frequency relationship is shown in Figure 2.1, where no discernible difference was observed at any temperature compared to the response at 37°C.

Thus no discernible alteration in the contractile characteristics of the soleus muscle was apparent over the temperature range of 35–39°C. In the experiments described below contractile characteristics were assessed with the muscle temperature maintained at 37 ± 0.2 °C.

### 2.2.2.2 Inherent variability in measurements of contractile characteristics.

The inherent variation of the contractile measurements were analysed by calculation of the coefficient of variation in the following manner:

a) WITHIN ANIMAL (intratest) variation assessed by analysis of the
     data collected after stimulation of the soleus muscle in the
     right and left limbs in each of three animals using the methods
     described above;

b) BETWEEN ANIMALS (intertest) variation, representing the
     biological variability was assessed by analysing the data
     collected from individual control animals measured on different
     days.

The within animal differences are shown in Table 2.4.

While it is not possible to assess the repeated measurement of a single muscle under the conditions of these experiments due to the possible effects of the fatigue test on subsequent assessment of skeletal muscle contractile characteristics, the assessment of between limb variation is the closest that can be achieved to determine within animal variation. The variation within an animal was between 2 & 10%. This therefore suggests that the techniques used in this study allow good reproducibility of measurement. Assessment of differences in the force– frequency curve was not appropriate since this is reliant upon the ability to detect forces which is determined above. Furthermore, stimulation at 200 Hz would result in a degree of fatigue which would directly effect the assessment of reliability.

The between animal variation is shown in Table 2.5. The biological variability is also between 7 & 18%. This variation is larger than that was observed from muscles taken from contralateral limbs of a single animal (Table 2.4), however these values are within an acceptable range for biological measurements. For example maximal tetanic tension 12%, fatigue at 100 Hz 59% (Russell *et al* 1984) and twitch tension 25% (Drew *et al* 1988). Clearly, for any difference in function to attain statistical significance it would need to exceed these limits for each variable. This may
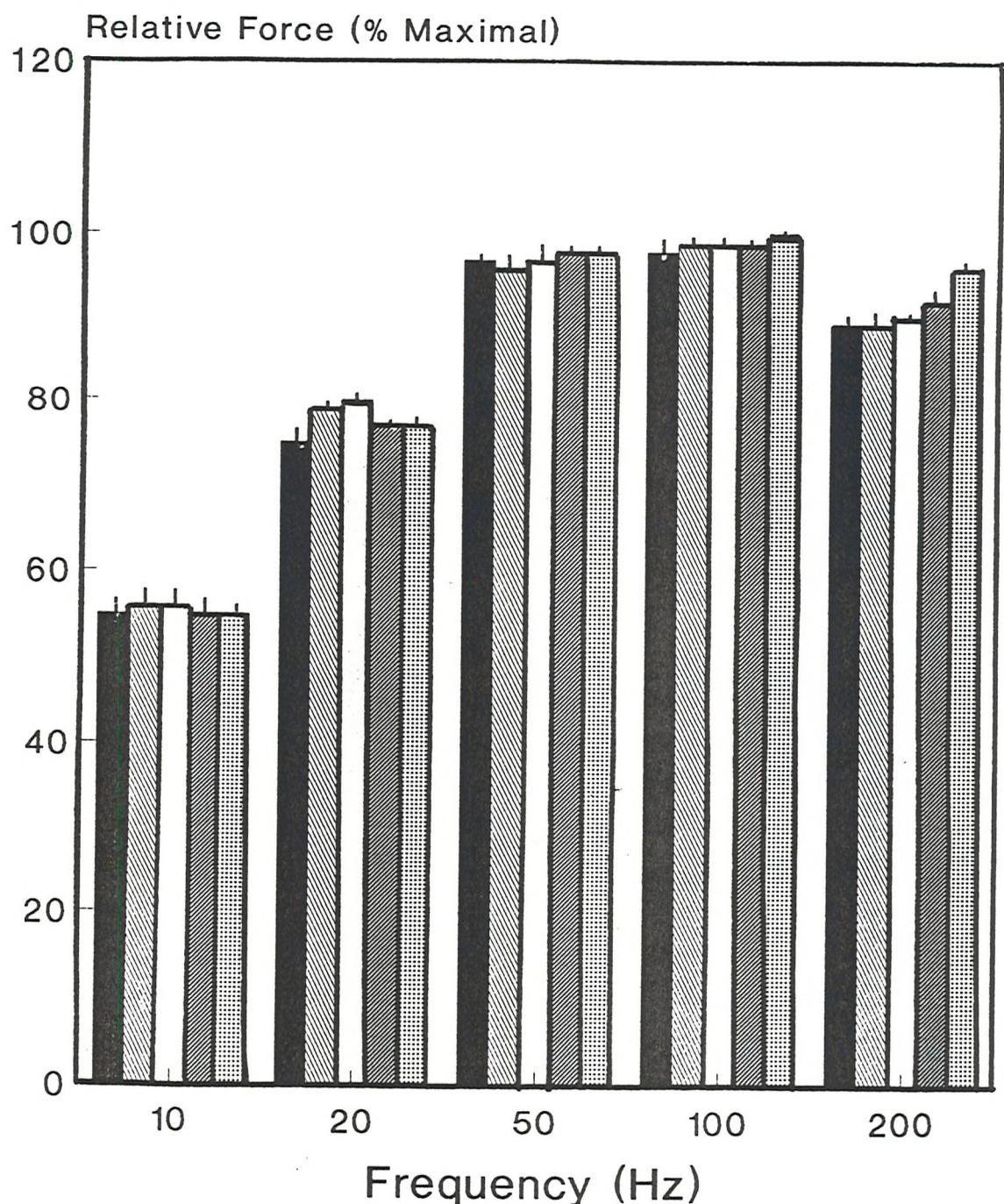
Figure 2.1. The influence of muscle temperature on the force–frequency relationship of the soleus muscle over the temperature range of 35–37°C.    (mean ± sem, n=4).
35°C; 36°C; 37°C; 38°C; 39°C.

Table 2.4 Within animal variability of the contractile characteristics of the soleus muscle.

| ANIMAL | TPT (ms) | | $\frac{1}{2}$RT (ms) | | PT (mN) | | FMAX (mN) | |
|--------|------|------|------|------|-----|-----|-----|-----|
| | R | L | R | L | R | L | R | L |
| 1 | 20.8 | 18.4 | 46.8 | 48.4 | 1.3 | 1.3 | 6.6 | 6.5 |
| 2 | 31.6 | 32.5 | 54.8 | 54.5 | 1.7 | 1.9 | 5.8 | 6.4 |
| 3 | 32.4 | 30.4 | 46.8 | 48.8 | 1.9 | 1.3 | 6.4 | 6.1 |
| GROUP MEAN | 27.6 | | 50.0 | | 1.4 | | 6.3 | |
| SD OF DIFFERENCE | 0.8 | | 1.0 | | 0.2 | | 0.2 | |
| CV(%)* | 2.1 | | 1.6 | | 10.1 | | 2.3 | |

TPT- Time to Peak Tension; $\frac{1}{2}$RT - Half Relaxation Time; PT- Peak Twitch Tension; FMAX - Maximal Tetanic Force.

CV - Coefficient of Variation.

* calculated from CV = [SD of difference] X [1/ $\sqrt{2}$] X [100/MEAN] (from Schantz 1986).

Table 2.5. The inherent variation in the contractile characteristics assessed between individual animals.

| ANIMAL | TPT (ms) | $\frac{1}{2}$RT (ms) | PT (mN) | FMAX (mN) |
|--------|----------|----------|---------|-----------|
| 1 | 36.4 | 45.5 | 1.3 | 5.5 |
| 2 | 35.1 | 40.0 | 1.9 | 5.1 |
| 3 | 31.3 | 52.8 | 1.5 | 5.2 |
| 4 | 33.6 | 47.6 | 1.9 | 5.4 |
| 5 | 35.5 | 51.0 | 1.5 | MD |
| 6 | 20.0 | 44.0 | 1.7 | 4.7 |
| 7 | 22.5 | 40.5 | 1.9 | 5.8 |
| 8 | 31.6 | 54.8 | 1.7 | 5.8 |
| 9 | 32.4 | 46.8 | 1.9 | 5.4 |
| 10 | 35.2 | 58.4 | 1.8 | 6.0 |
| | | | | |
| MEAN | 31.4 | 48.1 | 1.7 | 5.4 |
| SD | 5.6 | 6.1 | 0.2 | 0.4 |
| CV(%) | 17.8 | 12.7 | 11.7 | 7.4 |

KEY TPT - Time to Peak tension; $\frac{1}{2}$RT - Half relaxation Time, PT - Peak Twitch Tension; FMAX - Maximal Tetanic Tension; MD--MISSING DATA.

indicate that alteration in contractile characteristics of skeletal muscle remains undetected because it falls within the range of values described for normal function.

### 2.2.2.3 Burke's Paradigm.

In order to assess the ability of Burke's Paradigm to detect fatigue in whole skeletal muscles several procedures were conducted:

 i) to ensure reproducibility of measurements the coefficient of
    variation for fatigue index (the force generated at 2 minutes
    of stimulation compared to the initial force) for distinct
    muscles of different animals was calculated;

 ii) to ensure that this was not the result of changes external to
    the muscle the peak amplitude of the EMG was measured;

 iii) to ensure that fatigue did result in an alteration in the
    fatigue index the muscle was subjected to metabolic fatigue by
    blood flow occlusion.

### 2.2.2.3.i Coefficient of variability.

The coefficient of variation between measurements of fatigue index from the soleus, EDL and TA muscles of control animals is presented in Table 2.6. It was not considered reasonable to repeat fatigue measurements on a given muscle since the muscle recovery from the fatigue test may not be complete, hence the variation within individual muscles has not been assessed. The variation between animals is between 4.4% in the soleus muscle & 20.9% in the EDL. These values are below that described by other workers despite being greater in the EDL than the soleus or TA. As such they are within acceptable limits of measurement.

The profile of force generation obtained using Burke's Paradigm substantiates the choice of muscles to reflect the different fiber types (see Figure 2.2). Thus it was suggested that the soleus muscle would exhibit the characteristics of slow twitch type I fibers. The soleus muscle does exhibit these characteristics with a slow twitch time and fatigue resistance. The EDL is composed primarily of type IIB fibers and exhibits a quicker twitch time and rapid fatigue and the TA exhibits an intermediate response showing a fast twitch response and a degree of fatigue resistance.

Table 2.6.   The variation of fatigue measurements in rat muscles assessed by Burke's Paradigm.

| ANIMAL | SOL | EDL | TA |
|--------|------|------|------|
| 1 | 0.82 | 0.53 | 0.89 |
| 2 | 0.81 | 0.53 | 0.84 |
| 3 | 0.73 | 0.45 | 0.67 |
| 4 | 0.75 | 0.39 | 0.70 |
| 5 | 0.77 | 0.34 | 0.77 |
| 6 | 0.78 | 0.33 | 0.60 |
| | | | |
| MEAN | 0.78 | 0.43 | 0.75 |
| SD | 0.03 | 0.09 | 0.10 |
| COEFFICIENT OF VARIATION (%) | 4.4 | 20.9 | 14.6 |

KEY: Sol - Soleus; EDL - Extensor Digitorum Longus; TA - Tibialis Anterior.
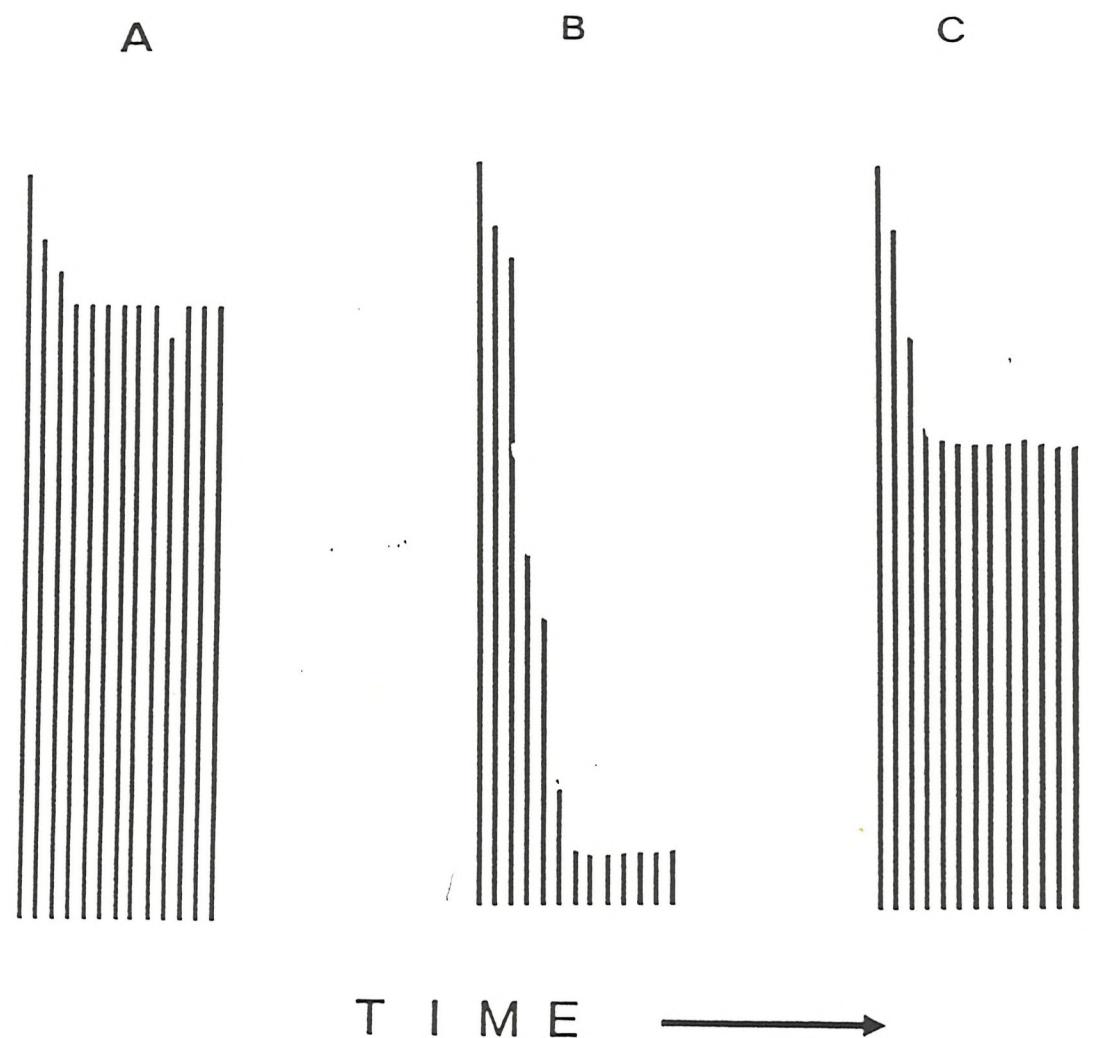
Figure 2.2. The force generation profile of the soleus (A),
extensor digitorum longus (EDL, B) and tibialis anterior
(TA, C) muscles of normal rats. These profiles exhibit the
primary force profiles expected from consideration of their
fiber type composition (see Burke *et al* 1973).

Table 2.7.  The peak amplitude of the EMG from three individual
soleus muscle of rats during stimulation using burke's paradigm.

|   | TIME (min) | PEAK AMPLITUDE (mV) | DIFFERENCE IN PEAK AMPLITUDE FROM TIME 0 (mV) | REDUCTION IN FORCE (%) |
|---|---|---|---|---|
| A | 0 | 4.6 | 0.0 | 0.0 |
|   | 1 | 4.7 | +0.1 | 24.0 |
|   | 2 | 4.5 | −0.1 | 43.0 |
|   | 3 | 4.4 | −0.2 | 47.0 |
|   | 4 | 4.4 | −0.2 | 53.0 |
| B | 0 | 3.1 | 0.0 | 0.0 |
|   | 1 | 3.5 | +0.4 | 42.3 |
|   | 2 | 3.4 | +0.3 | 44.6 |
|   | 3 | 3.2 | +0.1 | 50.0 |
|   | 4 | 3.1 | 0.0 | 46.2 |
| C | 0 | 4.0 | 0.0 | 0.0 |
|   | 1 | 4.0 | 0.0 | 18.2 |
|   | 2 | 4.1 | +0.1 | 20.0 |
|   | 3 | 4.0 | 0.0 | 24.2 |
|   | 4 | 4.1 | +0.1 | 34.2 |
| MEAN FOR GROUP | 0 | | 0.0 | 0.0 |
|   | 1 | | 0.16 | 28.2 |
|   | 2 | | 0.10 | 37.2 |
|   | 3 | | −0.03 | 42.4 |
|   | 4 | | −0.03 | 44.8 |

2.2.2.3.ii <u>EMG activity.</u>

In order to ensure that loss of force was not due to alterations in excitation–contraction coupling the muscles were connected to a storage oscilloscope using fine needle electrodes and the peak EMG amplitude recorded at intervals during stimulation. The results from the soleus muscle from 3 animals are presented in Table 2.7.

Peak EMG amplitude did not significantly alter during the course of stimulation. Thus, the loss of force generation noted during stimulation of Burke's Paradigm was not the result of changes external to the muscle.

2.2.2.3.iii <u>Burke's Paradigm as a detector of fatigue.</u>

In order to ensure that the equipment and procedures could detect fatigue, the fatigue characteristics of a soleus muscle of a control rat was measured before, during and after interruption of tissue perfusion.

During occlusion force loss was greater than 80% within 1 minute (shown diagramatically in Figure 2.3). Following 5 minutes of stimulation, reestablishing tissue perfusion resulted in a degree of recovery of force generation, which was not complete following a further three minutes.

In summary therefore, it would appear that Burke's Paradigm is an appropriate technique to detect fatigue in whole skeletal muscles.

2.3 <u>COLLECTION AND PREPARATION OF SAMPLES.</u>

Growth studies were made in all experiments. Those animals in the investigations of limitations in the composition of diet additionally underwent full carcus analysis.

2.3.1 <u>Whole Body.</u>

Snout–to–anus length of the anaesthetised rat was measured in fully extended rats using a ruler. Following collection of blood, muscles and liver (see below) and the gastrointestinal tract (GIT) was removed. Mesenteric fat attached to the GIT was dissected free and replaced into the body cavity. At the same time the body cavity was examined for signs of infection. The carcus was dried at 80°C to constant weight, powdered using a grinder and the resultant powder stored for determination of carcus energy by bomb calorimetry and lipid content by the method of Soxlet (Section 2.4.7). The body water content was calculated as the difference in weight
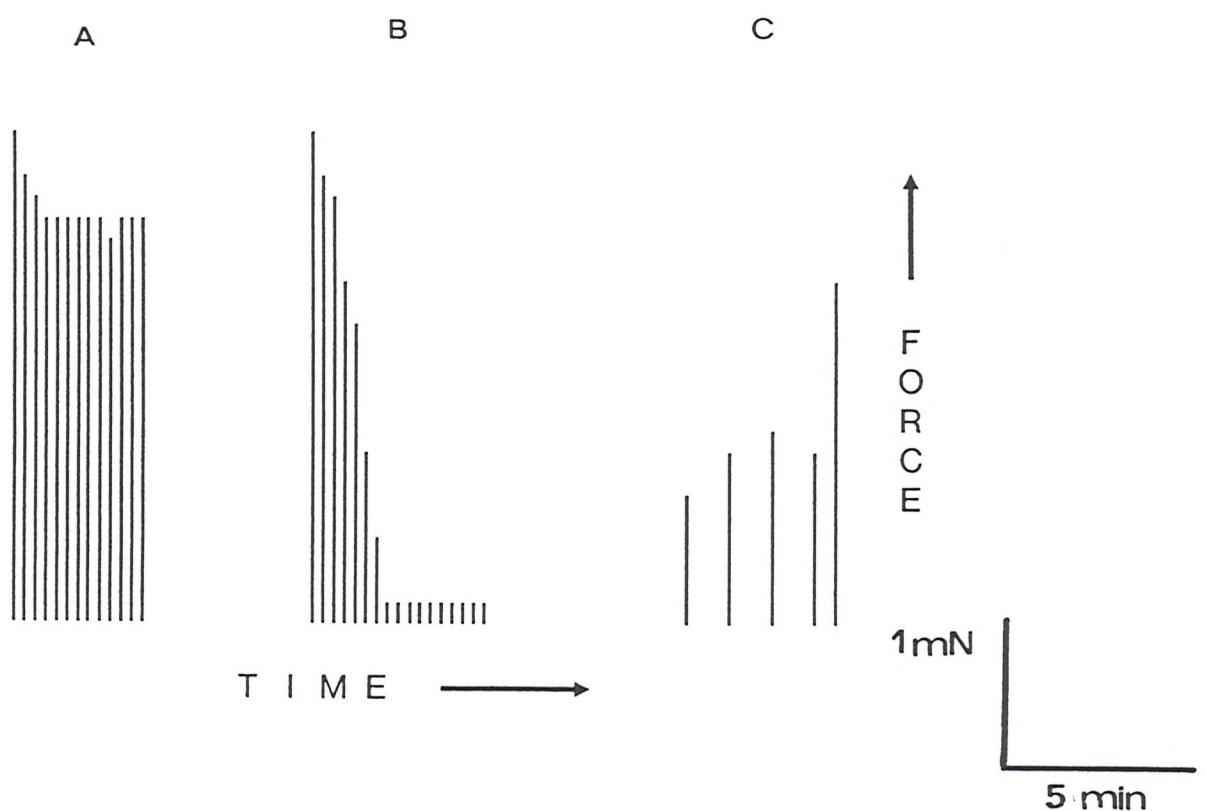
Figure 2.3. Force generation profiles of a soleus muscle during a fatigue trial using Burke´s paradigm (intermittent stimulatoin at 40 Hz for 5 minutes) of a control animal before (A), during (B) and after (C) ischemia caused by clamping of the arterial and venous vessels. For more details see text.

before and after drying.

### 2.3.2 Muscles.

The soleus, EDL and TA of the left limb were carefully isolated as described above, ablated and immediately frozen in liquid nitrogen. The muscles were lyophilised overnight (Genevac Ltd, Ipswich, UK) and stored at $-80°C$. Connective tissue and blood were dissected free from the samples manually prior to the samples being ground in an agate mortor and pestle. The ground tissue was extracted using 6 % perchloric acid (PCA) at 4 °C. Briefly, 100 $\mu$l of PCA were added per mg sample to the tubes which were being held over liquid nitrogen, thus producing a frozen sample. The samples were then aggitated for approximately 1 hour in the cold until the frozen sample had become a liquid. The reaction was neutralised by the slow addition of 25 $\mu$l of 2.1 M potassium carbonate. The Eppendorf tubes were then sealed and centrifuged for 30 seconds to precipitate potassium perchlorate. The supernatant was then removed and placed in fresh tubes for storage at $-80°C$ until used in subsequent assays.

The soleus, EDL and TA muscles of the right limb were dissected free, blotted, weighed and frozen in liquid nitrogen for later analysis of protein content (Section 2.4.4).

### 2.3.3 Blood.

Blood was obtained by cardiac puncture. For determination of blood lactate, 20$\mu$l was immediately mixed with 200$\mu$l of 6% perchloric acid and stored in liquid nitrogen. The remainder of the blood sample was allowed to clot and the serum separated by centrifugation. The serum was stored at $-20°C$ for measurement of the serum concentrations of albumin and zinc (Sections 2.4.5 & 2.4.6).

### 2.3.4 Liver.

The liver was removed, blotted dry, weighed and stored at $-80°C$ until a sample was used for determination of liver protein concentration (Section 2.4.4), lipid (Section 2.4.8) and zinc content (Section 2.4.6).

## 2.4 ASSAYS.

### 2.4.1 Determination of ATP and creatine phosphate (CP).

ATP and CP were determined in the muscle extracts by the method of Lowry and Passonneau [1972]. The principle of this reaction is:

$$\text{GLUCOSE} \xrightarrow[\substack{\text{HEXOSE}\\\text{KINASE}}]{\text{ATP} \quad \text{ADP}} \text{GLUCOSE-6-PHOSPHATE}$$

with

$$\text{NADP}^+ \longrightarrow \text{NADPH} + \text{H}^+$$

$$\text{PHOSPHOGLYCEROL}$$
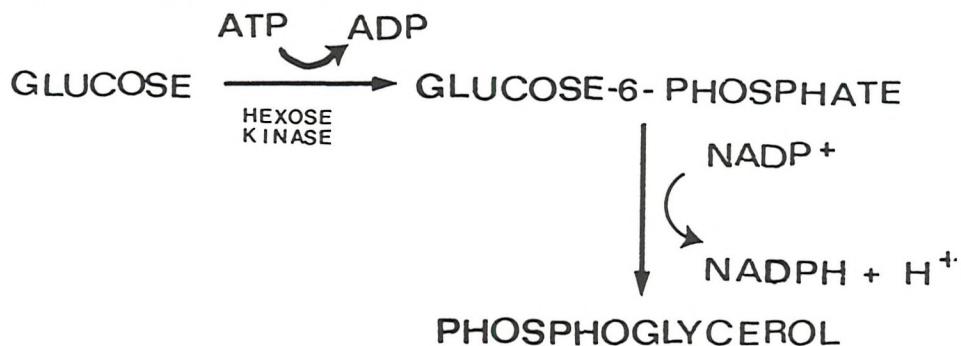
The thawed neutralised–PCA extract was diluted 1 in 6 with deionised distilled water and $10\mu l$ of this solution was reacted with one of three reaction mixtures.

The first reaction mixture provides the baseline measurement for the assay which represents the amount of glucose–6–phosphate in the sample. This solution consisted of 50mM Tris buffer (pH 8.1) containing ADP ($109\ \mu M$), glucose ($200\mu M$), NADP ($50\mu M$), DTT ($50\mu M$), $MgCL_2$ (50mM) and G6PDH (1.8U/ml). The second reaction mixture additionally contained 140mU/ml of hexokinase thus allowing measurement of the ATP content of the sample. The third reaction mixture consisted of the first solution plus 140 mU/ml hexokinase and $90\mu U/ml$ creatine kinase. This solution allows determination of both CP and ATP within the sample and thus the CP concentration can be obtained by subtraction.

ATP standards were prepared over the range of 0 to 200 $\mu M$ and sample blanks were measured for each reaction mixture. All samples were prepared in duplicate.

The tubes were mixed and incubated at room temperature until the reaction had run to completion, being checked by running a standard reaction mixture in a fluorimeter during this incubation. The reaction being stopped by the addition of an excess of 20 mM carbonate buffer (pH 10.0). The fluorescence of the samples was read at an excitation wavelength of 365 nm and an emission wavelength of 460 nm.

In order to correctly calculate the concentration of ATP and CP in the samples it was necessary to correct these readings for the various dilution steps, thus obtaining the results in mM ATP or CP per kg dry muscle.

## 2.4.2 Determination of free creatine.

The creatine content of the muscle was determined by the method of Lowry & Passonneau [1972]. The principle of this reaction is:

$$\text{CREATINE} + \text{ATP} \xrightarrow{\text{CREATINE KINASE}} \text{CREATINE PHOSPHATE} + \text{ADP}$$

$$\text{ADP} \xrightarrow{\text{PYRUVATE KINASE}} \text{ATP} + \text{PHOSPHOENOLPYRUVATE}$$

$$\text{ATP} + \text{PYRUVATE} \xrightarrow[\substack{\text{LACTATE} \\ \text{DEHYDROGENASE}}]{\text{NADPH} \quad \text{NADP}} \text{LACTATE}$$
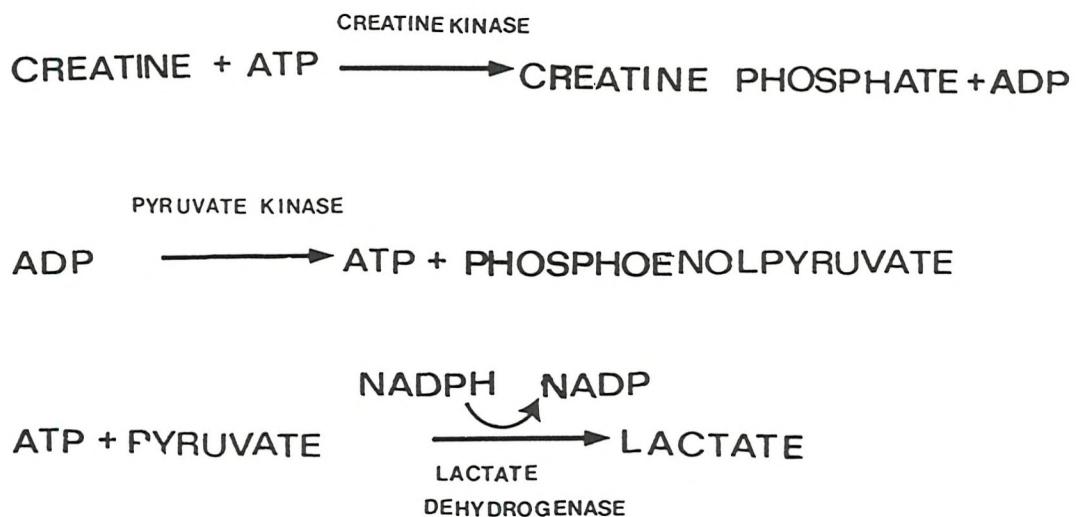
Briefly, $20\mu l$ of the thawed–neutralised extract were diluted 1 in 3 and reacted with one of three reaction mixtures.

The first reaction mixture provides the baseline measurement for the assay and consists of imidazole–HCl buffer (40mM, pH7.5) containing $MgCl_2$ (5mM), KCl (30 mM), NADH (10 $\mu$M), phosphoenolpyruvate (25 $\mu$M), ATP (200 $\mu$M) and lactate dehydrogenase (LDH, 0.2U/ml).

A second reaction mixture was prepared which additionally contained 0.75 U/ml of pyruvate kinase thus allowing the determination of ADP content. The third mixture, for the determination of ATP also contained 3.6 U/ml of creatine kinase.

Creatine standards were prepared over the range of 0–100$\mu$M. All samples were run in duplicate.

The tubes were mixed and incubated at room temperature until the reaction had run to completion, being checked by running a standard reaction mixture in a fluorimeter during this incubation. The reaction was stopped by the addition of an excess of 20 mM carbonate buffer (pH 10.0). The fluorescence of the samples was read at an excitation wavelength of 365 nm and an emission wavelength of 460 nm.

Correction of these creatine measurements was then carried out to give the results in mM creatine per kg dry muscle weight.

## 2.4.3 Determination of lactate.

The concentration of lactate in muscle extracts was determined by the method of Lowry & Passonneau [1972]. The principle of this reaction is:

$$NAD \quad NADH + H^+$$

$$LACTATE \longrightarrow PYRUVATE$$

LACTATE DEHYDROGENASE
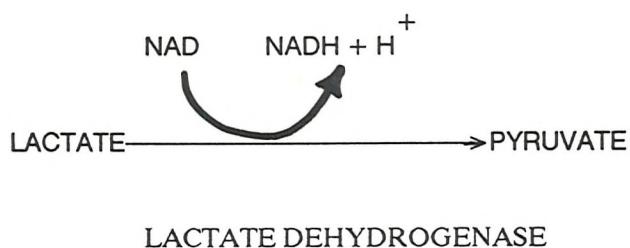
Briefly, 20$\mu$l of thawed neutralised$-$PCA extract were reacted with 400$\mu$l of a reaction mixture consisting of 1.1 M hydrazine buffer (pH 9.0) containing 500 $\mu$M NAD and 2mU/ml lactate dehydrogenase (LDH).

Lactate standards were prepared within the range of 0 $-$ 100 $\mu$M. All samples were prepared in duplicate. The tubes containing the samples and standards were mixed and incubated at room temperature until the completion of the reaction as demonstrated by a standard reaction run to completion in the fluorimeter during this incubation.

The reaction was stopped by the addition of an excess of 20mM carbonate buffer pH 10.0. The fluorescence of the samples was read at an excitation wavelength of 365nm and an emission wavelength of 460 nm.

## 2.4.4 Determination of protein concentration.

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall [1951]. Briefly, 0.1g of the non$-$lyophilised right limb was homogenised in 2% PCA. The pellet obtained from centrifugation was reacted with 0.2M sodium hydroxide at 80°C for 10 minutes. 100$\mu$l of the diluted solution was mixed with 1.0ml of a solution containing 2% sodium potassium tartarate, 1% copper sulphate and 2% sodium carbonate for 10 minutes at room temperature. 100$\mu$l of diluted Folin reagent was added and the samples reacted at 37°C for ten minutes. The colour produced could then be quantified by measurement of the absorbance at 750 nm.

## 2.4.5 Determination of serum albumin concentration.

Serum albumin concentration was determined by the method of McPherson & Everard [1972]. Briefly, 25$\mu$l of serum were mixed with 5ml of bromocreasol green reagent and the resultant colour quantified at 635nm on a spectrophotometer (LKB Ultrascope 450) against a series of standards over the range of 10$-$50mg/ml bovine serum albumin made up in deionised water.

## 2.4.6 Determination of zinc concentration.

Serum samples were diluted 1 in 5 with deionised water and measured for zinc concentration by atomic spectroscopy (Pye Unican Model 280) at 214nm. A series of standards were prepared over the range of 0.25 $-$ 1.0 $\mu$g/ml Zn2+ from zinc sulphate.

Liver samples were digested overnight in concentrated nitric acid and then assayed in a similar manner to the serum samples.

## 2.4.7 Determination of lipid by the method of Soxlet.

Dried samples were weighed into cellulose extraction thimbles (Whatman, Maidenstone, UK) and refluxed for 4 hours with petroleum ether (60$-$80$^\circ$C boiling point). The thimbles were dried and the loss of lipid during the extraction process determined by weighing.

## 2.4.8 Determination of lipid by the Fulch method.

Determination of lipid by the Fulch extraction was determined by the method outlined in Smith (1969).

Approximately 1g of tissue was homogenised in chloroform$-$methanol (1:1 v/v) for 5 minutes. The resultant homogenate was centrifuged to remove solid material and chloroform added to a final concentration of 2:1 (v/v). The homogenate was residued in vacuum at 40$^\circ$C. Free lipids were redissolved in chloroform$-$methanol (2:1 v/v). Sodium chloride (0.9%) was added to a final composition of 2:1:0.75, shaken vigorously, centrifuged and the upper aqueous layer removed. The lower chloroform layer was evaporated to dryness and the dried lipid weighed.

2.4.9 <u>Inherent variability within metabolite assays.</u>

The extent to which the variation in the ability to detect the products assayed by calculating the coefficient of variation of a sample assayed repeatedly in a single assay and a sample assayed repeatedly in assays completed on separate occasions and are shown in Tables 2.8 and 2.9.

Both the within and between test variability lies between 2 and 6%, indicating reliability of measurements between sampling. The variability in assessment of metabolites is small in comparison to the literature, for example PK activity 33% (Layman *et al* 1981), fractional degradation rate 13% (Kadawaki *et al* 1989), muscle ATP concentration 23% (Jepson *et al* 1987).

2.5 <u>STATISTICAL ANALYSIS.</u>

The results are presented as mean ± sem; where coefficients of variation are indicated the values are given as mean ± sd.

The statistical significance of differences between means was determined using unpaired Student's t−test. Significant difference was accepted at p<0.05. Additional analysis of the results was also performed using non−parametric statistics where the data did not appear to be normally distributed. This did not reveal any additional statistically significant differences between groups. Analysis of variance was not conducted as initial analysis revealed that the data did not satisfy the test of heterogeneity of variance.

Table 2.8. The variation associated with repeated analysis of a single muscle extract in a single assay (n=10).

| SAMPLE | ATP [!] | CP [!] | CR [!] | LACTATE [!] | PROTEIN (mg/g) |
|--------|------|------|------|---------|------------------|
| MEAN   | 39.7 | 43.3 | 62.3 | 1.6     | 192.9            |
| SD     | 1.1  | 1.9  | 0.5  | 0.1     | 4.4              |
| CV(%)  | 2.8  | 4.4  | 0.8  | 6.3     | 2.3              |

KEY: ATP – Adenosine Triphosphate; CP – Creatine Phosphate; CR – Free Creatine; CV – Coefficient of Variation.

! – all concentrations mmoles/kg dry muscle weight.

Table 2.9. The variability associated with repeated assessment of metabolite profile of a muscle extract between assays.

| ASSAY | ATP ! | CP ! | CR ! | LACTATE ! | PROTEIN (mg/g) |
|-------|-------|------|------|-----------|----------------|
| 1     | 35.6  | 44.5 | 62.5 | 1.7       | 192.9          |
| 2     | 34.9  | 43.0 | 62.9 | 1.6       | 201.4          |
| 3     | 32.5  | 41.8 | 60.1 | 1.6       | 208.9          |
| 4     | 36.8  | 46.1 | 64.6 | 1.7       | 216.5          |
| 5     | 34.9  | 46.1 | 63.1 | 1.7       | 203.9          |
|       |       |      |      |           |                |
| MEAN  | 34.9  | 44.3 | 62.9 | 1.7       | 205.0          |
| SD    | 1.6   | 1.9  | 1.6  | 0.05      | 7.8            |
| CV(%) | 4.5   | 4.3  | 2.5  | 2.9       | 3.8            |

KEY: ATP - Adenosine Triphosphate; CP - Creatine Phosphate; CR - Free Creatine; CV - Coefficient of Variation.

! - all concentrations in mmoles/kg dry muscle weight.

# CHAPTER 3.

## THE CHARACTERISATION OF NORMAL RAT MUSCLE FUNCTION.

The contractile characteristics of rat skeletal muscle have been reported by several workers (Table 1.3). The conditions under which these investigations were conducted were not standardised which probably explains the wide range in the reported measurements for named muscles in a given species. Additionally, these measurements of skeletal msucle contractile characteristics are not directly comparable due to a lack of information regarding animal and muscle weight and other dimentions (see Table 1.3). The effect of temperature on skeletal muscle contractile characteristics has previously been discussed (Section 2.2.2.1). The stimulation protocol, muscle length and anaesthesia, in *in situ* measurements may also influence the measured contractile characteristics of skeletal muscle.

The aim of this chapter was to assess the contractile characteristics of normal rat skeletal muscle under controlled conditions. Specifically, this investigation examined the time, force and fatigue characteristics of muscles selected to reflect the different muscle fiber types in order to report typical values of muscle function in normal male rats.

## 3.1 METHODS.

Six Wistar rats (initial body weight 176.1 g, sem 2.0g) were maintained individually in a room maintained at 23°C with a 12 hour light−dark cycle. Body weight and food intake was monitored at the same point in each light period.

On the day of study animals were anaesthetised using 0.44 mls Sagatal/kg body weight and prepared for measurement as described in Section 2.2. Assessment of contractile characteristics was conducted as reported in Section 2.2. Time to peak tension (TPT), half relaxation time (½RT) & peak twitch tension (PT) of the twitch and the maximal tetanic force (Fmax) & force frequency relationship of the muscles were determined.

## 3.2 RESULTS.

### 3.2.1 Typical weight of skeletal muscle.

The mass of the soleus, EDL and TA muscles are shown in Table 3.1. The soleus and EDL muscles were of approximately the same mass. The TA was approximately 4 fold heavier than either the soleus or EDL muscles.

Table 3.1. The mass of the soleus, extensor digitorum longus (EDL) and tibialis anterior (TA) muscles of male Wistar rats of mean body weight 176.1 g (n=6).

|  | mean | sem |
|---|---|---|
| SOLEUS MASS (mg) | 80.0 | 9.9 |
| (mg/100g BODY WEIGHT) | 45.4 | 3.1 |
| EDL MASS (mg) | 80.5 | 6.2 |
| (mg/100g BODY WEIGHT) | 45.5 | 2.6 |
| TA MASS (mg) | 319.0 | 45.7 |
| (mg/100g BODY WEIGHT) | 181.1 | 7.1 |

### 3.2.2. Twitch characteristics of normal rat skeletal muscle.

The twitch characteristics of the soleus, EDL and TA muscles of 6 male Wistar rats are presented in Table 3.2.

Time to peak tension of the soleus muscle was 32.8 ms. That of the EDL was 14.5 and TA 13.7 ms. Half relaxation time of the soleus muscle was slower than that of the EDL or TA muscles, being 49.6 ms compared to 14.4 and 14.6 ms for the EDL and TA muscles respectively.

Peak twitch tension of the soleus muscle was 0.9 mN. Peak twitch tension of the EDL and TA muscles was 2.8 mN and 4.8 mN respectively. When expressed per unit muscle weight, the force generated during the twitch was 20.7, 36.6 and 14.3 mN/g muscle for the soleus, EDL and TA muscles respectively.

### 3.2.3. Tetanic responses of rat skeletal muscle.

Maximal tetanic force was 3.9 mN in the soleus muscle and 11.2 mN in the EDL and 20.4 mN in the TA muscle (Table 3.3). When expressed per unit muscle, maximal force generation was 47.8, 138.6 and 60.5 mN/ g muscle for the soleus, EDL and TA muscles respectively (Table 3.3).

The force−frequency relationship of the soleus, EDL and TA muscles are presented in Figure 3.1. The force−frequency relationship were of similar shape but that of the soleus muscle was shifted to the lower frequency range. Thus maximal forces in the soleus muscle were generated at lower frequencies of stimulation than in the EDL and TA muscles.

### 3.2.4. Fatigue responses of normal rat skeletal muscle.

The fatigue index (i.e. force generated at 2 minutes of intermittent stimulation compared to the initial force) of the soleus muscle was 0.68 (Table 3.3). Thus, at 2 minutes of stimulation the soleus muscle produced 32% less force than at the commencement of stimulation. The profile of force generation over 5 minutes resembled that described in Figure 2.2A characteristic of slow twitch single motor units reported by Burke and colleagues (Burke *et al* 1973).

The EDL muscle exhibited a fatigue index of 0.38 (Table 3.3). Force generation at 2 minutes of stimulation was therefore 62% lower than at the commencement of stimulation. The profile of force generation over 5 minutes was typically associated with a rapid decline in force early in the stimulation sequence and was similar to the profile shown in Figure 2.2B characteristic to that described for type IIB fibers (Burke *et al* 1973).

Table 3.2.  The contractile characteristics of the soleus, extensor digitorum longus (EDL) and tibialis anterior (TA) muscles of normal male wistar rats (n=6).

|  |  | mean | sem |
|---|---|---|---|
| **SOLEUS** | | | |
| | TPT (ms) | 32.8 | 2.4 |
| | $\frac{1}{2}$RT (ms) | 49.6 | 6.7 |
| | PT (mN) | 0.9 | 0.1 |
| | PT (mN/g muscle) | 20.7 | 2.8 |
| **EDL** | | | |
| | TPT (ms) | 14.5 | 2.4 |
| | $\frac{1}{2}$RT (ms) | 14.4 | 2.5 |
| | PT (mN) | 2.8 | 0.5 |
| | PT (mN/g muscle) | 36.6 | 6.7 |
| **TA** | | | |
| | TPT (ms) | 13.7 | 0.6 |
| | $\frac{1}{2}$RT (ms) | 14.8 | 2.4 |
| | PT (mN) | 4.8 | 0.9 |
| | PT (mN/g muscle) | 14.3 | 2.7 |

KEY: TPT - Time to Peak Tension; $\frac{1}{2}$RT - Half Relaxation Time;

PT - Peak Twitch Tension;

Table 3.3  The tetanic responses of the soleus, extensor digitorum ongus (EDL) and tibialis anterior (TA) muscles of normal male wistar rats (n=6).

|  | mean | sem |
|---|---|---|
| **SOLEUS** | | |
| FMAX (N) | 3.9 | 2.8 |
| FMAX (N/g muscle) | 47.8 | 5.5 |
| FATIGUE INDEX $ | 0.68 | 0.05 |
| **EDL** | | |
| FMAX (N) | 11.2 | 2.7 |
| FMAX (N/g muscle) | 138.6 | 28.7 |
| FATIGUE INDEX $ | 0.38 | 0.04 |
| **TA** | | |
| FMAX (N) | 20.4 | 3.8 |
| FMAX (N/g muscle) | 60.5 | 10.9 |
| FATIGUE INDEX $ | 0.76 | 0.04 |

KEY: FMAX - Maximal Tetanic Force;

$ - Fatigue Index = Force at 2 minutes stimulation / initial force;
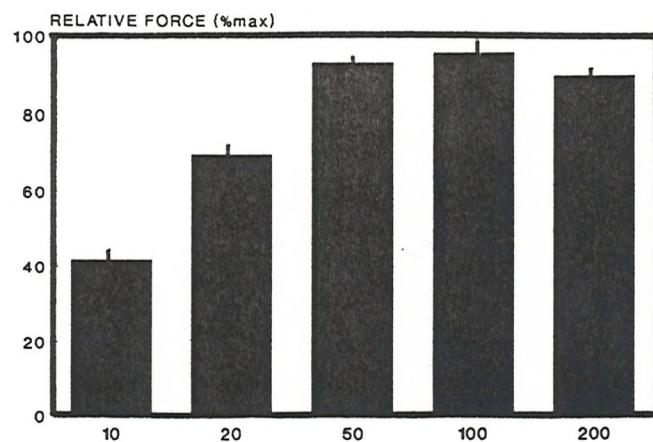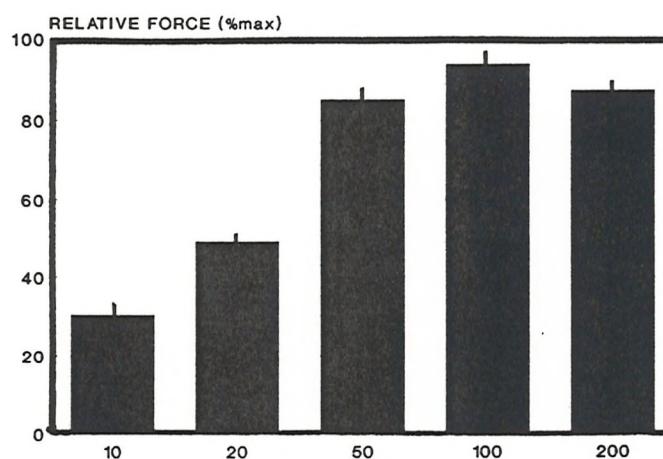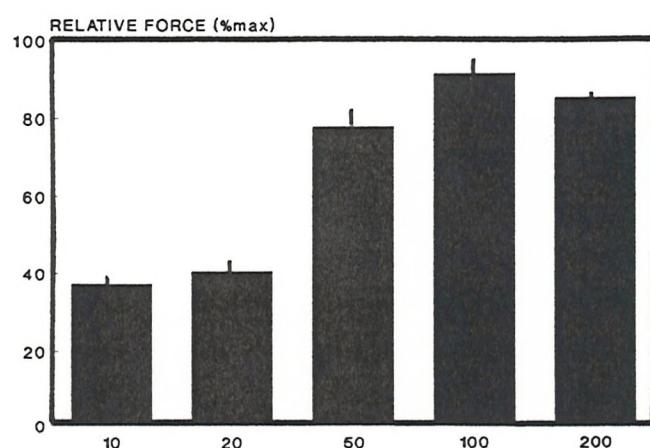
Figure 3.1. The force−frequency relationship of normal rat soleus (A), extensor digitorum longus (EDL, B) and tibialis anterior (TA, C) muscles (mean ± sem, n=6).

The TA muscle exhibited a fatigue index of 0.76 (Table 3.3). Force generation at 2 minutes of stimulation was 24% lower than that generated at the commencement of stimulation. The force profile of the TA muscle was different to that observed in the EDL muscle, resembling that previously described in Figure 2.2C. The force profile of the TA muscle during stimulation using Burke's Paradigm exhibited a rapid decline in force followed by maintenance of the force generated. This profile was intermediate of that obtained from single motor units of the type IIA and IIB classes (Burke *et al* 1973).

## 3.3. DISCUSSION.

The aim of this chapter was to characterise normal rat skeletal contractile characteristics. In particular the difference in the characteristics of the soleus, EDL and TA muscles were investigated.

It had been hypothesised that the soleus muscle would exhibit type I fiber characteristics due to the high proportion of these fibers which the soleus muscle is composed (Table 1.2). The EDL muscle was expected to exhibit characteristics of type IIB fibers and the TA a combination of type IIA and type IIB characteristics due to their respective fiber type compositions (Table 1.2).

Time to peak tension and half relaxation time measurements of the soleus, EDL and TA muscles taken from the literature appear to indicate a large range which may reflect differences in temperature, methodology and stimulation procedures. In general, the soleus muscle exhibited a slower time to peak tension and half relaxation time than either the EDL or TA muscles. This was also apparent in the measurements made in the animals of this study. The soleus muscle was approximately 2 times slower to contract and relax as the fast twitch muscles. The values for time to peak tension and half relaxation time of the soleus, EDL and TA muscles from this experiment was found to lie within the centre of the range reported in the literature (Table 1.3). The soleus muscle exhibited the widest range of values for time to peak tension and half relaxation time. This may reflect the greater susceptibility of the oxidative type I fibers to an interuption in blood flow which may occur during the surgical procedures. Great effort was made to minimise any such effect and measurements were not made in muscles which appeared ischemic (assessed subjectively, by a lack of the normal red colouration of the muscle). This is particularly important since the biological variability of these measurements was determined to be approximately 20% in a sample of 10 animals (Table 2.5). The variation in the measurements of the 6 animals reported in this chapter was greater than that value reported in Chapter 2, hence cahnges in skeletal msucle contractile characteristics may occur, but remain undetected as the values fall within the normal range.

Peak twitch tension in the soleus muscle was less than that in the EDL or TA muscles. It is not appropriate to compare these values with those reported in the literature because skeletal muscle mass is an important determinant of force generation (Section 1.3) and was not recorded in many of the reports in the literature. The TA muscle of rats in this study generated greater forces than the smaller soleus and EDL muscles. The force generated by the EDL muscle was greater than that of the soleus muscle despite being of similar mass. This would suggest that the type II fibers are capable of greater force generation than type I fibers. This has previously been indicated and may be due to the larger type II muscle fiber cross sectional area (Burke *et al* 1973; Sieck *et al* 1989). The angle of pennation may also play a role since fibers in the strap−like EDL muscle would tend to generate forces along the line of attachment. In the soleus muscle force generation is at an angle to the tendons resulting in a vectorial effect.

Maximal force generation in the TA muscle was approximately 5 & 2 fold greater than that in the soleus or EDL muscles. This may appear to be primarily due to the larger weight of the TA muscle, but since the soleus and EDL muscles were of similar weight, muscle size is not the sole determinant of force development. This difference in force generation did not disappear when corrected for muscle mass (i.e. per unit muscle) resulting in the relative force production of the EDL muscle becoming greater than either the soleus or TA. This may be due to the muscle fiber composition although the relevance of this observation remains unclear.

The force−frequency relationship of the soleus muscle achieved a greater proportion of the maximal response earlier than the EDL or TA muscles. This may reflect the lower frequency at which these muscles would be expected to receive neural input (10−20 Hz in slow twitch muscles, 40−50 Hz in fast twitch muscles, Adrian & Bronk 1929). No previous studies of the rat soleus, EDL or TA muscle force−frequency relationships have been made, although different muscles do appear to exhibit a similar shaped relationship (rat gastrocnemius − Russell *et al* 1984; hamster diaphragm, soleus and plantaris muscles − Drew *et al* 1988).

The fatigue of whole muscles in response to electrical stimulation has not previously been investigated using Burke's Paradigm (see Chapters 1 & 2). The reliability of this method in the assessment of fatigue has previously been described (Section 2.2.2.3). The fatigue profiles which were obtained in these normal rat muscles were similar to those predicted from the reported fiber type compositions (Table 1.2). The soleus muscle therefore exhibited a profile similar to that obtained following stimulation of single motor units of type I fibers, the EDL of type IIB fibers and the TA an intermediate response. These profiles were similar to those reported for the different single motor units of cat gastrocnemius muscle (Burke *et al* 1973).

3.4. <u>SUMMARY.</u>

This chapter has defined typical functional characteristics of the soleus, EDL and TA muscles of normal rats. The contractile characteristics have been described under carefully controlled conditions and appear to lie within the central range of values reported in the literature. The results confirm that the contractile characteristics of the soleus muscle as assessed by the techniques employed in this thesis are compatable with the soleus muscle being a slow twitch muscle which is fatigue resistant. Similarly, the EDL was assessed to be a fast twitch muscle which fatigues rapidly and the TA muscle a fast twitch muscle which exhibits little fatigue.

# CHAPTER 4.

# THE INFLUENCE OF LIMITATION OF FOOD INTAKE ON SKELETAL MUSCLE CONTRACTILE CHARACTERISTICS.

Malnutrition may result from several causes, fasting or starvation representing the extremes of this situation. These reductions in food intake result in changes in substrate metabolism to maintain essential body functions and has previously been described (see Section 1.2.2.1). Reductions in food intake of this nature are often accompanied by loss of lean body tissue (i.e. muscle) and is assumed to represent an impairment of skeletal muscle function. Little evidence of such impairment has been reported. This chapter will firstly describe the experiments conducted to investigate skeletal muscle function following acute food deprivation. Subsequently, this chapter will assess the contractile characteristics of skeletal muscle following small reductions in food intake over prolonged periods.

## 4.1 THE INFLUENCE OF ACUTE FOOD DEPRIVATION ON SKELETAL MUSCLE FUNCTION.

Few studies have investigated skeletal muscle function during or following fasting. Evidence for changes in skeletal muscle function following acute food deprivation has been summarised in Chapter 1. In general, fasting has been reported to be associated with reductions in force generation, slower relaxation and shifts in the force frequency relationship of the human adductor pollicis muscle (48 hours, Wootton *et al* 1987). This was also reported following electrical stimulation of the gastrocnemius muscle of the rat following 5 days starvation (Russell *et al* 1984). No changes in the twitch characteristics of the rat gastrocnemius muscle was reported (Russell *et al* 1984). The impairment of skeletal muscle function noted in the animal studies was suggested to result from impaired calcium handling due to reduced energy status of the gastrocnemius muscle (Russell *et al* 1984). It is unclear however, whether this was the result of reductions in muscle high energy compounds or due to the sustained contraction which the muscle underwent prior to sampling.

The aim of this study therefore, was to investigate the influence of acute food deprivation in the rat on the *in situ* contractile characteristics of muscles specifically selected to reflect the different skeletal muscle fiber types. Thus this study investigated the soleus (type I), extensor digitorum longus (EDL, type IIB) and the tibialis anterior (TA, type IIA/B) muscles following 5 days acute food deprivation.

4.1.1 <u>Methods.</u>

Twelve male Wistar rats (mean initial body weight 192.3 ± 2.2g) were allocated to one of two groups:

    a) CONTROL                     :fasted overnight (12−14 hours; n=6);

or  b) FASTED                       :fasted 5 days with free access to water (n=6);

All animals were maintained individually in a room maintained at 23°C with a 12 hour light−dark cycle. Body weight and food intake was monitored at the same point in each light period.

On the day of study animals were anaesthetised using 0.44 mls Sagatal/kg body weight and prepared for measurement as described in Section 2.1. Assessment of contractile characteristics was conducted as reported in Section 2.2. Time to peak tension (TPT), half relaxation time (½RT) & peak twitch tension (PT) of the twitch and the maximal tetanic force (Fmax) & force frequency relationship of the muscles were determined. Fatigue assessment was not conducted in these animals.

Following assessment of contractile characteristics the muscles of both hindlimbs were rapidly excised, weighed and stored as previously described (see Section 2.3.1) for determination of metabolite profile (see Section 2.4).

4.1.2 <u>Results.</u>

The influence of 5 days fasting on rat body and muscle mass, muscle contractile characteristics and metabolism is presented below. A complete discussion of the findings from this study can be found in Section 4.3. The TA muscle of one animal in the CONTROL group did not contract in response to electrical stimulation and data for the TA muscle in this group therefore reflects only 5 animals.

4.1.2.1 <u>Body and skeletal muscle weight.</u>

Food deprived animals lost weight rapidly over the first 3 days of the study, attaining a final body weight which was 20% lighter than that of CONTROL animals ($p < 0.01$, Table 4.1). Muscle weight was lower in the fasted animals: the soleus muscle was 3% lighter ($p > 0.05$), the EDL 26% lighter ($p < 0.05$) and the TA 12% lighter ($p < 0.05$). This resulted in muscle weight per unit body weight being 20% greater in the soleus muscle of fasted animals compared to muscles of CONTROL animals ($p < 0.05$, Table 4.1), 10 % greater in the TA muscle ($p > 0.05$) and 4% lighter in the EDL muscle ($p > 0.05$, Table 4.1).

Table 4.1.  Body and muscle weight of CONTROL and acutely food deprived male wistar rats.

| | CONTROL | | ACUTE FOOD DEPRIVATION | |
|---|---|---|---|---|
| | n=6 | | n=6 | |
| | mean | sem | mean | sem |
| BODY WEIGHT (g) | 172.7 | 4.5 | 137.9 | 2.9 ** |
| SOLEUS  WEIGHT | | | | |
| (mg) | 73.4 | 14.2 | 70.9 | 5.1 |
| (mg/100g body weight) | 42.6 | 3.1 | 51.2 | 3.8 * |
| EDL WEIGHT | | | | |
| (mg) | 77.7 | 6.3 | 57.6 | 6.3 * |
| (mg/100g body weight) | 43.2 | 2.8 | 41.4 | 4.2 |
| TA WEIGHT | | | | |
| (mg) | 330.8 | 13.1 | 292.3 | 8.1 * |
| (mg/100g body weight) | 192.6 | 8.8 | 211.4 | 6.9 |

KEY: * - p<0.05 v CONTROL; ** - p<0.01 v CONTROL.

### 4.1.2.2 Twitch characteristics.

Time to peak tension and half relaxation time of the soleus muscle of fasted animals was 18% faster than that of CONTROL animals ($p < 0.05$ and $p < 0.01$ respectively, Table 4.2). The time to peak tension and half relaxation time of the EDL and TA muscles of fasted animals were not different from that of muscles of CONTROL animals ($p > 0.05$, Table 4.2).

Peak twitch tension of the soleus, EDL and TA muscles were not different in the fasted animals from that of CONTROL animals when expressed in Newtons ($p > 0.05$, Table 4.2). Peak twitch tension per unit muscle weight in the muscles of fasted animals was also unaltered following fasting, except in the EDL muscle which exhibited a 74% greater relative force than the EDL muscle of CONTROL animals ($p < 0.01$, Table 4.2).

### 4.1.2.3 Tetanic responses.

Maximal tetanic force generation was not discernibly different in the soleus, EDL and TA muscles of fasted animals compared to muscles of CONTROL animals ($p > 0.05$, Table 4.2). However, when expressed per unit muscle the maximal tetanic force was greater in the EDL muscles of fasted animals compared to that of CONTROL animals (56%, $p < 0.05$, Table 4.2). No difference in the force generation per unit muscle was discernible in the soleus and TA muscles ($p > 0.05$).

The force–frequency relationship of the soleus, EDL and TA muscles of fasted animals was not different from that observed in the muscles of CONTROL animals (Figure 4.1).

### 4.1.2.4 Skeletal muscle metabolites.

The concentration of muscle metabolites were not discernibly different in all three muscles of the fasted animals from the muscles of CONTROL animals. However, there was a tendency for the ATP and creatine phosphate concentrations of the soleus muscle of fasted animals to be lower than that of CONTROL animals (Table 4.3). All three muscles exhibited a trend for higher muscle lactate concentration in the fasted condition, but this difference did not attain statistical significance.

TABLE 4.2. The contractile characteristics of the soleus, extensor digitorum longus (EDL) and tibialis anterior (TA) muscles of CONTROL and acutely food deprived male Wistar rats.

| | CONTROL !<br>n=6 | | ACUTE FOOD DEPRIVATION<br>n=6 | |
|---|---|---|---|---|
| | mean | sem | mean | sem |
| **SOLEUS** | | | | |
| TPT (ms) | 32.1 | 1.8 | 26.3 | 2.2 * |
| ½RT (ms) | 48.4 | 2.7 | 39.6 | 1.8 ** |
| PT (mN) | 1.6 | 0.3 | 1.3 | 0.2 |
| PT (mN/g muscle) | 21.1 | 2.8 | 18.9 | 3.3 |
| FMAX (mN) | 11.3 | 2.4 | 10.7 | 2.6 |
| FMAX (mN/g muscle) | 153.2 | 31.4 | 158.9 | 36.3 |
| | | | | |
| **EDL** | | | | |
| TPT (ms) | 14.4 | 0.5 | 14.5 | 0.9 |
| ½RT (ms) | 13.6 | 0.5 | 12.0 | 0.6 |
| PT (mN) | 2.4 | 0.3 | 3.3 | 0.3 ** |
| PT (mN/g muscle) | 34.2 | 4.3 | 59.7 | 5.1 |
| FMAX (mN) | 7.9 | 1.0 | 8.7 | 0.7 * |
| FMAX (mN/g muscle) | 103.7 | 12.5 | 162.7 | 19.7 |
| | | | | |
| **TA** | | | | |
| TPT (ms) | 13.7 | 0.7 | 12.5 | 0.3 |
| ½RT (ms) | 12.7 | 0.7 | 12.8 | 0.6 |
| PT (mN) | 7.9 | 1.3 | 6.3 | 0.5 |
| PT (mN/g muscle) | 20.6 | 3.2 | 21.6 | 1.4 |
| FMAX (mN) | 34.1 | 7.6 | 31.5 | 1.2 |
| FMAX (mN/g muscle) | 102.3 | 7.6 | 109.5 | 2.5 |

KEY: TPT - Time to Peak Tension; ½RT - Half Relaxation Time; PT - Peak Twitch Tension; FMAX - Maximal Tetanic Force;

! - n= 5 for CONTROL TA see text;

* - $p<0.05$ v CONTROL; ** - $p<0.01$ v CONTROL.

Table 4.3. The metabolite profile of the soleus, extensor digitorum longus (EDL) and tibialis anterior (TA) muscles of CONTROL and acutely food deprived male Wistar rats [*].

| | CONTROL n=6 | | ACUTE FOOD DEPRIVATION n=6 | |
|---|---|---|---|---|
| | mean | sem | mean | sem |
| **SOLEUS** | | | | |
| ATP | 32.9 | 5.8 | 22.5 | 4.0 |
| CP | 51.7 | 5.8 | 37.7 | 6.7 |
| Free Creatine | 90.1 | 10.6 | 82.9 | 9.5 |
| Total Creatine | 134.6 | 15.5 | 129.9 | 8.5 |
| Lactate | 15.6 | 4.3 | 16.5 | 4.7 |
| **EDL** | | | | |
| ATP | 34.9 | 2.1 | 33.2 | 2.0 |
| CP | 74.2 | 7.7 | 61.8 | 8.5 |
| Free Creatine | 73.6 | 12.6 | 85.6 | 14.1 |
| Total Creatine | 151.0 | 16.5 | 152.9 | 19.6 |
| Lactate | 11.8 | 1.7 | 15.4 | 3.7 |
| **TA** | | | | |
| ATP | 33.6 | 3.0 | 32.3 | 2.2 |
| CP | 62.8 | 5.2 | 57.8 | 6.7 |
| Free Creatine | 76.5 | 18.9 | 88.0 | 3.8 |
| Total Creatine | 139.1 | 19.2 | 128.0 | 15.8 |
| Lactate | 11.0 | 2.5 | 15.2 | 3.4 |

KEY: ATP - Adenosine Triphosphate; CP - Creatine phosphate

    * - All values are mM/kg dry wt of muscle.
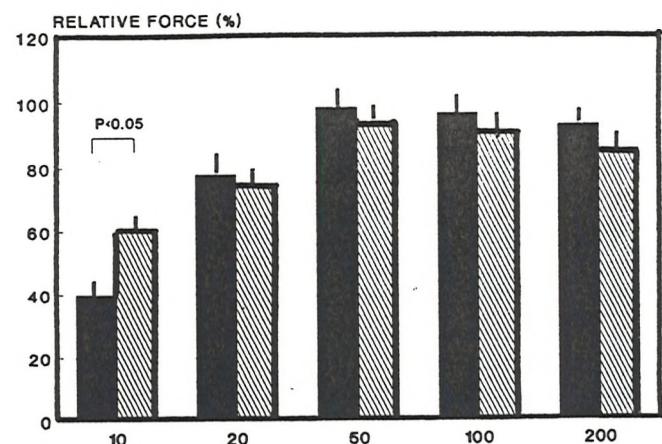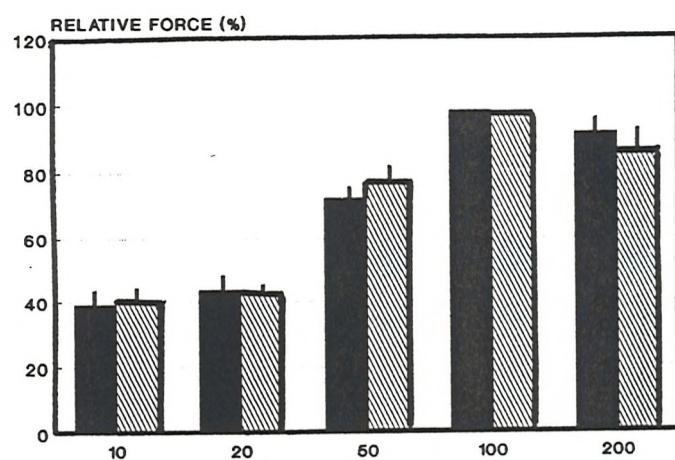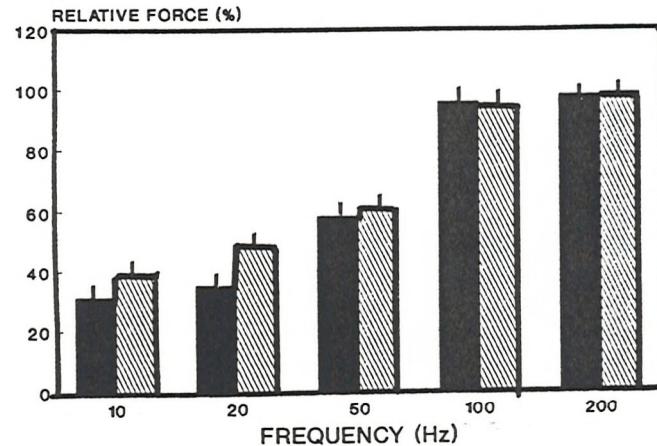
Figure 4.1. The force—frequency relationship of the soleus
(A), extensor digitorum longus (EDL, B) and tibialis anterior
(TA, C) muscles of CONTROL (■), and acute food deprived
(▨) male wistar rats. (n=6 except for CONTROL TA where n=5,
see text. Bars represent mean relative force generation ± 1 sem).

4.1.3 Summary of the influence of acute food deprivation on skeletal muscle contractile characteristics.

Force generation in muscles of fasted animals was preserved despite loss of skeletal muscle tissue. No impairments in skeletal muscle function were observed, although the time to peak tension and half relaxation time of the soleus muscle was faster following food deprivation when compared to CONTROL animals. For a more comprehensive discussion of the data see Section 4.3.

4.2 THE INFLUENCE OF CHRONIC FOOD RESTRICTION ON SKELETAL MUSCLE FUNCTION.

The previous section has indicated that acute food deprivation did not influence the contractile characteristics of skeletal muscles selected to reflect the different muscle fiber types. As discussed in Chapter 1, few investigators have attempted to characterise skeletal muscle contractile characteristics following chronic food restriction in humans (Lopes *et al* 1982; Russell *et al* 1983a,b) or in animals (Smart & Bedi 1982; Russell *et al* 1984).

In general, reduced force generation, slower relaxation, increased fatigue and a shift in the force–frequency relationship of the adductor pollicis of malnourished patients has been recorded (Lopes *et al* 1982; Russell *et al* 1983a,b; Bruce *et al* 1989). Similar trends have been reported in animal studies (Wareham *et al* 1982; Russell *et al* 1984; Drew *et al* 1988). Sieck and colleagues have reported little change in the contractile characteristics of muscle following food restriction, with enhanced fatigue resistance of the rat diaphragm muscle (Sieck *et al* 1989).

Rather than clarifying our knowledge these studies have further complicated our understanding of this area. Many of the workers noted above have suggested that the changes which they have observed may be due to atrophy of type II skeletal muscle fibers (Russell *et al* 1983a; Russell *et al* 1983b; Russell *et al* 1984; Sieck *et al* 1989) which would be consistent with observations made in humans (Heymsfield *et al* 1982; Russell *et al* 1983b). The influence of food restriction on skeletal muscle function and its relevance to type II fiber atrophy could either be assessed histologically or by investigating the contractile characteristics of skeletal muscles selected to reflect the different muscle fiber types.

The aim of this study therefore, was to assess the effect of food restriction for a period of 21 days at a level of 25% the intake of paired fed control animals on rat skeletal muscle contractile characteristics. Specifically this study investigated muscles selected to reflect the different muscle fiber types. Thus the soleus (type I), extensor digitorum longus (EDL, type IIB) and tibialis anterior (TA, type IIA/B) muscles were selected for investigation. This study therefore

compared the effects of food restriction on the contractile characteristics of rats with those of control animals, at the commencement of the study. In addition the contractile characteristics of skeletal muscles from animals consuming their normal diet for the period of the study was compared to the control animals and to those animals whose food intake was restricted.

4.2.1 Methods.

Thirty male Wistar rats (mean initial body weight 180.6 ± 2.2g) were allocated to one of three groups:

a) CONTROL     :fasted overnight (12−14 hours) at the
                commencement of the study period (n=10);

b) FED         :control animals consuming their normal diet of
                CRM(X) pellets (Table 2.1) *ad libatum* for 21
                days, followed by an overnight fast (12−14
                hours) prior to assessment of muscle function
                (n=10);

or c) FOOD RESTRICTED:receiving a quantity of food (CRM(X); Table
                2.1) equivalent to 25% that consumed by paired
                control FED animals on the previous day (n=10);

All animals were maintained individually in a room maintained at 23°C with a 12 hour light−dark cycle. Body weight and food intake was monitored at the same point in each light period.

On the day of study animals were anaesthetised using 0.44 mls Sagatal/kg body weight and prepared for measurement as described in Section 2.1. Assessment of contractile characteristics was conducted as reported in Section 2.2, thus the time to peak tension (TPT), half relaxation time (½RT) & peak twitch tension (PT) of the twitch and the maximal tetanic force (Fmax) & force frequency relationship were examined.

Following assessment of contractile characteristics the muscles of both hindlimbs were rapidly excised, weighed and stored as previously described (see Section 2.3.1) for determination of metabolite profile (see Section 2.4).

4.2.2 Results.

This section describes the effect of food restriction on rat body & muscle mass and muscle contractile characteristics & metabolism. Initially, the influence of food restriction will be assessed in comparison to those animals studied at the commencement of the study

(CONTROL). Secondly, the influence of normal growth and development will be assessed by comparing animals allowed free access to food for 21 days (FED) with the CONTROL animals. Finally, the influence of food restriction in age–matched animals will be assessed by comparison of food restricted animals with those allowed free access to food over the period of study (FED).

During anaesthesia 2 animals from the FOOD RESTRICTED group died, since animals in the FED group were paired the analysis below contains data only for 8 animals in each of these groups. The EDL muscle of one animal in this food restricted group did not respond to stimulation and the data from stimulation of the EDL from another animal was not adequately saved due to a computer malfunction. Hence the analysis for the EDL muscle of the FOOD RESTRICTED group represents only 6 animals.

### 4.2.2.1 The influence of food restriction on skeletal muscle contractile characteristics.

This section will compare the animals consuming 25% the intake of paired FED animals with the CONTROL animals at the commencement of the study (i.e. FOOD RESTRICTED v CONTROL).

### 4.2.2.1.i Body and skeletal muscle weight.

Animals restricted in food intake exhibited a rapid loss of body weight over the first three days of study with a slower but progressive loss of weight over the subsequent 18 days reaching a body weight 12% lighter than their starting weight ($p < 0.01$, Table 4.4). These changes in body weight over the course of the study did not appear to be associated with changes in muscle weight (Table 4.4). The soleus muscle of food restricted animals was 15% lighter than that of CONTROL animals while the EDL was 6% heavier and the TA 15% heavier at the end of 21 days ($p > 0.05$, Table 4.4). Expressed per unit body weight, however, only the EDL muscle contributed a greater proportion of total body mass (20%, $p < 0.01$, Table 4.4).

### 4.2.2.1.ii Twitch responses.

Time to peak tension of the soleus and TA muscles of food restricted animals was 22% and 24% slower than that of muscles of CONTROL animals ($p < 0.05$, Table 4.5). Time to peak tension of the EDL muscle of food restricted animals was 16% slower, but this difference did not attain statistical significance ($p > 0.05$, Table 4.5). Half relaxation time of all three muscles of the food restricted animals was unchanged from that measured in muscles of CONTROL animals (Table 4.5). Peak twitch tension in Newtons was unchanged following food restriction in the soleus, EDL and TA muscles, resulting in a general increase in the relative force generation per unit muscle – 89% in the soleus muscle ($p < 0.05$, Table 4.5), 20% in the EDL ($p > 0.05$) and 44% in

the TA muscle (p>0.05).

4.2.2.1.iii <u>Tetanic responses.</u>

Maximal tetanic tension in Newtons was generally raised following food restriction, although this difference attained statistical significance only for the TA muscle (36%, p<0.05, Table 4.5). The relative maximal tetanic tension was raised in all three muscles following food restriction in comparison to CONTROL muscles, this difference attained statistical significance only for the TA muscle (46%, p<0.05, Table 4.5). A tendency for a shift in the force frequency curve of the fast twitch muscles of food restricted animals was noted, attaining statistical significance for the EDL muscle (Figure 4.2b). There was no difference in the force−frequency relationship of the soleus muscle of food restricted animals compared to muscles of CONTROL animals (Figure 4.2a).

4.2.2.1.iv <u>Skeletal muscle metabolites.</u>

There was a tendency for the ATP concentration of the soleus muscle of food restricted animals to be 30% lower compared to CONTROL muscles, but this difference did not attain statistical significance (p>0.05, Table 4.6). The concentration of ATP in the EDL and TA muscles of food restricted animals was not different from that of muscles from CONTROL animals (p>0.05). The concentration of free creatine within the muscles of food restricted animals was not different from that of muscles from CONTROL animals (Table 4.6). The concentration of creatine phosphate in the EDL muscle of food restricted animals was 35% lower than that of the EDL of CONTROL animals (p<0.05, Table 4.6). No differences in creatine phosphate concentration was discernible in the soleus and TA muscles of food restricted animals compared to muscles of CONTROL animals.

Muscle lactate concentration was 20−62% lower in the muscles following food restriction (p<0.05, Table 4.6), but this may be the result of a relatively high lactate concentration in the muscles of CONTROL animals.

Table 4.4.  Body and muscle weight of control animals and animals consuming their normal diet for 21 days (FED) or following restriction of food intake to 25% that of paired fed animals on the previous day (CHRONIC FOOD RESTRICTED)

| | CONTROL | | FED | | CHRONIC FOOD RESTRICTION | |
|---|---|---|---|---|---|---|
| | n=10 | | ! n=8 | | ! n=8 | |
| | mean | sem | mean | sem | mean | sem |
| BODY WEIGHT (g) | 169.0 | 2.6 | 320.7 | 5.4 ** | 149.6 | 3.6 **╫ |
| SOLEUS WEIGHT (mg) | 75.5 | 4.5 | 129.8 | 7.9 ** | 64.3 | 5.1 ╫ |
| (mg/100g body weight) | 44.6 | 2.5 | 40.6 | 2.0 | 43.2 | 3.7 |
| EDL WEIGHT (mg) | 77.4 | 4.3 | 128.4 | 10.3 ** | 82.2 | 2.8 ╫ |
| (mg/100g body weight) | 45.8 | 2.2 | 40.1 | 2.9 | 55.4 | 2.0 **╫ |
| TA WEIGHT (mg) | 302.3 | 33.2 | 555.1 | 27.7 ** | 346.7 | 14.9 ╫ |
| (mg/100g body weight) | 197.8 | 9.0 | 173.8 | 7.9 ** | 197.2 | 30.4 |

KEY: ** - P< 0.01 v CONTROL; ╫ - p<0.01 v FED.

! - two animals in FOOD RESTRICTED group died, hence n=8 for this group and paired FED animals, see text

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Table 4.5. The contractile characteristics of soleus, extensor digitorum longus (EDL) and tibialis anterior (TA) muscles of control animals and animals following consumption of their normal diet for 21 days (FED) or following restriction of food intake to 25% that of paired fed animals on the previous day (CHRONIC FOOD RESTRICTED)

| | CONTROL | | FED | | CHRONIC FOOD RESTRICTION | |
|---|---|---|---|---|---|---|
| | n=10 | | n=8 [!] | | n=6 [!] | |
| | mean | sem | mean | sem | mean | sem |
| **SOLEUS** | | | | | | |
| TPT (ms) | 30.1 | 2.1 | 36.0 | 0.9* | 36.7 | 1.9* |
| ½RT (ms) | 57.0 | 4.5 | 61.4 | 5.6 | 64.2 | 8.6 |
| PT (mN) | 1.5 | 0.3 | 1.8 | 0.2 | 1.9 | 0.4 *$ |
| PT (mN/g muscle) | 18.7 | 2.2 | 15.1 | 1.7 | 35.3 | 7.8 *$ |
| FMAX (mN) | 8.0 | 1.2 | 10.7 | 0.9 | 8.7 | 1.6 $ |
| FMAX (mN/g muscle) | 101.3 | 11.7 | 78.1 | 5.1 | 157.1 | 38.2 $ |
| **EDL** | | | | | | |
| TPT (ms) | 14.2 | 0.5 | 16.0 | 0.7* | 16.6 | 0.5 |
| ½RT (ms) | 13.4 | 0.6 | 13.5 | 1.0 | 13.8 | 0.5 |
| PT (mN) | 2.3 | 0.3 | 5.3 | 0.5* | 4.0 | 1.6 |
| PT (mN/g muscle) | 27.8 | 2.6 | 39.1 | 3.3** | 33.6 | 4.8 $ |
| FMAX (mN) | 7.6 | 0.7 | 15.9 | 1.3* | 8.5 | 1.5 $ |
| FMAX (mN/g muscle) | 93.7 | 5.5 | 115.1 | 4.8 | 100.3 | 14.9 |
| **TA** | | | | | | |
| TPT (ms) | 12.5 | 0.7 | 16.4 | 0.9* | 15.5 | 0.5* |
| ½RT (ms) | 12.5 | 0.7 | 13.1 | 0.8 | 13.4 | 1.3 |
| PT (mN) | 6.1 | 1.1 | 7.7 | 0.2 | 7.8 | 1.9 |
| PT (mN/g muscle) | 15.6 | 2.6 | 13.3 | 0.5* | 22.6 | 5.8 |
| FMAX (mN) | 27.1 | 0.8 | 46.9 | 5.8 | 36.9 | 3.2 |
| FMAX (mN/g muscle) | 71.7 | 9.8 | 85.1 | 12.41 | 104.7 | 7.6* |

KEY: TPT - Time to Peak Tension; ½RT - Half Relaxation Time; PT - Peak Twitch Tension; FMAX - Maximal Tetanic Force;
* - $p<0.05$ v CONTROL; ** - $p<0.01$ v CONTROL; $ - $p<0.05$ v FED;
!- n=8 in FED and FOOD RESTRICTED groups due to death of two animals under anaesthesia, addtionally 2 EDL muscles of FOOD RESTRICTED group not reported, see text.
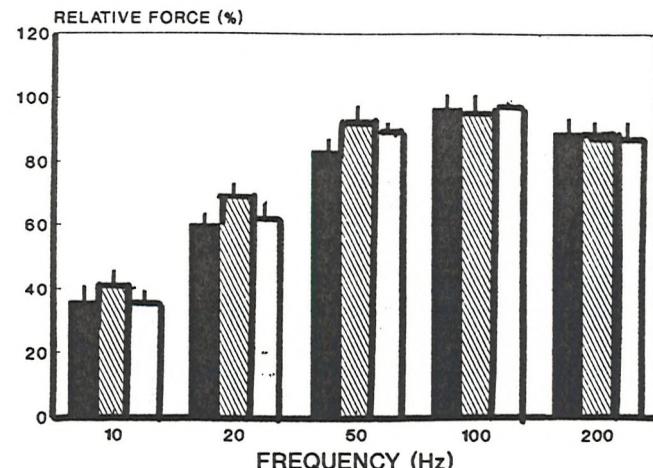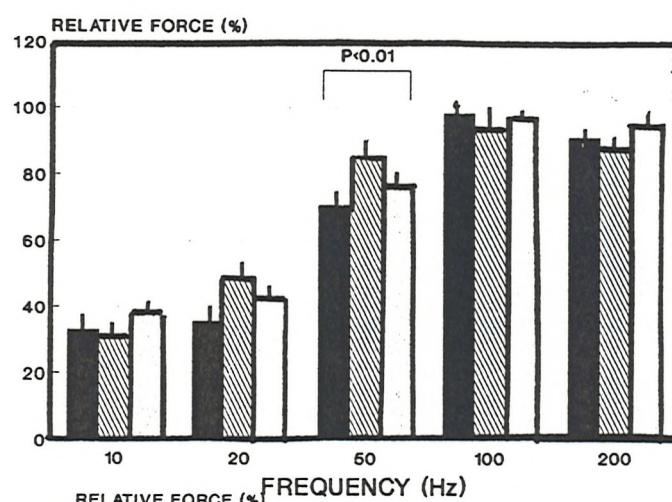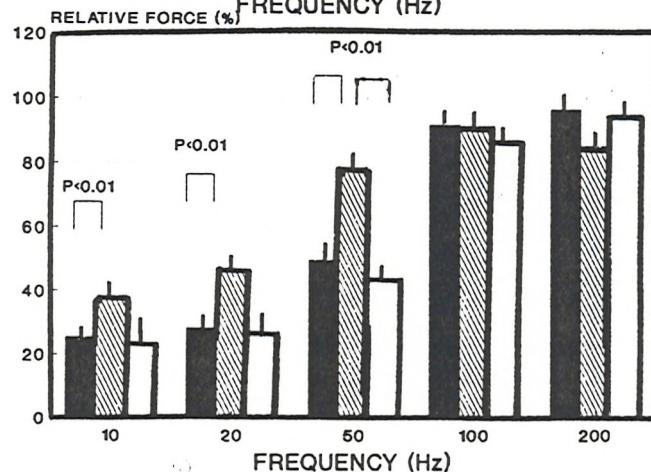
Figure 4.2. The force–frequency relationship of the soleus (A), extensor digitorum longus (EDL, B) and tibialis anterior (TA, C) muscles of CONTROL (■ n=10), FED (▨, n=8) and Food restricted (FR,□, n=8 except for EDL where n=6 – see text) male wistar rats. (Bars represent mean relative force generation ± 1 sem).

Table 4.6. The metabolite profile of soleus, extensor digitorum longus (EDL) and tibialis anterior (TA) muscles of control animals and animals following consumption of their normal diet for 21 days (FED) or following restriction of food intake to 25% that of paired fed animals on the previous day (ACUTE FOOD RESTRICTED). All values are mmoles/kg dry muscle weight.

| | CONTROL | | FED | | CHRONIC FOOD RESTRICTION | |
| | n=10 | | n=8 | | n=8 [!] | |
| | mean | sem | mean | sem | mean | sem |
|---|---|---|---|---|---|---|
| **SOLEUS** | | | | | | |
| ATP | 32.9 | 5.8 | 22.7 | 1.7 | 21.4 | 0.9 |
| CP | 51.7 | 5.8 | 42.5 | 5.8 | 48.5 | 8.7 |
| Free Creatine | 90.1 | 10.6 | 80.6 | 20.9 | 97.1 | 17.1 |
| Total Creatine | 134.6 | 15.5 | 131.8 | 19.3* | 156.8 | 20.7* |
| Lactate | 15.6 | 4.3 | 6.6 | 0.4 | 5.9 | 0.7 |
| **EDL** | | | | | | |
| ATP | 34.9 | 2.1 | 37.1 | 2.9 | 35.8 | 3.9* |
| CP | 74.2 | 7.7 | 56.9 | 9.9 | 48.3 | 8.1 |
| Free Creatine | 73.6 | 12.6 | 100.9 | 12.9 | 89.5 | 10.0 |
| Total Creatine | 151.0 | 16.5 | 157.9 | 12.1* | 137.8 | 11.9* |
| Lactate | 11.8 | 1.7 | 6.2 | 1.0 | 6.8 | 0.7 |
| **TA** | | | | | | |
| ATP | 33.6 | 3.0 | 36.9 | 5.0 | 34.3 | 4.5 |
| CP | 62.8 | 5.2 | 49.4 | 7.3 | 53.5 | 6.7 |
| Free Creatine | 76.5 | 18.9 | 87.9 | 9.8 | 88.2 | 13.6 |
| Total Creatine | 139.1 | 19.2 | 140.3 | 14.0 | 145.1 | 14.5 |
| Lactate | 11.0 | 2.5 | 8.1 | 0.4 | 8.9 | 1.4 |

KEY:ATP - Adenosine Triphosphate; CP - Creatine Phosphate; * - $P<0.05$ v CONTROL.
! - n= 6 in EDL of FOOD RESTRICTED group, see text.

### 4.2.2.2 The influence of normal growth on skeletal muscle function.

This section will compare animals consuming their normal diet for 21 days with the control animals at the commencement of the study (i.e. FED v CONTROL).

#### 4.2.2.2.i Body and skeletal muscle weight.

Animals consuming their usual laboratory diet grew normally over the 21 days of study attaining a body mass which was 89% heavier than at the start of the study ($p < 0.01$, Table 4.4). These changes in body weight over the course of the study were associated with changes in muscle weight as presented in Table 4.4. The soleus muscle of FED animals was 72% heavier, the EDL 66% and the TA 84% heavier than muscles from CONTROL animals ($p < 0.01$). Muscle weight per unit body weight of FED animals was 10% lower in the soleus muscle, 12% lower in the EDL and TA muscles compared to CONTROL animals (Table 4.4). This difference attained statistical significance only in the case ot the TA muscle ($p < 0.05$, Table 4.4).

#### 4.2.2.2.ii Twitch characteristics.

Time to peak tension in the muscles of FED animals was slower compared to the muscles of CONTROL animals, being 20% slower in the soleus muscle, 13% slower in the EDL and 31% slower in the TA ($p < 0.01$, Table 4.5). No alteration in the half relaxation time of the soleus, EDL or TA muscles was observed in the FED animals when compared to the muscles of CONTROL animals ($p > 0.05$, Table 4.5).

Peak twitch tension was generally greater in the larger muscles of the FED group compared to CONTROL; force generation was 20% greater in the soleus muscle ($p > 0.05$, Table 4.5), 130% greater in the EDL ($p < 0.05$, Table 4.5) and 26% greater in the TA ($p < 0.05$, Table 4.5). When expressed per unit muscle mass, however, the force generation of the larger muscles of the FED animals was comparable to that of the CONTROL muscles in the case of the soleus and TA but raised in the EDL muscle (40%, $p < 0.05$, Table 4.5).

#### 4.2.2.2.iii Tetanic responses.

Maximal tetanic force in Newtons was greater in all three muscles of the FED group compared to the muscles of CONTROL animals (33% soleus, $p > 0.05$; 109% EDL, $p < 0.01$; 73% TA, $p < 0.05$; Table 4.5). When expressed per unit muscle mass, however, this enhanced force generation was not different to that of muscles from CONTROL animals ($p > 0.05$, Table 4.5) except for the EDL muscle which exhibited a 23% greater relative force generation ($p < 0.05$, Table 4.5).

There was no difference in the force—frequency relationship of the soleus and EDL muscles of FED animals compared to CONTROL animals (p>0.05, Figure 4.2). The TA muscle of FED animals exhibited raised relative force generation at all frequencies up to 50 Hz (60%, p<0.01, Figure 4.2).

### 4.2.2.2.iv Skeletal muscle metabolites.

The muscle concentration of ATP, creatine phosphate and free creatine was not different in the soleus, EDL or TA muscles of FED animals compared to those of CONTROL animals. There was a tendency for a lower concentration of ATP in the soleus muscle, but this difference did not attain statistical significance (p>0.05, Table 4.6). Muscle lactate concentration was 57%, 48% and 26% lower in the soleus, EDL and TA muscles of FED animals compared to the muscles of of the CONTROL animals (p<0.05 for the soleus and EDL, TAble 4.6), but may be the result of a relatively high lactate concentration in the muscles of the CONTROL animals.

### 4.2.2.3 Comparison of the difference in muscle function between animals food restricted and those consuming their normal diet.

This section will compare the animals which were food restricted for 21 days with the paired FED animals having free access to their normal diet for this 21 day period (i.e. FED v FOOD RESTRICTION).

### 4.2.2.3.i Body and muscle weights.

Body weight of the food restricted animals was 47% lighter than that of animals which grew normally (i.e. the FED group; p<0.01, Table 4.4). The soleus muscle was 50% lighter in the food restricted animals than in the FED group, while that of the EDL was 64% lighter and the TA 62% lighter (p<0.05, Table 4.4). This resulted in an increased muscle mass per unit body weight in all three muscles of the food restricted animals compared to the FED group. This difference attained statistical significance only for the EDL muscle (38%, p<0.01, Table 4.4).

### 4.2.2.3.ii Twitch Characteristics.

Time to peak tension of the soleus, EDL and TA muscles of food restricted animals was not different from that of CONTROL animals (p>0.05, Table 4.5). No alteration in the half relaxation time of the soleus, EDL and TA muscles of food restricted animals was noted compared to FED animals (p>0.05, Table 4.5). Peak twitch tension was not different in the soleus, EDL and TA muscles of the food restricted animals than in the FED group (p>0.05, Table 4.5). When expressed per unit muscle, the soleus and TA muscles exhibited a 134% and 170% greater

relative force generation (p<0.05 & p<0.01 respectively, Table 4.5).

4.2.2.3.iii <u>Tetanic Responses.</u>

Maximal force generation in Newtons was 13% lower in the soleus muscle (p>0.05), 46% lower in the EDL muscle (p<0.05) and 21% lower in the TA muscle of food restricted animals compared to animals consuming their normal food intake (p<0.05, Table 4.5). When expressed per unit muscle weight however, the maximal tetanic force of the EDL and TA muscles of the food restricted animals was 13% lower and 23% higher than the muscles of the FED animals respectively, but these differences in force generation did not attain statistical significance (p>0.05, Table 4.5). Relative maximal force generation was 101% greater in the soleus muscle of food restricted animals when compared to CONTROLs (p<0.05, Table 4.5).

The force−frequency relationship was not different for the soleus and EDL muscles of food restricted animals when compared to those animals exhibiting normal growth (i.e. the FED group). The relative force generation in the TA muscle of the food restricted animals was however, approximately 50% lower than that of the FED group (P<0.01, Figure 3.2).

4.2.2.3.iv <u>Muscle Metabolites.</u>

The muscle concentrations of ATP, creatine phosphate, free creatine and lactate were not different in the soleus, EDL or TA muscles of food restricted animals from that measured in FED muscles (p>0.05, Table 4.6).

4.3 <u>DISCUSSION.</u>

The aim of this investigation was to specifically examine the influence of the limitation of food supply in rats on the contractile characteristics of skeletal muscles selected to reflect the different muscle fiber types. In this way it was intended to determine the peripheral adaptations which occur to malnutrition (i.e. those occurring within the muscle itself). Despite a reduction in the overall supply of nutrients in a balanced form the only impairment of contractile characteristics observed appeared to be a slowing of twitch time in the food restricted and FED animals, hence this may be an age related effect.

Following fasting and food restriction a reduction in body weight associated with a cessation of growth resulted in a general maintenance of skeletal muscle mass and function. Force generation per unit muscle was enhanced following limitation of food supply. Greater force generation at intermediate frequencies of electrical stimulation in fast twitch muscles was

apparent, but did not always attain statistical significance. No change in the muscle metabolite profile was noted.

### 4.3.1 Skeletal muscle mass.

The skeletal muscle mass following acute food deprivation was lower than that of animals at the commencement of the study, particularly in the fast–twitch muscles.  This suggests fasting did not initiate considerable wasting of all skeletal muscle mass. The relative EDL muscle weight remained unaltered following 5 days fasting while that of the soleus and TA muscles was raised indicative of a selective preservation of these muscles following 5 days of fasting.  This was also apparent for the EDL muscle of food restricted animals where raised relative muscle mass was observed.  Thus the requirements of the body for energy and nitrogen must be met from other endogenous sources during food restriction.  Normal growth in the animals as evidenced by the FED group did not result in selective changes in muscle weight, thus all muscles exhibited relative muscle mass slightly lower, but comparable, to CONTROL animals.

From the studies reported in this chapter it was not possible to determine whether the differences in muscle mass were the result of changes in water, glycogen, triglyceride or protein content.  As noted previously water comprises up to 80% of normal muscle (see Chapter 1) and the loss of other components may not represent large changes in muscle mass.

### 4.3.2 Force generation.

Preservation of functional capacity of skeletal muscle was the most striking observation of these studies.  This is most obvious in the case of fasted animals where force generation per unit muscle was preserved or enhanced despite the muscles being up to 26% lighter than those of CONTROL animals. It would appear that this reduction in muscle weight represents a cessation of growth since animals which were given some food (the restricted group) exhibited a general maintenance or a small increase in muscle weight.

The mechanism by which this reduction in muscle weight may have occurred without loss of force generation remains unclear.  It is possible that the loss of muscle protein, which could explain the observation of smaller muscles in the fasted and food restricted animals, does not represent those proteins which comprise the contractile machinery.  It is unclear which proteins could be lost without an alteration in function.  It is also possible that the smaller mass of the muscles represents loss of contractile proteins, but that this results in a closer packing of fibers along the direction of force generation resulting in maintenance of force generation.  Similarly, it is possible that the loss of protein allows a greater interaction of crossbridges within the fibers.

The observation of greater absolute force generation in the animals of the FED group compared to that of CONTROL, food deprived or food restricted animals is consistent with the increased muscle mass of these animals. This greater force generation was normalised when expressed per unit muscle, except in the EDL muscle where greater relative force generation was observed when compared to FED animals. Since no histological sections were studied from these animals it is unclear whether the increased force generation is associated with hypertrophy or hyperplasia. The soleus and TA muscles from fasted and food restricted animals were capable of generating as much force as the muscles of the FED animals, consuming their normal diet, while the EDL muscle was able to generate greater forces. Hence, the limit on the force generated appears to be that of size, ultimately this represents the number of crossbridges able to form (see Chapter 1).

The selective loss of type II muscle fibers following undernutrition has been reported in the literature in both malnourished humans (Heymsfield *et al* 1982) and animal models of undernutrition (Russell *et al* 1984). It may be expected that muscles exhibiting selective atrophy of type II fibers might come to exhibit twitch characteristics similar to the slow–twitch muscles (see below).

### 4.3.3 Force–frequency relationship.

The force–frequency relationship of muscles from fasted rats was unaltered in comparison to the muscles of CONTROL animals. The soleus muscle of food restricted animals did not exhibit changes in the force–frequency relationship when compared to either CONTROL or FED animals. The EDL and TA muscles exhibited shifts in the force–frequency relationship at around 50 Hz which has previously been taken to indicate an impaired skeletal muscle function. This would suggest a great capacity of the soleus muscle to accommodate changes resulting from reduced food intake. The changes in the force–frequency relationship of the fast–twitch muscles were not consistent and it is therefore possible that the fast twitch muscles of some animals may also have demonstrated a capacity for accommodation. A primary mechanism involved in this accommodation of nutritional stress may be the cessation of growth, since where growth was noted in the food restricted animals larger alterations in the force–frequency relationship were observed: particularly for the TA muscle. Thus the peak twitch force and maximal force able to be generated (Fmax) may increase when compared to CONTROLS but the tetanic contractions occur earlier. This may be to maintain function of skeletal muscles at the frequency at which the muscles would normally receive neuronal impulses, around 10 Hz for slow twitch muscles and 40 Hz for fast twitch muscles (Adrian & Bronk 1929).

As noted earlier, a possible mechanism involved in the loss of muscle tissue following limitation of food supply is the atrophy of type II fibers. If this were to occur it may be expected that fast–

twitch muscles might come to exhibit a more slow—type profile. The force—frequency relationship of the fast—twitch EDL and TA muscles did exhibit raised relative force generation at intermediate frequencies of stimulation (Figures 3.1b,c & 3.2b,c). This may reflect a change in the fiber composition of the muscle such that it becomes composed of a greater proportion of slow twitch fibers. However, it is difficult to envisage an atrophy of a significant number of fibers resulting in a muscle whose composition has changed to contain mainly type I fibers. Similarly, enhanced relative force generation would be expected to be associated with a lower absolute maximal force generation (Fmax) if the type II fibers atrophied. In fact the opposite was observed.

It should be noted however, that the rat gastrocnemius muscle has been reported to exhibit both reduced maximal tetanic force and a shift in the force—frequency relationship following both food restriction and deprivation (Russell *et al* 1984). It is possible that the lack of agreement of this study with that of Russell and co—workers is the difference in the muscles studied and the methodology employed. The gastrocnemius muscle is a large muscle of mixed fiber composition. It is unclear from the studies of Russell and colleagues the magnitude of the reported atrophy of the rat gastrocnemius muscle following food deprivation and restriction (Russell *et al* 1984). From the data presented in this chapter it is clear that muscles of different fiber composition differ in their susceptibility to atrophy. Thus the soleus muscle was selectively preserved in the face of food deprivation. A similar observation is apparent in Layman and colleagues study of partially food restricted rats, where the soleus muscle loss relatively less mass than the gastrocnemius, EDL, biceps branchii and psoas muscles (Layman *et al* 1981). Thus it is possible that the rat gastrocnemius muscle studied by Russell and colleagues had atrophied greatly. In this case it would be expected that a lower force would be generated when compared to that of age—matched animals. Since Russell and associates did not determine the contractile characteristics of the gastrocnemius muscle at the commencement of the study it is unclear whether this represents an impairment of muscle function or a preservation of function as described by the data presented in this chapter.

Despite the alterations in the force—frequency relationship the ability of soleus, EDL and TA muscles to perform work would appear to be maintained following both fasting and food restriction, and may reflect the underlying metabolic profile of the muscles.

4.3.4 <u>Adaptive capacity.</u>

It would appear that rats are able to accommodate limitations in the amount of food supplied in such a manner that muscle function is preserved. This will allow essential work related activities, such as food gathering, to occur. This adaptation was associated with an immediate cessation of growth, but the provision of protein and energy does allow the laying down of small amounts

of lean tissue as observed in the food restricted animals. Other adaptations may occur at levels above that of the skeletal muscle which will further maintain normal physical working capacity under these conditions. As such these results may reflect the maintenance of skeletal muscle function in malnourished humans despite the reports of lowered physical working capacity (Spurr *et al* 1984). Adaptations at the central and cardiovascular levels would be expected to be particularly important in determining the physical working capacity of an individual considering the apparent preservation of skeletal muscle at the expense of other body tissues.

Since the skeletal muscle contractile characteristics of rats following food deprivation and restriction were not impaired, any impairments in physical working capacity may not be related to the ability of skeletal muscle to perform work. Thus, the reduced force generation in food restricted animals compared to the FED group may not reflect impaired function but a lack of development. The slower time to peak tension and half relaxation time in the food restricted and FED animals would appear to be an age–related, developmental effect. This prolongation in twitch time was only just greater than the inherent variability reported in Chapter 2, hence these responses may still reflect normal muscle function.

## 4.4 SUMMARY

Food deprivation resulted in muscle atrophy although the slow twitch soleus muscle exhibited a selective preservation of muscle weight. Food restriction resulted in a degree of growth, noted as raised muscle weight, although this was not large. Force generation was preserved or enhanced in the face of limitations of food intake although a tendency for raised relative force generation at intermediate frequencies of stimulation was noted in the fast–twitch muscles. Twitch time was prolonged in the food restricted and FED animals, but this may reflect an age–related effect.

# CHAPTER 5.

# THE INFLUENCE OF ALTERATIONS IN FOOD COMPOSITION ON RAT SKELETAL MUSCLE FUNCTION – THE CONSUMPTION OF LOW PROTEIN DIETS.

Skeletal muscle wasting is a clear sign of malnutrition. Several workers have suggested that this may result in a lowered physical working capacity in malnourished individuals (see Chapter 1; Viteri 1971; Maksud *et al* 1976; Spurr *et al* 1977; Spurr *et al* 1984; Spurr & Reina 1990). No studies to date have investigated the ability of muscle to produce mechanical work following limitation of the composition of food available. This is due to the difficulty in separating the influence of motivation and the cardiovascular system from the peripheral (i.e. muscle) response to malnutrition.

Independent assessment of the peripheral response to consumption of diets limited in composition can be achieved using the techniques developed for the investigation of muscle function during fasting and food deprivation, reported in Chapter 2. Only a small alteration in skeletal muscle function could be attributed to the limitation of food intake (Chapter 4), other than that associated with a cessation of growth or the age–related slowing of twitch time. It would therefore appear that a lower physical working capacity in malnourished individuals may require specific changes at the central, cardiovascular and peripheral levels to occur. These changes would be difficult to assess independently in malnourished individuals and thus the development of animal studies would be of prime interest.

The diets of many developing countries are based on one or a few staple products which may result in the limitation of protein availability. This appears to be particularly associated with the clinical conditions of marasmus and kwashiorkor (Roussow 1989). While it is unclear whether a diet becomes limiting in protein before it becomes limiting in energy (Waterlow 1986; Jackson 1990) many investigators have attempted to reproduce these conditions in animal models, without success (Golden 1985 and Chapter 1).

Animals in these studies were stunted and wasted, though not all of the clinical signs of marasmus and kwashiorkor were evident (Edozein 1968; Coward & Lunn 1981; Anthony & Edozein 1975; Lowrey *et al* 1962; Ausman *et al* 1989; Section 1.2.2.3). This stunting and wasting appears to correlate well with the lower documented stature of individuals of developing countries compared to that of well developed nations (see Spurr 1987).

This study was therefore conducted to investigate the contractile characteristics of rat skeletal muscle in response to the consumption of a diet restricted in protein content. Since atrophy of type II fibers has been reported during loss of muscle tissue, this study specifically investigated

muscles selected to reflect the different muscle fiber types: soleus (type I), extensor digitorum longus (EDL, type IIB) and tibialis anterior (TA, type IIA,B). This chapter will firstly describe the growth and appearance of normally growing   animals and those consuming one of the two low protein diets.   Subsequently, the influence of these diets on skeletal muscle contractile characteristics will be discussed.

5.1 <u>METHODS.</u>

Twenty four male Wistar rats (mean initial weight 100.3 ± 1.7g) were allocated to one of four groups:

A) CONTROL                          :animals weighing 97 ± 4.7 g at time of study (n=6);

B) FED                              :animals consuming a normal diet containing 15% protein, 580 mg sodium/Kg diet and 2930 mg potassium/kg diet *ad libatum* for 21 days (n=6);

C) Low Protein, Low Electrolyte (LPLE):
animals consuming a diet containing 0.5% protein, 580 mg sodium/Kg diet and 2930 mg potassium/kg diet for 21 days (n=6).

Protein−energy malnutrition is associated with reduced body potassium (Golden & Jackson 1985).   Reduced total body potassium may be associated with the oedema of the kwashiorkor−like state of protein−energy−malnutrition (Anthony & Edozein 1975).   Thus, in an attempt to maintain the sodium and potassium intake of animals consuming low protein diets a further group was studied:

D) Low Protein, High Electrolyte (LPHE):
animals consuming a diet containing 0.5% protein, 4900 mg sodium/Kg diet and 4900 mg potassium/kg diet for 21 days (n=6);

Consumption of diets with high sodium and potassium content has previously been reported to result in the production of oedema in rats (Anthony & Edozein 1975).

The exact composition of the diet for the animals of the growth study was reported in Table 2.2.

All animals were maintained individually in a room maintained at 23°C with a 12 hour light−dark cycle.   Body weight and food intake was monitored at the same point in each light period.

On the day of study animals were anaesthetised using 0.44 mls Sagatal/kg body weight and prepared for measurement as described in Section 2..1. Assessment of contractile characteristics was conducted as reported in Section 2.2. Time to peak tension (TPT), half relaxation time (½RT) & peak twitch tension (PT) of the twitch and the maximal tetanic force (Fmax) & force frequency relationship were determined. Body temperature was measured as described in Section 2.1.2.

Following assessment of contractile characteristics the muscles of both hindlimbs were rapidly excised, weighed and stored as previously described (see Section 2.3) for determination muscle protein concentration (see Section 2.4). In addition serum albumin and zinc concentrations were determined (see Section 2.4), and full carcus analysis completed (see Section 2.3.1 & 2.4).

## 5.2 THE EFFECT OF CONSUMPTION OF LOW PROTEIN DIETS ON GROWTH AND DEVELOPMENT OF MALE WISTAR RATS.

This section will describe the influence of consumption of low protein diets on the amount of food consumed, body composition and condition of male wistar rats. Subsequent sections will describe the influence of these diets on skeletal muscle contractile characteristics.

### 5.2.1 Results.

#### 5.2.1.1 Food Intake.

The average daily food intake over the three week period is shown in Table 5.1. Animals consuming the low protein, low electrolyte diet exhibited a reduced food intake of 64% compared to animals consuming the 15% protein diet (FED, $p < 0.01$). Animals consuming the low protein, high electrolyte diet exhibited similar reductions in daily food intake (65%, $p < 0.01$). This resulted in a 66% and 67% lower energy intake per day in animals consuming either low protein, low electrolyte or low protein, high electrolyte diets respectively, compared to the FED group ($p < 0.01$). When expressed per unit body weight per day, animals in the low protein, low electrolyte and low protein, high electrolyte groups consumed more energy – but the difference in energy intake did not attain statistical significance ($p > 0.05$).

Protein intake of animals consuming the low protein, low electrolyte and low protein, high electrolyte diets was only 1.2% of that consumed by the FED animals ($p < 0.01$). Daily sodium and potassium intake in the low protein, low electrolyte animals was 36% of that of animals in the FED group ($p < 0.01$). In animals consuming the low protein, high electrolyte diet, however, sodium intake was three fold greater than that of FED animals ($p < 0.01$) while potassium intake

Table 5.1. Food consumption in rats fed diets containing 15% protein (FED) or diets low in protein (0.5%) containing high (LPHE) or low (LPLE) electrolyte contents. (n=6 in each group).

| | FED | | LPHE | | LPLE | |
|---|---|---|---|---|---|---|
| | mean | sem | mean | sem | mean | sem |
| Food Intake (g/d) | 17.6 | 0.5 | 6.3 ** | 0.7 | 6.4 ** | 0.5 |
| (g/g body weight/d) | 0.077 | 0.002 | 0.088 | 0.009 | 0.089 | 0.01 |
| Energy Intake (kJ/d) | 331.4 | 8.7 | 108.7 ** | 12.2 | 113.1 ** | 8.2 |
| (kJ/g body weight/d) | 1.44 | 0.04 | 1.52 | 0.16 | 1.58 | 0.18 |
| Protein Intake (g/d) | 2.64 | 0.07 | 0.032 ** | 0.04 | 0.032 ** | 0.02 |
| Sodium intake (mg/d) | 1.02 | 0.03 | 3.11 ** | 0.35 | 0.37 | 0.03 **$ |
| Potassium Intake (mg/d) | 5.16 | 0.14 | 3.11 * | 0.35 | 1.87 | 0.14 **$ |

KEY: * - p<0.05 v FED; ** - p<0.01 v FED; $ - p<0.05 v LPHE  by students t test.

was 60% that of the FED group (p<0.01). Thus animals consuming the low protein, high electrolyte diet consumed 8 times more sodium and 1.6 times more potassium per day than animals consuming the low protein, low electrolyte diet.

Animals of the low protein, low electrolyte group consumed less energy, protein, sodium and potassium than the animals in the FED group while the animals consuming the low protein, high electrolyte diet consumed less energy, protein and potassium but more sodium than the FED animals. Despite differences in the sodium and potassium content of the diets, the protein and energy consumption of animals on both low protein diets was similar. Despite the potassium consumption of the low protein, high electrolyte group being greater than that observed in the low protein, low electrolyte group it did not reach the levels consumed by the animals of the FED group.

5.2.1.2 <u>Body weight and appearance.</u>

Table 5.2 shows the mean body weight of the animals in this study. Animals in the FED group (15% protein, *ad libatum*) exhibited a linear growth pattern resulting in a 2.3 fold increase in weight over the 21 day period (p<0.01, Table 5.2 & Figure 5.1). These animals also exhibited a 1.4 fold increase in snout−to−anus length resulting in a body mass index which was approximately 1.3 times greater than the animals at the commencement of the study (CONTROL, p<0.01, Table 5.2). In contrast, animals consuming the low protein, low electrolyte diet exhibited a 29% reduction in body weight (p<0.01) without any change in snout−to−anus length, resulting in a 24% reduction in body mass index (p<0.01). Animals consuming the low protein, high electrolyte diet similarly exhibited a 30% lower body weight (p<0.01), no change in snout−to−anus length and a 26% reduction in body mass index (p<0.01).

Carcus energy of the FED group was 3 times greater than that of CONTROL animals (p<0.05, Table 5.2). The carcus energy of the animals in the low protein, low electrolyte group and low protein, high electrolyte group was 21% and 35% lower than that of CONTROL animals respectively (p<0.05, Table 5.2). Carcus energy of the low protein, high electrolyte animals was 18% lower than that of the animals in the low protein, low electrolyte group. Since body weight changes of the two groups compared to the CONTROL animals were of similar magnitudes this may be indicative of greater changes in the protein and fat content of the animals in the high electrolyte group than that seen in the animals consuming the low protein, low electrolyte diet.

Hair loss was apparent in animals consuming both low protein, low electrolyte and low protein, high electrolyte diets, as was the production of "red tears". Visible oedema was not present in any of the animals studied.
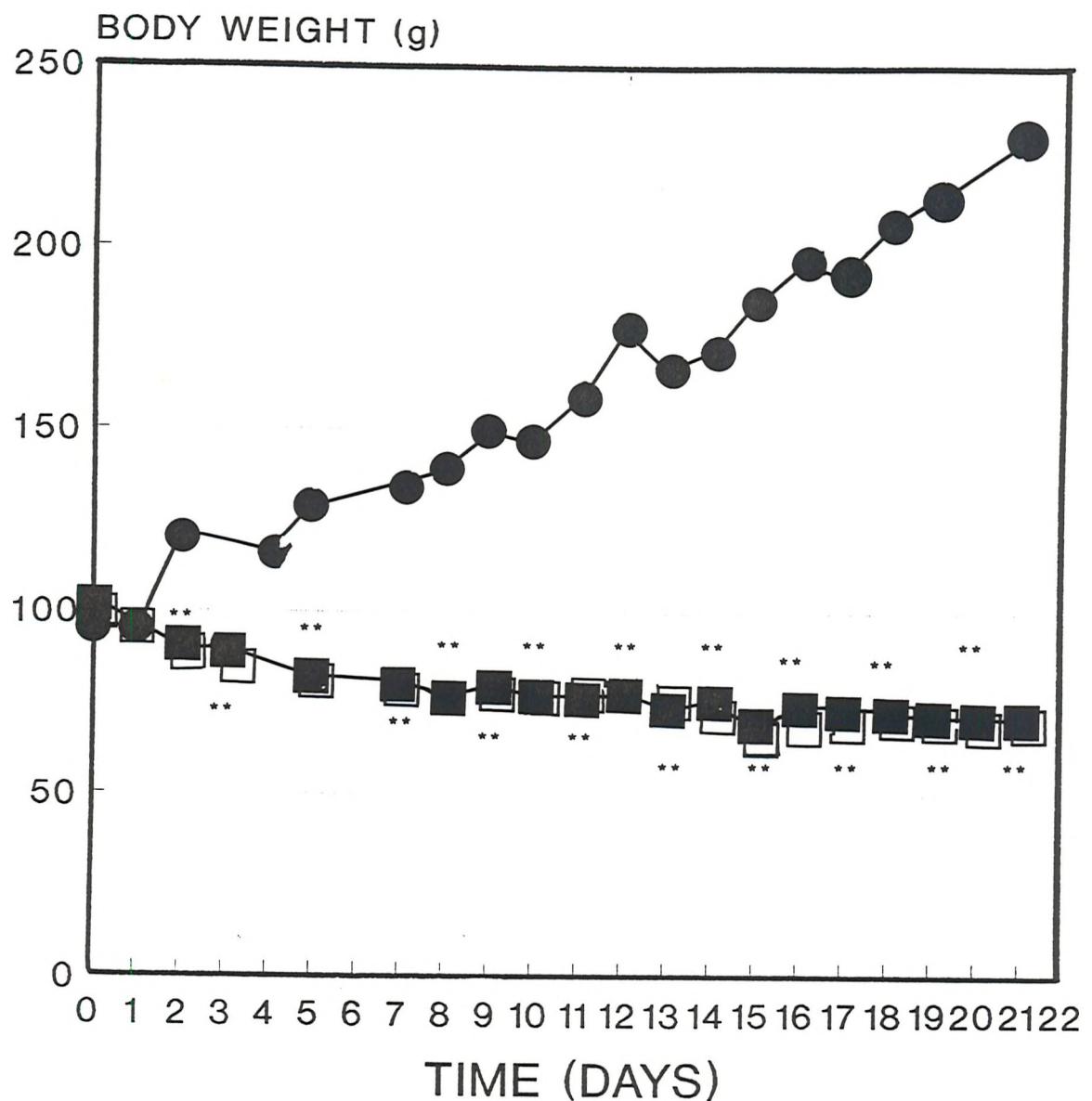
Figure 5.1. Diagram to show the growth of animals consuming (i) a normal protein containing diet (FED, ●, n=6) and (ii) a low protein diet (0.5%, LPLE,■,n=6; LPHE,□, n=6). Points represent mean values, sems within size of symbols. ** − p<0.01 v FED.

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Table 5.2. Body composition of rats studied at the commencement of the investigation (CONTROL) or following consumption of diets containing 15% protein (FED) or 0.5% protein diets containing high (LPHE) or low (LPLE) concentrations of electrolytes (n=6 in each group).

| | CONTROL | | FED | | LPHE | | LPLE | |
|---|---|---|---|---|---|---|---|---|
| | mean | sem | mean | sem | mean | sem | mean | sem |
| Body Weight (g) | 102.0 | 3.3 | 230.0 | 5.6 * | 71.7 | 0.9 **$$ | 73.0 | 2.9 **$$ |
| Snout to Anus Length (mm) [†] | 149.3 | 0.2 | 200.5 | 2.9 ** | 145.7 | 1.5 $$ | 144.8 | 1.2 **$$ |
| Body Mass Index | 45.7 | 0.3 | 57.3 | 1.4 * | 33.7 | 0.8 **$$ | 34.7 | 1.0 **$$ |
| Carcus Energy (MJ) | 1.61 | 0.14 | 4.82 | 0.20 * | 1.04 | 0.02 *$ | 1.27 | 0.11 *$ |
| Rectal Temperature | 36.4 | 0.5 | 37.9 | 0.3 | 34.7 | 0.7 *$$ | 35.5 | 0.7 $ |

KEY: $\dagger$ - Body mass index = body weight/ (snout-to -anus length)$^2$;
* - p<0.05, ** - p<0.01 v CONTROL; $ - p<0.05 v FED; $$ - p<0.01 v FED by students t-test.

Thus, animals consuming the low protein, low electrolyte diet were stunted and wasted compared to age-matched animals (FED group) and wasted compared to CONTROL animals. Animals consuming the low protein, high electrolyte diet were similarly stunted and wasted compared to the FED group and wasted compared to CONTROL animals, but did not appear to differ from the animals consuming the low protein, low electrolyte group.

### 5.2.1.3 Body temperature.

Body temperature of the animals in the low protein, low electrolyte group was 2.5% lower than that of CONTROL animals ($p > 0.05$, Table 5.2), while that of animals in the low protein, high electrolyte group was 4.7% lower ($p < 0.01$, Table 5.2). The body temperature of FED animals was 4.3% higher than that of CONTROL animals ($p < 0.05$, Table 5.2). Thus, both groups of animals consuming low protein diets exhibited body temperatures significantly lower than FED animals (6-8%, $p < 0.01$, Table 5.2).

### 5.2.1.4 Skeletal muscle.

Muscle mass and protein content of the soleus, EDL and TA muscles of CONTROL, FED and animals consuming low protein diets are presented in Table 5.3.

Growth in the FED animals was apparent as a raised muscle mass: 2.6 fold in the soleus muscle ($p < 0.01$), 2.0 fold in the EDL and TA muscles ($p < 0.01$) compared to the muscles of CONTROL animals. Total muscle protein was more than 2 fold greater in all three muscles of the FED group compared to CONTROLs ($p < 0.01$, Table 5.3).

Animals consuming the low protein, low electrolyte diet exhibited a 15% lower soleus muscle weight ($p < 0.01$), 29% lower EDL weight ($p < 0.01$) and a 25% lower TA muscle weight ($p < 0.01$) compared to the muscles of CONTROL animals. This resulted in a lower total muscle protein content of 36% in the soleus muscle ($p > 0.05$), 34% in the EDL muscle ($p > 0.05$) and 32% in the TA muscle ($p > 0.05$) compared to CONTROL animals.

Animals consuming the low protein, high electrolyte diet exhibited similar muscle mass to those animals consuming the low protein, low electrolyte diet, being 26% lighter than the soleus and EDL muscles of CONTROL animals and 26% lighter than the TA muscle of CONTROL animals ($p < 0.01$). The protein content of these muscles for animals of the low protein, high electrolyte group were 3% lower for the soleus muscle ($p > 0.01$), 37% lower in the EDL ($p > 0.05$) and 14% lower in the TA muscle ($p > 0.05$) than CONTROL animals.

Table 5.3. Organ weight and protein content of rats studied at the commencement fo the investigation (CONTROL), or in rats consuming normal (15% protein, FED) diets or low protein diets containing high (LPHE) or low (LPLE) electrolyte content (n=6 in each group)

| | CONTROL | | FED | | LPHE | | LPLE | |
|---|---|---|---|---|---|---|---|---|
| | mean | sem | mean | sem | mean | sem | mean | sem |
| Soleus muscle Weight (mg) | 44.4 | 3.7 | 117.4** | 7.7 | 32.8 | 1.9**$$ | 37.7 | 0.4**$$ |
| (mg/100g body weight) | 43.3 | 28.8 | 51.0 | 2.1** | 46.2 | 2.7**$$ | 51.2 | 3.8**$$ |
| EDL muscle Weight (mg) | 49.7 | 5.3 | 100.6 | 4.9 | 36.6 | 2.7 | 35.4 | 2.8 |
| (mg/100g body weight) | 48.2 | 3.5 | 43.5 | 2.1** | 53.8 | 3.2**$$ | 48.6 | 3.0**$$ |
| TA muscle weight (mg) | 215.6 | 12.4 | 437.4 | 14.9* | 153.9 | 9.0$ | 160.9 | 6.0$ |
| (mg/100g body weight) | 210.6 | 5.8 | 191.7 | 6.0** | 218.1 | 7.0*$ | 220.9 | 10.7*$ |
| Liver Weight (g) | 3.9 | 0.2 | 9.9 | 0.3* | 2.5 | 0.06*$ | 2.8 | 0.15$ |
| (g/100g body weight) | 3.7 | 0.1 | 4.3 | 0.1 | 3.8 | 0.8 | 3.5 | 0.9$ |
| TOTAL PROTEIN CONTENT: | | | | | | | | |
| Liver (g) | 0.60 | 0.05 | 1.8 | 0.13** | 0.38 | 0.02$ | 0.57 | 0.04$ |
| Soleus (mg) | 10.0 | 1.0 | 21.8 | 3.9** | 9.7 | 1.2$$ | 6.4 | 1.0$$ |
| EDL (mg) | 11.0 | 2.0 | 25.1 | 3.3** | 6.9 | 1.2$$ | 7.2 | 1.3$$ |
| TA (mg) | 50.0 | 9.0 | 137.8 | 2.8 | 42.8 | 8.2$$ | 33.8 | 1.7$$ |

KEY: * - p<0.05, ** - p<0.01 v CONTROL; $ - p<0.05, $$ - p<0.01 v FED by students t-test.

Despite these differences in muscle mass following consumption of 15% protein diets or consumption of low protein diets no difference was apparent in the relative weight of the muscles (i.e. per unit body weight, Table 5.3).

Thus animals consuming either low protein, low electrolyte or low protein, high electrolyte diets appear to exhibit muscle atrophy. This results in a lower skeletal muscle mass than age–matched animals (FED) and was generally reflected by a lower protein content of the muscles. However, since the soleus muscle of the animals consuming the low protein, high electrolyte diet contained less protein than that of animals consuming low protein, low electrolyte diets it is unclear as to which components of skeletal muscle are lost or whether the magnitude of loss is similar for both situations.

5.2.1.5 Liver.

Liver weight of the FED group was approximately 2.5 fold greater than that of the CONTROL animals, consistent with the 2–2.5 fold increase in body weight and muscle weight described above (Table 5.3, $p < 0.01$). The protein concentration of the liver was not discernibly different from CONTROL values (data not shown), but total liver protein content was raised 3 fold (Table 5.3, $p < 0.01$). In contrast, the liver of animals consuming the low protein, low electrolyte diet was 28% lighter than that of CONTROL animals ($p < 0.05$) which was associated with a 5% reduction in total liver protein content ($p < 0.05$). Liver weight of animals consuming the low protein, high electrolyte diet was 36% lighter than that of CONTROL animals ($p < 0.05$), with a total protein content 37% lower than livers of CONTROL animals ($p > 0.05$).

Liver zinc concentration was not discernibly different in all animals studied, but total liver zinc was two fold greater in the FED animals compared to the CONTROLS (Table 5.4, $p < 0.01$), 53% lower in animals consuming the low protein, low electrolyte diet and 49% lower in animals consuming the low protein, high electrolyte diet ($p < 0.01$, Table 5.4).

Liver lipid concentration was not discernibly different from CONTROL in the FED and low protein, low electrolyte groups but was 45% greater in the low protein, high electrolyte group (Table 5.4, $p < 0.05$). Since liver weight was greater in the FED animals and lower in the animals consuming either of the low protein diets, total liver lipid was 2.5 times higher in the FED animals (v CONTROL, $p < 0.01$), 31% lower in the low protein, low electrolyte animals ($p < 0.05$) and 14% lower in animals consuming the low protein, high electrolyte diet ($p > 0.05$). Thus while the liver lipid content of the low protein groups were not discernibly different, a tendency towards greater liver lipid content in the high electrolyte group was evident.

Table 5.4. Zinc, lipid and albumin concentrations of animals at the commencement of study (CONTROL) and following consumption of 15% protein diets (FED) or diets of low protein (0.5%) containing high (LPHE) or low (LPLE) electrolyte content (n=6 in each group).

| | CONTROL | | FED | | LPHE | | LPLE | |
|---|---|---|---|---|---|---|---|---|
| | mean | sem | mean | sem | mean | sem | mean | sem |
| Liver Zinc Concentration ($\mu$g/g) | 4.05 | 0.51 | 3.14 | 0.22** | 3.27 | 0.31**$$ | 2.68 | 0.32**$$ |
| Total Liver Zinc Content ($\mu$g) | 15.6 | 2.4 | 31.5 | 2.9 | 7.9 | 0.8 | 7.4 | 0.9 |
| Liver Lipid Concentration (mg/g) | 179.3 | 30.5 | 166.0 | 33.7 | 259.6 | 41.8*$ | 156.9 | 14.7*$ |
| Total Liver Lipid (g) | 0.65 | 0.16 | 1.61 | 0.29** | 0.56 | 0.08**$ | 0.45 | 0.06*$ |
| Serum Albumin Concentration (mg/ml) | 35.5 | 2.2 | 33.7 | 3.8 | 24.9 | 2.6**$$ | 22.2 | 0.9**$$ |
| Serum Zinc Concentration ($\mu$g/ml) | 1.7 | 0.24 | 2.1 | 0.20 | 1.8 | 0.45 | 1.2 | 0.25 |

KEY: * - $p<0.05$, ** - $p<0.01$ v CONTROL; $ - $p<0.05$, $$ - $p<0.01$ v FED by students t-test.

### 5.2.1.6 <u>Other biochemical variables.</u>

Serum albumin concentration was unaltered following 21 days feeding the 15% protein diet (i.e. the FED group) but was approximately 33 and 38% lower in the animals consuming the low protein, low electrolyte and low protein, high electrolyte diets when compared to animals of the CONTROL and FED groups (p<0.01, Table 5.4).

Serum zinc concentration was 24% greater in the FED group compared to CONTROL animals, 29% lower in animals of the low protein, low electrolyte group and unaltered in animals consuming the low protein, high electrolyte diet. These differences did not attain statistical significance (Table 5.4).

### 5.2.2 <u>DISCUSSION.</u>

The aim of the present study was to induce severe stunting and wasting in an animal model of malnutrition due to limitations in the composition of the diet.

The animals fed low protein diets in this study were clearly stunted and wasted. This appears to be the result of a reduction in both protein and energy intake. Muscle and liver weight were similarly reduced in the animals consuming the low protein diets. The animals of the FED group, however, exhibited normal growth over the 21 day period resulting in a 2– 2.5 fold increase in body and organ weight and a 1.5 fold increase in length.

### 5.2.2.1 <u>Food Intake.</u>

Alterations in the dietary composition of the food presented is associated with reduced food consumption (Pi–Sunyer 1990), in particular this has been seen for low protein diets. The present study observed a 64% reduction in food intake over the period of investigation. This is greater than reported in similar studies conducted by other workers. For example, Anthony & Edozein (1975) reported the normal increase in food intake of male Sprague–Dawley rats consuming their normal (18% protein) diets of 14 g/d increasing to 23 g/d over a period of 9 weeks. Over the same period of time rats fed diets containing 3% protein consumed 12.5 g/d falling to 11.5 g/d. Food consumption in rats consuming a 0.5% protein diet fell from 8.6 to 6.9 g/d by 9 weeks (Anthony & Edozein 1975). Heard and colleagues have reported a 50% reduction in food intake of rats fed a low protein diet (8.3 g/d of a 4% casein diet; Heard *et al* 1977). This greater reduction in food intake could result from the diet being unpalatable. This however, is considered unlikely since the animals consuming the 15% protein diet did not alter their daily food intake and grew normally. This reduced food intake in the animals consuming

the low protein diets therefore would appear to be an adaptation to the protein or energy content of the diet in relation to body requirements.

Despite a reduced energy intake in the animals consuming low protein diets, the energy intake per unit body weight was similar to that of FED animals. This has previously been reported by ANthony & Edozein (1975). In the present study, the animals would be expected to be consuming an amount of energy near to that needed for maintenance requirements. Grande and colleagues have estimated that the basal metabolic rate may be reduced up to 15% following adaptation to starvation (Grande, Anderson & Keys 1958). Animals in this study were consuming 1.5 kJ/g/day in the FED and low protein groups by the end of the 21 day period of study (1.44 $\pm$ 0.04 kJ/g/day FED; 1.52 $\pm$ 0.16 kJ/g/day low protein, high electrolyte; 1.58 $\pm$ 0.18 kJ/g/day low protein, low electrolyte). Clearly, this is not an energy intake equivalent to the maintenance requirements of the animals in this study since they are still losing small amounts of body weight and the animals are still active. Similarly, Duffy and colleagues have reported that calorie restricted animals spent greater periods of time eating per meal than *ad libatum* fed rats and exhibited higher motor activity scores than the *ad libatum* rats (Duffy, Feuers, Leakey, Nakamura, Turturro & Hart 1989). Some degree of reduction in energy requirements can be determined, however, from the lower rectal temperatures recorded in the protein deficient animals of this study and that reported by Duffy and colleagues (Duffy *et al* 1989).

It is unclear from this study which component of the diet was limiting. Obviously, the energy intake was reduced and protein intake was negligible. But few diets have been shown to be limiting in protein before being deficient in energy (Waterlow 1986; Jackson 1990). Consumption of a low protein diet with normal electrolyte concentrations results in a reduction in both sodium and potassium intake. In an attempt to induce oedema in the rat model of malnourishment, Anthony & Edozein (1975) increased the electrolyte content of the diets fed to rats. In the present study a similar raised electrolyte content resulted in a significant elevation of sodium intake but reduction in potassium intake per day of the low protein, high electrolyte animals compared to CONTROL animals.

Potassium deficiency has been shown to result in significant and immediate cessation of growth of the rat (Dorup & Clausen 1989). Whether the levels of potassium intake in the low protein groups of this study were at a level significantly low to reproduce this potassium deficiency is unclear. The development of oedema in rats on these diets would therefore appear to be the consequence of impaired renal handling of sodium, although renal functional studies were not carried out in these experiments. No data is available to indicate if such a mechanism is partially responsible for the development of oedema in the clinical situation.

In the case of the animals consuming the low protein, low electrolyte diet the reduction in potassium intake was twice that of the animals consuming the low protein, high electrolyte diet, yet this did not appear to result in greater reductions in body weight. Thus either the growth of the animals is already limited by the 35% reduction in potassium intake of the low protein, high electrolyte animals and further reduction of potassium intake has little effect on growth or, more likely, there is an interrelated deficiency of more than one element of the diet resulting in impaired handling of nutrient intake.

## 5.2.2.2 Body weight.

The loss of body weight of the animals consuming the low protein diets was most rapid over the first three days of the study. This pattern of weight loss was similar to that seen in rats on an intake of food 25% that of their normal intake (see Section 4.2). Following this period of three days the reduction in body mass began to slow with indications of a plateau being attained (see Figure 5.1). This level of equilibration was at a higher weight in those animals of the food restriction study (Chapter 4.2) than the animals consuming low protein diets (Figure 5.1). This would be consistent with a greater reduction in food intake and hence energy in the protein deficient animals. This pattern of weight loss followed by a period of maintenance of body weight has previously been reported. Thus, rats fed a 3% lactalbumin diet initially lost weight but returned to their starting weight within 5 weeks of consuming the low protein diet (Anthony & Edozein 1975). Those consuming 2% protein diets lost over 18 g in one week and then maintained their body weight while those consuming 0.5% lactalbumin diets showed a progressive weight loss for the continuation of the study (Anthony & Edozein 1975). Thus in the present study it would be expected that the period of body weight stability had not yet been reached and that continued loss of body mass would have occurred had the experiment continued.

Snout−to−anus length of the animals in the food restricted study was not recorded so it is difficult to compare the lack of growth in this manner. Stunting in the animals of the protein deficient groups was observed. Thus animals consuming the low protein, low electrolyte and low protein, high electrolyte diets exhibited the same snout−to−anus length as CONTROL

animals, while the FED group increased in length.

Hair loss in animals on low protein diets has previously been described (Edozein 1968; Anthony & Edozein 1975; Ausman *et al* 1989; Roussow 1989) and is likely to represent a reduction in the maintenance of hair protein turnover. The "red tears" reported in animals of both the low protein groups is the consequence of the chromodacryorrhea eminating from the Haderian gland, closely associated with the lacrymal apparatus (Anthony & Edozein 1975). The phenomenon of red tear secretion occurs in rats that have been subjected to a variety of forms of stress (Collins 1957) and is not thought to be part of the specific response to low protein diets.

Oedema, one of the signs of kwashiorkor was not observed during the present investigation. It has been reported in rats fed low protein diets for periods greater than 3 months (Anthony & Edozein 1975), pigs fed wheat gluten diets for 14 weeks (Lowrey *et al* 1962), squirell monkeys fed low protein diets for 6 weeks (Ausman *et al* 1989) and a variety of studies of children summarised by Roussow (1989).

It would therefore appear that the production of oedematous malnutrition may require a longer period of development than seen in the present study when consuming a low protein diet. Alternatively, the diet which was consumed was of an incorrect composition to produce oedematous malnutrition, that the sodium and potassium content of the diet is not related to the development of oedema or a combination of these possibilities.

The present investigation therefore did not reproduce a kwashiorkor like state but this is likely to be due to the shortened period over which the study was conducted in comparison to these other studies. However, in discussion with the Home Office it was concluded that it was unreasonable to allow greater loss of body weight than that observed following 21 days consumption of the low protein diets used in this study.

5.2.2.3 Skeletal muscle mass.

The loss of skeletal muscle tissue in animals consuming the low protein diets was of a similar order of magnitude in the soleus muscle as that seen following chronic food restriction (Chapter 4.2). In contrast to the situation of chronic food restriction, however, where the EDL and TA muscles exhibited a 6% and 15% growth respectively, muscles of the animals consuming either low protein diets were lighter by 29% and 25% respectively. The lower skeletal muscle mass of the animals food deprived and restricted was attributed to lack of growth, in this study of protein deprivation, the greater loss of muscle tissue is likely to reflect both a lack of growth and a net degradation of muscle tissue. As the protein concentration of the muscles studied did not

change it is likely to reflect a general loss of muscle protein rather than a specific component of the muscle. McCance & Widdowdson (1956) investigated the composition of rat skeletal muscle following consumption of a casava flour–based diet. In this study, they were unable to detect changes in either muscle composition or protein concentration which would agree with the above suggestion. However, pigs fed a wheat gluten diet exhibited atrophy of skeletal muscle with partial or complete loss of cross striations (Lowrey *et al* 1962). Considerable inconsistencies therefore appear to exist between studies which may be related to the diet composition, species under investigation and muscles examined.

### 5.2.2.4 Liver weight.

Greater liver weight during growth in the FED group, as with other organ weights , has resulted in a 2.5 fold heavier liver than at the commencement of study (v CONTROL). This increase in weight therefore parallels that of the body generally, representing normal growth over the 21 days of study.

The liver weight of the animals consuming the low protein diets was significantly lower than at the commencement of the study (v CONTROL) and, although the loss of liver tissue is slightly greater than that of the muscles studied, reflects the general loss of body mass. Such reports are in agreement with studies of rats fed casava flour–based diets (McCance & Widdowson 1956), pigs fed wheat gluten diets (Lowrey *et al* 1962) and rats fed a 0.5% lactalbumin diet (Anthony & Edozein 1975). This loss of liver mass was not different to CONTROL animals when expressed per unit body weight and it paralleled the change in body weight as reported for muscle (see Section 5.2.2.2). This is in agreement with the study of McCance & Widdowson where liver weight was reported to occupy that mass expected for weight–matched animals (McCance & Widdowson 1957). This also agrees with the studies of Lowrey *et al* (1962) and Anthony & Edozein (1975) previously described.

The liver weight of the animals consuming the low protein, high electrolyte diet was greater than those of the low protein, low electrolyte group and may reflect the tendency towards lipid accumulation, as would be expected from the study of Lowrey *et al* (1962) and Anthony & Edozein (1975). The liver lipid content of the low protein, high electrolyte group was reduced to a lesser extent than that of the low electrolyte group. The difference in the lipid content did not however attain statistical significance. Fatty liver may have developed if the experiment had been allowed to continue for longer.

5.2.2.5 <u>Other biochemical variables.</u>

In the present study, serum albumin concentration was approximately 30% lower in the protein deficient animals compared to both the CONTROL and FED animals. Reduced circulating serum albumin is a characteristic feature of malnutrition and has been reported in both man and in animal models (Dean & Schwartz 1959; Baptist, deSilva & Sideek 1959; Lowrey *et al* 1962; Edozein 1968; Anthony & Edozein 1975; Heard *et al* 1977; Ausman *et al* 1989; Roussow *et al* 1989). Thus, the animals consuming low protein diets exhibited another characteristic of malnutrition.

5.2.3. <u>Summary.</u>

In summary therefore, the animals fed low protein diets in the present study exhibited clear indications of malnutrition with reduced body and organ weight, stunting and reduced serum albumin concentrations. The inclusion of supplementary electrolytes in the low protein, high electrolyte diet did not result in exacerbation of changes in body or organ weight, snout-to-anus length or other variables measured which were associated with malnutrition.

5.3. <u>THE INFLUENCE OF THE CONSUMPTION OF LOW PROTEIN DIETS ON THE *IN SITU* CONTRACTILE CHARACTERISTICS OF RAT SKELETAL MUSCLE.</u>

To date, no investigation of the changes in the contractile characteristics of muscle of individuals or animals consuming diets deficient in protein has been conducted. The malnourishment described in Chapter 5.2 resulting from the consumption of low protein diets is clearly different from that described following acute or chronic limitations in food intake (Chapter 4). This section will describe the contractile characteristics of rat skeletal muscles following the consumption of low protein diets. Firstly, an assessment of the influence of normal growth and skeletal muscle contractile characteristics will be discussed in relation to those animals consuming the 15% protein diet. Secondly, the influence of the protein deficient diets on skeletal muscle contractile characteristics will be described. Finally, a comparison will be made between those animals growing normally (the FED group) and those restricted in protein intake.

5.3.1. <u>Results.</u>

5.3.1.1 <u>Skeletal muscle function following growth and the consumption of diets of normal protein content.</u>

The contractile characteristics of skeletal muscles of animals of the FED group compared to CONTROL animals at the commencement of the study are presented in Tables 5.5. and 5.6 (i.e. FED v CONTROL).

5.3.1.1.i <u>Twitch Characteristics.</u>

Peak twitch tension was 111% greater in the soleus muscle of FED animals compared to the CONTROL animals ($p < 0.05$), that of the TA muscle 171% greater ($p < 0.05$) and that of the EDL muscle 29% lower ($p > 0.05$). This difference in force generation was consistent with the muscle mass in the soleus and TA, but not the EDL, previously described (Table 5.3), hence the peak twitch tension per unit muscle was not different from that of CONTROL animals (Table 5.5).

Time to peak tension and half relaxation time were unaltered in the muscles of animals in the FED group compared to CONTROL animals, except for the TA muscle where time to peak tension was 23% faster ($p < 0.05$).

5.3.1.1.ii <u>Tetanic Responses.</u>

Maximal force generation in Newtons was 2.9 times greater in the soleus muscle of FED animals compared to CONTROL muscles ($p < 0.05$), 32% greater in the EDL muscle ($p > 0.05$) and 98% greater in the TA muscle ($p < 0.05$). When expressed per unit muscle weight, however, there was a 50% greater relative force generation in the soleus muscle of FED animals compared to CONTROL muscles ($p > 0.05$), a 80% increase in the TA muscle ($p < 0.05$) and a 32% greater relative force generation in the EDL muscle ($P > 0.05$, Table 5.6).

The force−frequency relationship of the soleus, EDL and TA muscles of animals in the FED group was not different to that of CONTROL muscles ($p > 0.05$, Figure 5.2).

No difference in the fatigue susceptibility of muscles of the FED group was observed compared to the muscles of CONTROL animals ($p > 0.05$, Table 5.6, Figure 5.3).

Table 5.5. The effect of low protein diets on the twitch characteristics of the soleus, extensor digitorum longus (EDL) and tibialis anterior (TA) muscles of rats at the commencement of study (CONTROL), in rats fed a normal, 15% protein diet (FED), in rats consuming a low protein, low electrolyte diet (LPLE) and in rats fed a low protein, high electrolyte diet (LPHE) for 21 days (n= 6 in each group).

| | | CONTROL | | FED | | LPLE | | LPHE | |
|---|---|---|---|---|---|---|---|---|---|
| | | mean | sem | mean | sem | mean | sem | mean | sem |
| **SOLEUS** | | | | | | | | | |
| TPT | (ms) | 31.0 | 1.4 | 32.2 | 1.9 | 46.6 | 5.2 *$ | 40.1 | 2.1 *$ |
| 1/2RT | (ms) | 32.2 | 2.1 | 33.8 | 5.5 * | 46.8 | 3.3 $ | 44.8 | 6.9 $ |
| PT | (mN) | 0.9 | 0.07 | 1.9 | 0.4 | 1.0 | 0.2 $ | 0.9 | 0.2 $ |
| PT (mN/g muscle) | | 20.7 | 2.8 | 15.9 | 3.4 | 25.1 | 7.0 | 26.0 | 6.7 |
| **EDL** | | | | | | | | | |
| TPT | (ms) | 15.9 | 0.4 | 15.6 | 0.9 | 15.8 | 0.5 * | 16.8 | 0.42 |
| 1/2RT | (ms) | 13.5 | 0.9 | 12.7 | 1.6 | 10.4 | 0.3 | 12.9 | 0.9 |
| PT | (mN) | 2.8 | 0.5 | 2.0 | 0.5 | 2.6 | 0.4 *$$ | 2.4 | 0.4 *$$ |
| PT (mN/g muscle) | | 36.6 | 6.7 | 24.7 | 6.7 | 72.4 | 8.5 | 75.5 | 11.2 |
| **TA** | | | | | | | | | |
| TPT | (ms) | 16.5 | 1.2 | 12.7 | 1.0 * | 14.3 | 0.5 | 15.9 | 2.2 |
| 1/2RT | (ms) | 11.7 | 0.8 | 10.9 | 1.3 * | 12.7 | 0.6 $$ | 13.2 | 2.3 *$ |
| PT | (mN) | 4.8 | 0.9 | 13.0 | 1.0 * | 6.5 | 0.5 *$ | 7.7 | 1.0 *$ |
| PT (mN/g muscle) | | 14.3 | 3.7 | 20.5 | 4.2 | 35.1 | 4.5 | 44.3 | 4.3 |

KEY: * - P<0.05 v CONTROL; $ - P<0.05, $$ - p<0.01 v FED by students t- test.

Table 5.6. Maximal tetanic tension and fatiguability of the rat soleus, extensor digitorum longus (EDL) and tibialis anterior (TA) muscles of rats at the commencement of study (CONTROL), in rats fed a normal, 15% protein diet (fed), in rats consuming a low protein, low electrolyte diet (LPLE) (n=6) and in rats fed a low protein, high electrolyte diet (LPHE) for 21 days. (n=6 for each group).

| | CONTROL | | FED | | LPLE | | LPHE | |
|---|---|---|---|---|---|---|---|---|
| | mean | sem | mean | sem | mean | sem | mean | sem |
| **SOLEUS** | | | | | | | | |
| Fmax (mN) | 3.9 | 0.4 | 11.2** | 1.2 | 5.0 | 0.4 $$ | 5.6 | 1.4 $ |
| Fmax (mN/g muscle)| | 47.8 | 5.5 | 71.9 | 18.0 | 139.4 | 4.7 *$ | 164.9 | 42.3 *$ |
| Fatigue Index | 0.78 | 0.02 | 0.73 | 0.09 | 0.78 | 0.06 | 0.73 | 0.10 |
| **EDL** | | | | | | | | |
| Fmax (mN) | 11.2 | 2.7 | 14.8 | 4.3 | 8.2 | 1.6 | 8.1 | 1.1 |
| Fmax (mN/g muscle)| | 138.6 | 28.7 | 171.6 | 40.6 | 233.7 | 37.9 | 218.6 | 33.3 |
| Fatigue Index | 0.41 | 0.05 | 0.44 | 0.10 | 0.22 | 0.09 | 0.31 | 0.08 |
| **TA** | | | | | | | | |
| Fmax (mN) | 20.4 | 3.8 | 40.3* | 7.5* | 21.8 | 3.3 $ | 18.9 | 2.8 $ |
| Fmax (mN/g muscle)| | 60.5 | 10.9 | 108.8 | 6.7 | 117.2 | 23.7 | 106.6 | 1.8 *$ |
| Fatigue Index | 0.75 | 0.04 | 0.80 | 0.08 | 0.60 | 0.14 | 0.42 | 0.14 |

| - Fatigue Index is the ratio of force generated at 2 minutes to that at 0 time.

* - $p < 0.05$, ** - $p < 0.01$ v CONTROL; $ - $p < 0.05$, $$ - $p < 0.01$ v FED by students t-test.
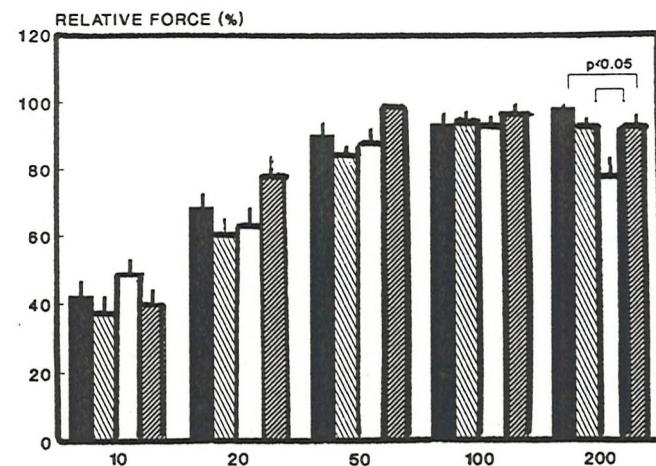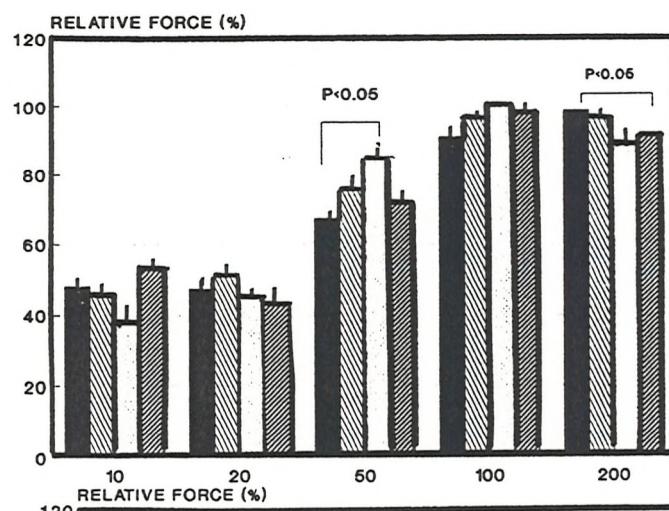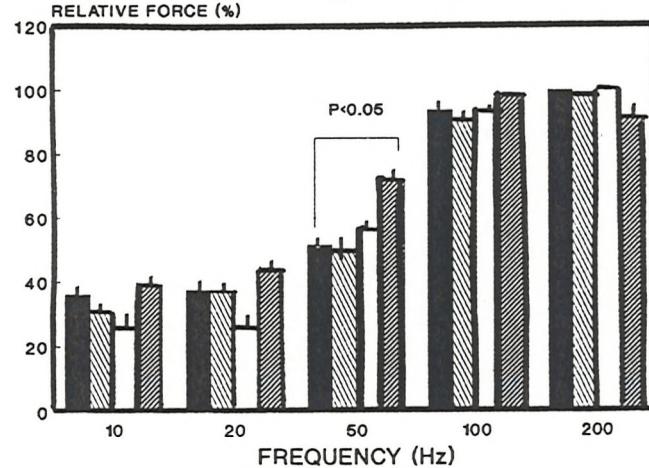
Figure 5.2. The force–frequency relationship of the soleus (A), extensor digitorum longus (EDL, B) and tibialis anterior (TA, C) of CONTROL animals (■, n=6), and in animals following consumption of a normal 15% protein diet (◪, n=6) or following consumption of a low protein, low electrolyte (◨, n=6) or low protein, high electrolyte (▯, n=6) diet for 21 days. (Bars represent mean ± sem).
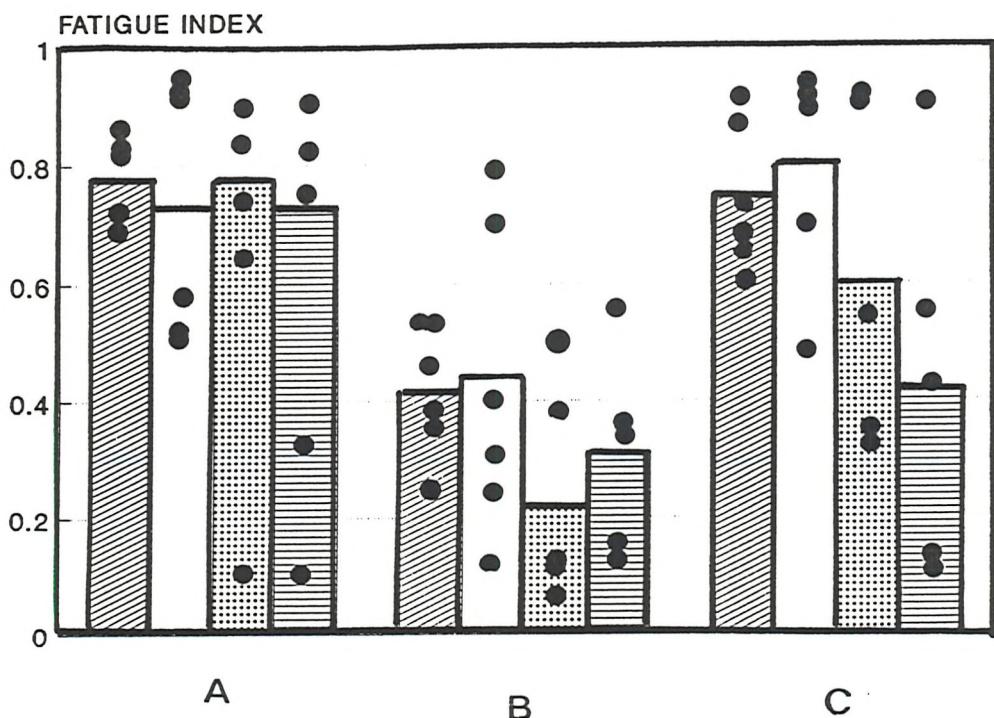
Figure 5.3. The fatigue characteristics of the soleus (a), extensor digitorum longus (b) and tibialis anterior (c) muscles of CONTROL male wistar rats (▨ ) and following consumption of a normal protein containing diet (FED, ☐ ), consumption of a low protein, low electrolyte diet (LPLE, ▦ ) and a low protein, high electrolyte diet (LPHE, ▤ ). (n= 6 except soleus of LPLE n=5, EDL of LPHE n=5 and TA of LPHE n=4 – see text. Bars represent the mean value of the group).

5.3.1.2 <u>Skeletal muscle function following consumption of low protein diets.</u>

The contractile characteristics of animals consuming the low protein, low electrolyte and low protein, high electrolyte diets compared to CONTROL animals are presented in Tables 5.5 and 5.6 (i.e. LPLE and LPHE v CONTROL). The differences between the low protein, low electrolyte and low protein, high electrolyte groups did not attain statistical significance (p>0.05, Table 5.5).

The soleus muscle of one animal consuming the low protein, low electrolyte diet exhibited an inappropriate low force generation during assessment of fatigue and was not included in the analysis of fatigue index (i.e. n=5). In the low protein, high electrolyte group the EDL of one animal did not generate force during assessment of fatigue despite allowing longer rest periods and was not included in the analysis of fatigue. In addition two animals in the low protein, high electrolyte group died during assessment of TA fatigue index and hence n=4 for the TA of this group.

5.3.1.2.i <u>Twitch characteristics.</u>

Peak tension in Newtons of the soleus and EDL muscles was not different in animals consuming low protein, low electrolyte diets from muscles of CONTROL animals (p>0.05, Table 5.5). Peak twitch tension in the TA muscle of animals consuming low protein, low electrolyte diets was 35% greater than that of CONTROL muscles (p>0.05). Skeletal muscle mass was lighter in animals consuming the low protein, low electrolyte diet therefore force generation per unit muscle was not different to muscles of CONTROL animals in the soleus muscle (p>0.05), but raised in the EDL (98%, p<0.05, Table 5.5) and TA (146%, p<0.05, Table 5.5) muscles.

Animals consuming the low protein, high electrolyte diet exhibited a peak twitch tension which was 60% greater in the TA muscle (p<0.05) but was unchanged in the soleus and EDL muscles compared to muscles of CONTROL animals (p>0.05, Table 5.5). When expressed per unit muscle weight, however, the relative force generation was 26% greater in the soleus muscle (p>0.05), 106% greater in the EDL muscle (p<0.05) and 210% greater in the TA muscle (p<0.05) than that measured in the muscles of CONTROL animals.

Thus, despite a loss of skeletal muscle tissue, the muscles of animals consuming the low protein, low electrolyte and low protein, high electrolyte diets were able to generate equivalent or greater forces when compared to CONTROL muscles.

The soleus muscle of animals consuming the low protein, low electrolyte diet was 50% slower to contract and 45% slower to relax than the soleus muscle of CONTROL animals (p<0.05, Table 5.5). The soleus muscle of the animals consuming the low protein, high electrolyte also exhibited a slower contraction and relaxation compared to that of CONTROL animals, being 29% and 39% slower respectively (p<0.05, Table 5.5). Half relaxation time of the EDL muslce of animals consuming the low protein. low electrolyte diet was 23% sloweer than CONTROL muscles (p<0.05, Table 5.5). No alteration in the time to peak tension or half relaxation time of the EDL of animals in the low protein, high electrolyte group was observed (p>0.05, Table 5.5). No difference was discernible in the time to peak tension or half relaxation time of the fast−twitch TA muscles of animals consuming either low protein diet when compared to CONTROL animals (p>0.05, Table 5.5).

### 5.3.1.2.ii Tetanic Responses.

Maximal force generation in Newtons was 28% and 44% greater in the soleus muscle of animals consuming either the low protein, low electrolyte or low protein, high electrolyte diets respectively (p>0.05, Table 5.6). The TA muscle of animals consuming the low protein, low electrolyte and low protein, high electrolyte diets exhibited a 7% increase in force generation (p>0.05, Table 5.6). The EDL muscle of animals consuming either low protein diet generated less force, but this reduced force generation did not attain statistical significance (27 & 28%, p>0.05). When expressed per unit muscle, however, force generation of the soleus muscle of animals consuming the low protein, low electrolyte diet was 192% greater than CONTROL muscles (p<0.05), that of the EDL was 69% greater (p>0.05) and that of the TA 94% greater (p<0.05). Relative force generation was also greater in the muscles of animals consuming the low protein, high electrolyte diet: 245% in the soleus muscle, 58% in the EDL (P>0.05) and 76% in the TA (p<0.01).

The force−frequency relationship was unaltered following consumption of the low protein diets in the soleus muscle (Figure 5.2a, p<0.05) in the EDL muscle of animals consuming the low protein, high electrolyte diet (Figure 5.2b, p<0.05) and in the TA muscle of animals consuming the low electrolyte diet (Figure 5.2c, p<0.05). The EDL muscle of animals consuming the low protein, low electrolyte diet and TA muscles of animals consuming the low protein, high electrolyte diet exhibited a 15% greater relative force generation at 50 Hz compared to CONTROL animals (Figures 5.2b & 5.2c, p<0.05).

Some, but not all, muscles from animals consuming the low protein, low electrolyte and high electrolyte diets exhibited increased fatigue susceptibility, but this difference attained statistical significance only in the TA muscle of animals consuming the low protein, high electrolyte diet when compared to CONTROL (p>0.01, Figure 5.3 & Table 5.6). Variability in the fatigue index

of animals consuming the low protein diets was large, with the muscles of some animals exhibiting similar fatigue characteristics to CONTROL muscles while other exhibited a fatigue index which was more than 50% lower than CONTROL values (Figure 5.3).

### 5.3.1.3 Skeletal muscle function following consumption of low protein diets assessed against well-nourished aniamls consuming a diet of normal protein content.

This section will compare the contractile characteristics of animals consuming the 15% protein diet (FED) with those animals consuming the low protein, low electrolyte and low protein, high electrolyte diets (i.e. LPLE and LPHE v FED). The contractile characteristics of these animals is presented in Tables 5.5 and 5.6.

### 5.3.1.3.i Twitch characteristics.

Peak twitch tension in Newtons of the animals consuming the low protein, low electrolyte diet was 47% lower in the soleus muscle than that of FED animals ($p < 0.05$), 30% greater in the EDL ($p > 0.01$) and 50% lower in the TA muscle ($p < 0.01$). Relative force generation of these muscles was 58% greater in the soleus muscle ($p > 0.05$), 193% greater in the EDL ($p < 0.01$) and 71% greater in the TA muscle ($p < 0.05$) compared to that generated by muscles from FED animals.

Animals consuming the low protein, high electrolyte diet exhibited a 52% lower force generation in the soleus muscle compared to the FED animals ($p < 0.05$), a 20% greater force generation in the EDL muscle ($p > 0.05$) and a 41% lower force generation in the TA muscle ($p < 0.05$). When expressed per unit muscle weight, however, the soleus muscle exhibited a 64% greater relative force compared to the muscles of the FED animals ($p < 0.05$) while the EDL and TA muscles exhibited a 206% and 116% increase in relative force generation ($p < 0.05$ & $p < 0.01$ respectively). Thus despite loss of skeletal muscle mass (Table 5.3) the muscles of animals consuming low protein, low or high electrolyte diets were able to generate greater relative forces than muscles form animals which had exhibited normal growth.

No differences in the time to peak tension or half relaxation time were noted for the fast-twitch muscles of the FED animals and the low protein, low electrolyte or high electrolyte groups. In the case of the soleus muscle, however, time to peak tension was 40% slower in both low protein groups compared to that of FED animals ($p < 0.05$) and half relaxation time was 33% slower in the high electrolyte group ($p < 0.05$).

5.3.1.3.ii <u>Tetanic Responses.</u>

Maximal force generation in Newtons of the soleus, EDL and TA muscles of animals consuming the low protein, low electrolyte diet was 55%, 45% and 46% lower respectively than that generated in Newtons by muscles of FED animals. When expressed per unit muscle weight force generation by muscles of animals in the low protein, low electrolyte group were 94%, 36% and 8% greater in the soleus, EDL and TA muscles respectively than those of FED animals. This difference attained statistical significance only for the soleus muscle ($p > 0.05$, Table 5.6).

In animals consuming the low protein, high electrolyte diet a similar trend was noted with absolute force generation being 50% lower in the soleus muscle compared to that of FED animals ($p < 0.05$), 45% lower in the EDL ($p > 0.05$) and 53% lower in the TA ($p < 0.05$, Table 5.6). Relative force generation was, however, 130% greater in the soleus muscle of animals consuming the low protein, high electrolyte diet compared to CONTROL muscles, 27% greater in the EDL and 2% lower in the TA. This difference attained statistical significance only for the soleus muscle of animals consuming the low protein, high electrolyte diet compared to FED animals ($p < 0.05$, Table 5.6).

Thus, despite reduced muscle mass of animals consuming both low protein diets, force generation was preserved. The capacity to generate force increased, being most notable in the muscles of animals consuming the low protein, high electrolyte diet.

Muscles from animals consuming the low protein, low electrolyte and high electrolyte diets generally fatigued more rapidly than muscles of FED animals (Figure 5.3 and Table 5.6). The variability within the muscles of these low protein fed animals was large. Some muscles exhibited a fatigue index which was greater than 50% lower than that of FED muscles (Figure 5.3). Statistical significance was attained only for the TA muscle of animals consuming the low protein, high electrolyte diet ($p < 0.05$, Table 5.6, Figure 5.3).

Thus it would appear that force generation in skeletal muscles of animals consuming protein deficient diets is preserved despite a loss of skeletal muscle mass. The twitch time of fast−twitch muscles is unaltered following consumption of protein deficient diets, but the soleus muscle exhibited slower time to peak tension and half relaxation time. Increased fatigue susceptibility may be present in the muscles of some animals, but not those of others. Force generation in the animals consuming protein deficient diets was not impaired when compared to those animals studied at the commencement of the study (the CONTROL group).

5.3.2 <u>Discussion.</u>

The aim of the present study was to investigate the contractile characteristics of skeletal muscle following consumption of low protein diets which resulted in severe wasting and stunting.

The animals fed low protein diets in the present study exhibited clear indications of malnutrition with reduced body and organ weight, stunting and reduced serum albumin concentrations. The addition of sodium and potassium to the protein deficient diets did not alter this effect.

Generally, the twitch time of the slow−twitch soleus muscle became prolonged following consumption of the low protein diets but maintenance of force generation was noted. Despite observed slowing of muscle relaxation, no alteration was detected in fatiguability or force generation at higher frequencies of stimulation. In the fast−twitch muscles, no impairment in time to peak tension and half relaxation time was observed in animals consuming low protein diets. Preservation of force generating capacity was noted, but alteration in the force−frequency curve of the fast−twitch muscles could be detected for both the EDL and TA muscles.

Increased fatiguability of the fast twitch muscles was evident in the case of some animals but not others and attained statistical significance for the TA muscle of animals consuming the low protein, high electrolyte diet. Thus the fast− and slow− twitch muscles appear to respond differently to diets poor in protein.

5.3.2.1 <u>Force generation.</u>

The loss of muscle mass would be expected to reduce the force generating capacity of the muscle. The animals consuming the low protein diets exhibited preserved or enhanced absolute peak twitch tension generation in the soleus, EDL and TA muscles compared to the CONTROL animals. This resulted in greater relative force generation (i.e.per unit muscle) in all three muscles despite the considerable loss of muscle mass. This has not previously been reported in protein deficiency, but was apparent in the data presented for food restricted animals (Chapter 4.2).

Maximal force generation in absolute terms was either not different or greater in the soleus, EDL and TA muscles of the animals consuming the low protein diets compared to CONTROL despite the reduction in muscle mass. This maximal force production was, however, lower than that measured for the animals of the FED group and may be accounted for by the growth of muscle tissue alone. Animals of the low protein group exhibited enhanced relative force

production since force generation was maintained despite the loss of muscle tissue. This effect has been described in the EDL muscle of acute food deprived animals and in chronic food restriction (see Chapter 4) and may therefore be a universal response to a reduction in food intake or a reduction in muscle mass. The component of the diet responsible for this effect cannot be identified from these experiments.

The mechanism by which the muscles are able to maintain force generation despite loss of muscle tissue remains unclear. Since loss of muscle tissue is not associated with lower force generation it may be that loss of muscle tissue is related to non–contractile machinery proteins. Hence the protein constituting the contractile machinery remains intact and the force generation in absolute terms is maintained at the CONTROL level. This would be possible since the magnitude of force generation is dependent upon the number of cross bridges formed during contraction (see Chapter 1). Hence loss of contractile proteins had not occurred following consumption of low protein diets, the number of cross bridges able to attach remains unaltered. The changes in triglyceride, glycogen and water content of the muscles was not assessed in the present study. It is possible that reductions in these components did occur, thus allowing preservation of muscle protein content. The literature would suggest that this hypothesis is incorrect since selective atrophy of type II muscle fibers has been reported (Heymsfield *et al* 1982; Russell *et al* 1984). In this case it would have to be suggested that the loss of contractile machinery does occur but due to the altered dimentions of the skeletal muscle the packing of the remaining fibers becomes closer. Although no histological sections were examined in this study, this closer packing of fibers could then allow greater interaction of the cross–bridges during contraction and hence maintenance of force generation is achieved in this manner.

### 5.3.2.2 Speed of contraction and relaxation.

The soleus muscle of the animals consuming the low protein diets exhibited both slower contraction (TPT) and relaxation (½RT) resulting in prolongation of the twitch. Thus the slow twitch muscle has become slower. It is surprising that the only ′deficit′ observed appears to be present in the soleus muscle since it is believed that slow twitch muscles are generally more resistant to change than fast–twitch muscles, for example in terms of protein turnover (see Millward 1970). Slowing of the fast–twitch muscle contraction and relaxation was apparent in the protein restricted animals although it did not attain statistical significance, the difference from the CONTROL and FED animals, remaining undetected because the value lay within the normal range (see Chapter 2). Hence there may be evidence of a impairment in twitch time but its influence was not sufficiently large to lie outside a normal range. Since both contraction and relaxation involve calcium movement it may be that the soleus muscle has incurred a deficit or has become inefficient in handling calcium (e.g. Russell *et al* 1984).

Ultimately, the two processes of contraction and relaxation can be differentiated in that the release of calcium during the contraction phase is due to excitation–contraction coupling responses and relaxation due to sequestration of calcium by the sarcoplasmic reticulum. Slower contraction may result from reduced release of calcium either due to alteration in the membrane potential resulting in prolongation of the time before the action potential can be elicited in the muscle membrane, slower propogation of the potential via the transverse tubule system within the muscle, a reduced release of calcium per impulse or a combination of these effects.

Relaxation of skeletal muscle is an energy–dependent function. As such it could be suggested that reduced energy intake in the animals consuming the low protein diets has resulted in reduced ATP availability and hence a slower rate of calcium sequestration over the sarcoplasmic reticulum. This would be expected to be apparent in both slow– and fast–twitch muscles and hence it should be expected that a slower relaxation would be discernible in all three muscles studied. This was not observed. It is possible, though unlikely, that a slower rate of calcium sequestration is more discernible in the slow–twitch soleus muscle due to a prolongation of a relatively slower process resulting in a difference which is more detectable.

### 5.3.3.3 Force–frequency relationship.

Despite slower contraction and relaxation in the slow–twitch soleus muscle preservation of function at higher frequencies of stimulation was apparent. In the fast–twitch muscles, however, preservation of twitch characteristics did not necessarily maintain the force–frequency relationship of the muscles at higher stimulation frequencies. Thus consumption of low protein diets has resulted in a greater relative force production at 50 Hz. That the force–frequency relationship appears to shift at 50 Hz in the fast–twitch muscles without loss of peak twitch tension or maximal force generated suggests that the total capacity to generate force is unaltered following consumption of low protein diets, but that the manner in which this is achieved may become altered. This alteration appears to occur over a frequency range at which the muscle would normally be expected to receive neuronal input. This may therefore be an important physiological adaptation to maintain normal functional capacity within the muscle. Thus, fast–twitch muscles are rarely required to function at either low frequencies or at the very high frequencies over which the force–frequency curve was drawn and thus do not exhibit alteration in function at these extremes. The manner in which the force–frequency curve shifts may be indicative of the selective loss of type II muscle fibers and would be in agreement with the reports of such occurrences in the literature.

### 5.3.3.4 Fatigue.

Fatiguability in the soleus, EDL and TA muscles of the animals in the low protein group was generally increased, although only attained statistical significance in the TA muscle of animals consuming the low protein, high electrolyte diet. It is clear from the fatigue index of these muscles that in the case of the EDL and TA in particular some muscles maintain normal fatigue characteristics while other exhibit significant increases in their susceptibility to fatigue (Figure 5.3). This tendency for increased fatiguability may be important in the endurance of individuals in the work environment and represent the major impairment at the peripheral level for the working individual. The mechanism(s) of fatigue have previously been discussed (Chapter 1). How these relate to the observed variability in the fatigue index of animals consuming the low protein diets remains unclear. Despite the demonstration of maintained EMG amplitude in the muscles of CONTROL animals during stimulation using Burke's Paradigm (Section 2.2.2.3), it is not clear whether this occurs in the protein restricted animals. Assuming that peak EMG activity remains constant it would then suggest that the tendency for increased fatigue associated with the consumption of low protein diets lies within skeletal muscle. It remains a possibility that reduced ATP supply or impaired calcium handling may be implicated (Russell *et al* 1984) but, no evidence in these studies was collected which could confirm or refute this suggestion.

Providing animals with the low protein, high electrolyte diet was an attempt to ensure that the reduced intake of micronutrients was not the specific limiting nutrient for growth. It is well established that growth in the young is sensitive to potassium deficiency (Alleyne *et al* 1977; Dorup & Clausen 1989). This effect may be related to inhibition of protein synthesis (Cannon, Frazier & Hughes 1952; Leach, Dam, Zeigler & Norris 1959). It should be noted that the intake of potassium by the animals consuming the low protein, high electrolyte diet of this study was still lower than in those consuming the normal protein (15%) diet. Thus it would appear more likely that the intake of potassium relative to some other factor/nutrient is more important.

### 5.3.3.5 Limitation of function.

By far the most surprising observation from this study is the preservation of force generation in muscles when compared to those of weight– matched, control animals. This represents a reduced force generation in absolute terms in comparison to age matched, well–nourished controls (i.e. the FED group) and would therefore suggest agreement with reports of reduced physical working capacity (see Chapter 1 and Spurr 1987). The apparent tendency for increased fatiguability is also in agreement with these reports. Spurr and co–workers have indicated a reduced physical working capacity of malnourished Columbian sugar cane cutters (summarised in Spurr 1987). However, in ambulatory humans the peripheral (i.e. muscle)

phenomenon is profoundly influenced by central (motivational) and cardiovascular factors such that the pattern of activity during the day may alter (see Chapter 1 & Ferro—Luzzi 1989,1990).

Thus it can be concluded that alteration of the contractile characteristics of skeletal muscle does occur during accomodation of nutritional insults when the level of insult is greater than that which can be accommodated simply by cessation of growth (i.e. acute food deprivation, Chapter 4). This adaptation does not necessarily represent an impairment of function, but rather occurs as a prerequisite to maintaining the functional characteristics of skeletal muscles in face of deficiencies in the composition of food supplied.

5.4 <u>SUMMARY.</u>

Changes in skeletal muscle contractile characteristics were evident following consumption of low protein diets. This resulted in slowing of soleus twitch time, shifts in the force—frequency relationship and increases in fatigue susceptibility. When compared to age—matched animals reduced force generation was noted, but this was thought to be due to growth/ development of the muscles in the heavier animals. Thus the capacity to generate force was preserved or enhanced following consumption of low protein diets. The increased susceptibility to fatigue may represent a major challenge to malnourished individuals with respect to performance of mechanical work. This is discussed further in the general discussion (Chapter 8).

CHAPTER 6.

THE EFFECT OF ENDOTOXIN ADMINISTRATION ON RAT SKELETAL MUSCLE
FUNCTION AND METABOLISM.

6.1 <u>INTRODUCTION.</u>

Muscle weakness and lethargy are often reported during infections, for example influenza. No
evidence exists as to whether this reflects infection–mediated changes in muscle metabolism or
muscle mass. Metabolic related events may include alterations in tissue perfusion (Fish *et al*
1986; Burnier *et al* 1988), alterations in cellular calcium concentrations (Nicholas *et al* 1974),
plasma glucose concentration (Southorn & Thompson 1986; Ward *et al* 1987) and plasma and
muscle lactate concentration (Ward *et al* 1987). Such suggestions are supported by the study of
Drew and associates who investigated the *in vitro* functional properties of the plantaris, soleus
and diaphragm muscles of hamsters during infection with *Leishmania donovani* (Drew *et al* 1988).
These muscles exhibited reduced maximal tetanic force generation following 8–12 weeks of
infection. This reduction in tetanic force was greatest in the fast–twitch muscles (Drew *et al*
1988).

Changes in muscle mass have not been reported in the literature during and following infection.
Reductions in muscle mass associated with endotoxin administration in animals has been
reported (Long *et al* 1982; Baracos *et al* 1983; Ruff & Secrist 1984; Wan *et al* 1984; Wan &
Grimble 1986; Southorn & Thompson 1986; Wan & Grimble 1986; Fish *et al* 1986; Jepson *et al*
1988; Bibby & Grimble 1989). Yet, the reduced muscle mass associated solely with reduced
food intake did not result in impaired skeletal muscle function (see Chapters 4 & 5).

The only study to date which has investigated the influence of infection on skeletal muscle
function is that of Drew and associates (Drew *et al* 1988). Their investigation reported a long
term parasitic infection which resulted in a 40% reduction in body mass and a 25% lower muscle
mass in hamsters. The *in vitro* twitch characteristics of hamsters infected with *L.donovani* were
not different from non–infected animals but maximal force development was lower, resulting in a
shift in the force–frequency relationship (Drew *et al* 1988). The changes in *in vitro* muscle
contractile characteristics following long term parasitic infections may not be related to the flu–
like syndrome noted above in which muscle weakness is reported. A common pathway in the
response to bacterial infections is believed to involve endotoxin (Beisel 1977; Chapter 1). The
endotoxin effects are probably mediated through interleukin–1 (IL–1) and tumour necrosis
factor (TNF) and animal models utilizing endotoxin administration have been widely investigated
as previously described in Chapter 1. No investigations have been reported of the influence of
endotoxin, IL–1 or TNF on skeletal muscle contractile characteristics. Evidence that contractile

events may be impaired in endotoxic shock is implied from studies of smooth muscle contraction. The contraction force of vascular smooth muscle has also been reported to be lowered by endotoxin when assessed *in vitro* (Hess & Briggs 1971; Hulsman *et al* 1981; Pomerantz, Casey, Fletcher & Ramwell 1982; Wakabayshi *et al* 1987). Others failed to see significant alterations in contractile force of vascular smooth muscle following endotoxin administration (Vargas & Beck 1957; Weiner & Zweifach 1966; Kutner & Cohen 1966; Priano, Wilson & Traber 1970).

The metabolic responses to administration of *E.coli* endotoxin are well documented (Beisel 1977; Wan 1987). Two phases are of interest. Firstly the febrile phase which occurs within 2 hours of endotoxin administration, with body temperature returning towards normal by 8 hours (Bibby & Grimble 1989). Secondly, the period over which changes in protein turnover have been reported, requiring approximately 24 hours (Wan 1987). A variety of routes, dosages and sequences of endotoxin administration have been reported in the literature (see Chapter 1). These include single dose administration (Wan 1987); continuous infusion (Southorn & Thompson 1986) and repeated administration (Ward *et al* 1987). It is not possible to complete true chronic administration of endotoxin because the effects diminish after the second or third challenge (Beisel 1977). The aim of the present study therefore, was to examine the skeletal muscle contractile characteristics at 8 hours following endotoxin administration (at the end of the febrile response) and 8 hours following a second challenge with endotoxin occurring 24 hours after the initial insult. It was hypothesised that this protocol of further challenging the response to endotoxin would exacerbate alterations in the body's ability to accommodate changes in metabolic regulation.

Since endotoxin administration is reported to reduce food intake (see Chapter 1), it is necessary to ensure that any changes in food consumption in response to endotoxin administration did not influence the measurements made. As such, food was removed from all animals over the 8 hour period immediately prior to the assessment of skeletal muscle function.

In order to determine whether the alteration in skeletal muscle contractile characteristics was due to metabolic or mass effects, muscle ATP, creatine, creatine phosphate and lactate concentrations were determined. In addition, blood lactate, serum albumin and zinc concentrations were measured to characterise the development of the response to endotoxin administration.

6.2 <u>METHODS.</u>

Sixty eight male Wistar rats (mean weight 175.8 ± 0.9g) were initially divided into two groups:
a) 30 animals in which assessment of skeletal muscle contractile characteristics was conducted;

b) 30 animals used for the assessment of body and skeletal muscle metabolite profile.

The terms "ACUTE" and "CHRONIC" are used below to define the 8 hour period following a single challenge with endotoxin and the 32 hour period during which the animals received two challenges with endotoxin respectively. The reader will recognise that this is not a classical definition of these terms but these terms will be used in order to enable a less complicated discussion of the findings of this study.

Animals were further subdivided into the following groups:

I) ACUTE CONTROL
:receiving saline injections intraperitonealy (ip) at 0 hours with assessment of muscle contractile characteristics at 8 hours during which no food was available (n=6);

II) ACUTE ENDOTOXIN
:receiving *E.coli* endotoxin (1.2 mg/Kg body weight) ip at 0 hours with assessment of muscle function at 8 hours during which time no food was available (n=6);

III) CHRONIC CONTROL
:receiving saline injections ip at 0 and 24 hours during which time food was available *ad libatum*. Functional assessment of skeletal muscle contractile characteristics was made at 32 hours, 8 hours after the last administration of endotoxin during which time no food was made available (n=6);

IV) CHRONIC ENDOTOXIN
:receiving *E.coli* endotoxin (1.2 mg/Kg body weight) ip at 0 and 24 hours during which time food was available *ad libatum*. Functional assessment of skeletal muscle contractile characteristics was made at 32 hours, 8 hours after the last administration of endotoxin during which time no food was made available (n=6);

V) PAIR FED CONTROL :receiving saline injections at 0 and 24 hours. These animals were provided with the quantity of food consumed by paired endotoxin–treated animals (group IV) during the first 24 hours of the study. Assessment of skeletal muscle contractile characteristics was conducted at 32 hours, 8 hours following the last endotoxin administration, following which no food was made available (n=6).

All animals were maintained individually in a room maintained at 23°C with a 12 hour light–dark cycle and free access to water. Body weight and food intake was monitored at the same point in each light period.

On the day of study animals were anaesthetised using 0.44 mls Sagatal/kg body weight and prepared for measurement as described in Section 2.1. Assessment of contractile characteristics was conducted as reported in Section 2.2 thus the time to peak tension (TPT), half relaxation time (½RT) & peak twitch tension (PT) of the twitch and the maximal tetanic force (Fmax) & force frequency relationship were determined.

Following assessment of contractile characteristics the muscles of both hindlimbs were rapidly excised, weighed and stored as previously described (see Section 2.3) for determination of metabolite profile and muscle protein concentration (Section 2.4). Other events associated with endotoxin administration include the reduction in the serum concentrations of albumin and zinc, increased liver weight and raised temperature associated with the febrile response (see Biesel 1977). These variables were monitored in the animals of this study in order to reflect the positive response of animals to endotoxin. Details of these measurements have been previously described (see Section 2.4).

## 6.3 <u>RESULTS.</u>

The results will be presented in two parts. Firstly, the response of body and muscle will be presented following 8 hours of endotoxin administration (Section 6.3.1). Secondly, the influence of a second challenge with endotoxin 24 hours after the first challenge will be discussed, where assessment of skeletal muscle contractile characteristics occurred at 32 hours following the first administration of endotoxin (Section 6.3.2).

### 6.3.1 Acute endotoxin administration.

This section presents the findings of the investigation of animals 8 hours after the administration of endotoxin at which time the febrile response is believed to have been completed (i.e. ACUTE).

#### 6.3.1.1 Body and organ weight.

No difference in body weight was observed 8 hours following endotoxin administration ($p > 0.05$, Table 6.1). Administration of endotoxin did not result in discernible difference in the muscle mass ($p > 0.05$, Table 6.1) or protein concentration (data not shown) hence, total protein content of the soleus, EDL or TA muscles was not different to that of muscles in the ACUTE CONTROL group ($p > 0.05$, Table 6.1). Liver weight and protein content of endotoxin−treated animals was not different from that of CONTROL animals ($p > 0.05$, Table 6.1).

#### 6.3.1.2 Contractile characteristics.

No difference in time to peak tension and half relaxation time was discernible in any of the muscles of the endotoxin−treated animals compared to CONTROL ($p > 0.05$, Table 6.2). Peak twitch tension was lower in the soleus muscle (35%, $p > 0.05$), raised in the EDL and TA muscles (105% & 48%) of endotoxin treated animals compared to CONTROLs, attaining statistical significance only in the EDL muscle ($p < 0.005$, Table 6.2). Maximal tetanic tension was not different in the muscles of endotoxin treated animals compared to CONTROL animals in absolute terms ($p > 0.05$, Table 6.2). When expressed per unit muscle, however, maximal force generation was lower in the soleus and TA muscles (36% & 32%) and increased in the EDL (41%), but these differences did not attain statistical significance ($p > 0.05$, Table 6.2).

No difference was observed in the force−frequency relationship of the soleus muscle of endotoxin−treated animals compared to CONTROL (Figure 6.1a). The EDL exhibited reduced relative force generation at all frequencies of stimulation (Figure 6.1b) and the TA raised relative force generation at all frequencies (Figure 6.1c). These differences did not attain statistical significance.

No change was apparent in the fatigue susceptibility of muscles from endotoxin−treated animals (Figure 6.2).

Table 6.1. Body and organ weight and organ protein content of the wistar rat 8 hours following administration of saline (ACUTE CONTROL) or *E.coli* endotoxin (ACUTE ENDOTOXIN).

| | ACUTE CONTROL (n=6) | | ACUTE ENDOTOXIN (n=6) | |
| --- | --- | --- | --- | --- |
| | mean | sem | mean | sem |
| Final Body Weight (g) | 171.8 | 2.7 | 170.7 | 2.6 |
| MUSCLE WEIGHT (mg) | | | | |
| Soleus | 67.1 | 3.4 | 64.9 | 5.2 |
| EDL | 73.9 | 3.4 | 85.1 | 7.9 |
| TA | 332.5 | 8.9 | 331.8 | 7.5 |
| Liver Weight (g/kg body weight) | 40.5 | 1.4 | 39.9 | 1.2 |
| TOTAL PROTEIN CONTENT (mg) | | | | |
| Soleus | 8.1 | 0.6 | 7.4 | 1.1 |
| EDL | 9.7 | 0.8 | 10.5 | 0.7 |
| TA | 52.2 | 3.2 | 43.6 | 3.4 |
| Liver (g) | 1.2 | 0.3 | 1.2 | 0.3 |

KEY: EDL – Extensor digitroum longus; TA – Tibialis Anterior.

Table 6.2. The contractile characteristics of wistar rats 8 hours following the administration of saline (ACUTE CONTROL) or *E.coli* endotoxin (ACUTE ENDOTOXIN).

| | ACUTE CONTROL n=6 | | ACUTE ENDOTOXIN n=6 | |
|---|---|---|---|---|
| | mean | sem | mean | sem |
| **SOLEUS** | | | | |
| TPT (ms) | 35.2 | 1.9 | 35.3 | 1.1 |
| ½RT (ms) | 39.8 | 2.9 | 39.3 | 3.9 |
| PT (mN) | 1.1 | 0.1 | 1.7 | 0.3 |
| PT (mN/g muscle) | 13.8 | 2.7 | 19.2 | 4.9 |
| Fmax (mN) | 5.2 | 0.6 | 4.6 | 1.1 |
| Fmax (mN/g muscle) | 77.6 | 9.5 | 49.5 | 12.8 |
| **EDL** | | | | |
| TPT (ms) | 17.4 | 1.3 | 17.7 | 0.8 |
| ½RT (ms) | 13.6 | 0.7 | 13.7 | 0.8 |
| PT (mN) | 2.2 | 0.6 | 4.5 | 0.7 * |
| PT (mN/g muscle) | 28.3 | 6.1 | 57.9 | 9.3 * |
| Fmax (mN) | 12.0 | 2.3 | 16.3 | 1.8 |
| Fmax (mN/g muscle) | 148.8 | 24.8 | 210.1 | 22.9 |
| **TA** | | | | |
| TPT (ms) | 14.9 | 0.5 | 14.4 | 1.8 |
| ½RT (ms) | 11.7 | 1.3 | 14.0 | 1.4 |
| PT (mN) | 5.9 | 2.1 | 8.7 | 1.7 |
| PT (mN/g muscle) | 17.9 | 2.1 | 26.9 | 7.2 |
| Fmax (mN) | 30.2 | 5.9 | 29.2 | 2.9 |
| Fmax (mN/g muscle) | 130.4 | 10.78 | 90.6 | 15.9 |

KEY: TPT - Time to Peak Tension; ½RT - Half Relaxation Time; PT - Peak Twitch Tension; Fmax - Maximal Tetanic Force.

* - $p < 0.05$ v ACUTE CONTROL.
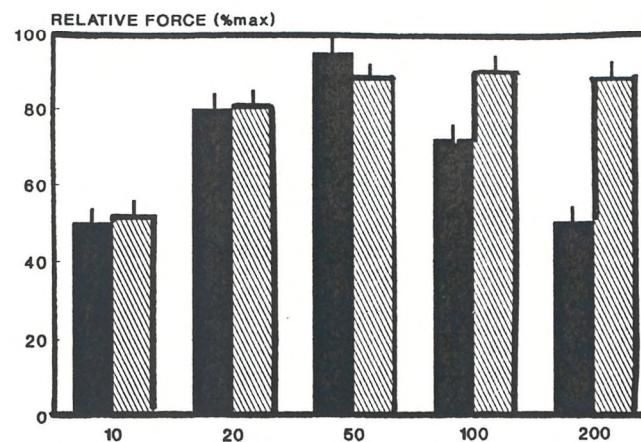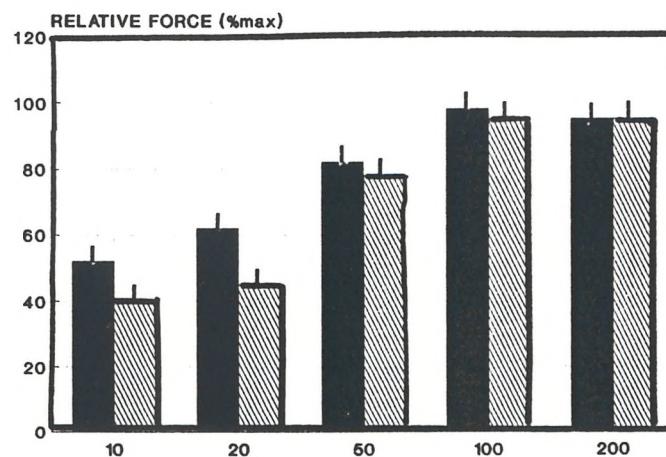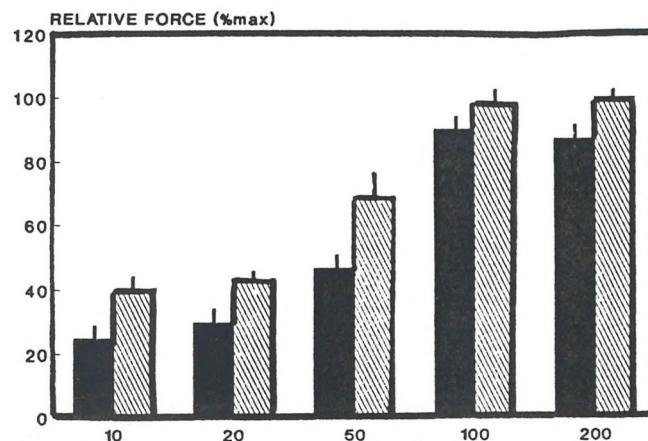
Figure 6.1. The force—frequency relationship of the soleus (a),
extensor digitorum longus (b) and tibialis anterior (c) muscles
of CONTROL animals (ACUTE CONTROL,■, n=6) and 8 hours
following administration of *E.coli* endotoxin (ACUTE ENDOTOXIN,

▨   , n=6). Bars represent mean ± sem.
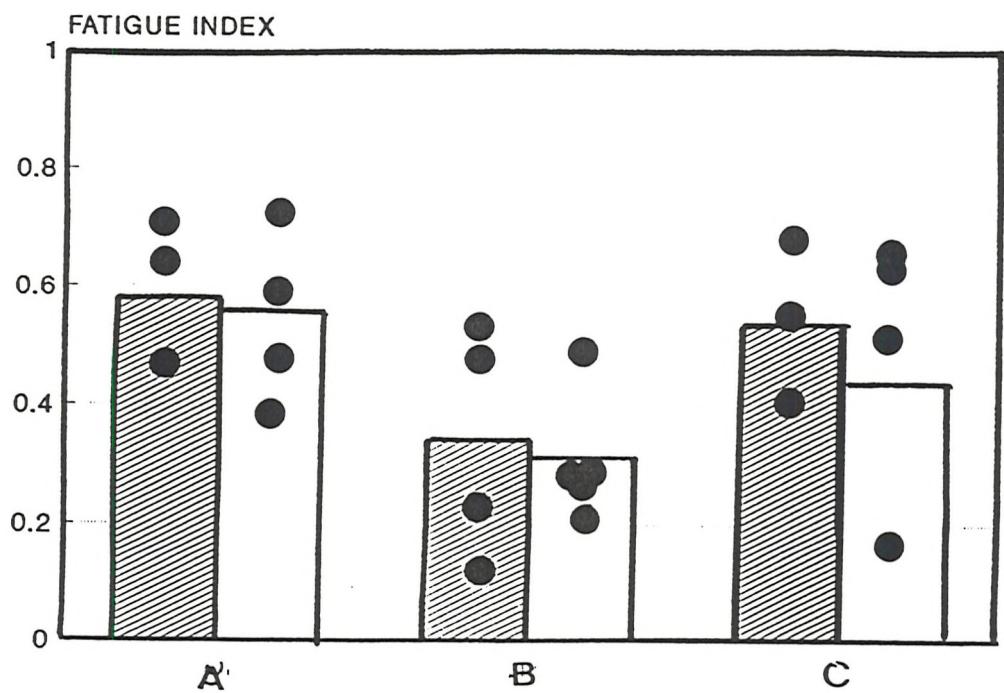
Figure 6.2. The fatigue characteristics of the soleus (A), extensor digitorum longus (EDL, B) and tibialis anterior (TA, C) muscles of CONTROL animals (ACUTE CONTROL,☑, n=6) and 8 hours following administration of *E.coli* endotoxin (ACUTE ENDOTOXIN,☐, n=6). Bars represent mean value.

6.3.1.3 <u>Skeletal muscle, serum and blood metabolite concentrations.</u>

Blood lactate concentration was 4 mmoles/l greater in the animals 8 hours following administration of endotoxin compared to the animals receiving saline, but this difference did not attain statistical significance ($p>0.05$, Table 6.3). Serum albumin concentration was 17% lower than that of CONTROL animals following 8 hours of endotoxin administration ($p<0.01$, Table 6.3). Serum zinc was also lower in the animals having received endotoxin (59%, $p<0.01$, Table 6.3).

No difference in the muscle concentration of ATP, creatine phosphate or free creatine was observed 8 hours following endotoxin administration ($p>0.05$, Table 6.3). Total creatine concentration of the soleus muscle of the ACUTE ENDOTOXIN group was lower than that of the ACUTE CONTROL group but this difference did not attain statistical significance (24%, $p>0.05$, Table 6.3). No difference in the concentration of muscle lactate was observed between the EDL and TA muscles of endotoxin treated animals and the CONTROL group ($p>0.05$, Table 6.3). The lactate concentration of the soleus muscle of the ACUTE CONTROL animals was higher than would be normally expected . This difference in lactate concentration did not attain statistical significance due to the large sem associated with this measurement in the ACUTE CONTROL group.

6.3.1.4 <u>Summary of changes in skeletal muscle function following 8 hours (acute) endotoxin administration.</u>

The administration of endotoxin resulted in reduced serum concentrations of zinc and albumin and an indication of early lactic acidosis by 8 hours as reported by previous investigators (e.g. Southorn & Thompson 1988, see also Chapter 1). No impairment in skeletal muscle contractile characteristics could be discerned at this early time point in the response to endotoxin. The findings are discussed further in the chapter discussion below (see Section 6.3.5).

6.3.2 <u>Chronic endotoxin administration.</u>

This section will discuss the skeletal muscle function 8 hours after a second administration of endotoxin, given 24 hours after the initial injection. Thus skeletal muscle contractile characteristics were assessed 32 hours following the initial challenge with endotoxin. One animal in the CHRONIC ENDOTOXIN group died under anaesthesia, hence data for the contractile characteristics reflects 5 animals in this group. Two animals in the PAIR FED group accidentally received endotoxin at 24 hours and hence have been removed from the analysis of this chapter, therefore n=4 in this group.

Table 6.3. Blood, Serum and Muscle Metabolite Profiles of wistar rats 8 hours following the administration of saline (ACUTE CONTROL, n=6) or *E.coli* endotoxin (ACUTE ENDOTOXIN, n=6).

| | ACUTE CONTROL | | ACUTE ENDOTOXIN | |
|---|---|---|---|---|
| | mean | sem | mean | sem |
| Blood Lactate (mmoles/l) | 6.5 | 2.3 | 10.3 | 2.4 ** |
| Serum Albumin (mg/ml) | 41.0 | 1.3 | 34.2 | 1.3 ** |
| Serum Zinc (µg/ml) | 1.66 | 0.10 | 0.68 | 0.03 |
| MUSCLE METABOLITES $ | | | | |
| **SOLEUS** | | | | |
| ATP | 22.4 | 2.2 | 21.7 | 4.1 |
| CP | 38.7 | 4.9 | 39.0 | 9.2 |
| Creatine | 67.9 | 11.5 | 40.9 | 8.3 |
| Total Creatine | 110.0 | 15.9 | 82.6 | 12.1 |
| Lactate | 30.7 | 11.9 | 18.8 | 3.7 |
| **EDL** | | | | |
| ATP | 32.1 | 4.5 | 31.2 | 2.2 |
| CP | 56.1 | 11.2 | 54.6 | 6.9 |
| Creatine | 58.1 | 10.2 | 62.3 | 15.6 |
| Total Creatine | 114.2 | 18.6 | 116.9 | 14.3 |
| Lactate | 17.1 | 5.9 | 21.8 | 3.4 |
| **TA** | | | | |
| ATP | 32.3 | 2.4 | 38.8 | 2.6 |
| CP | 68.8 | 14.2 | 62.4 | 8.6 |
| Creatine | 70.7 | 9.1 | 59.0 | 11.8 |
| Total Creatine | 141.2 | 13.4 | 111.1 | 15.4 |
| Lactate | 24.3 | 6.5 | 24.1 | 5.4 |

KEY: EDL - Extensor Digitorum Longus; TA - Tibialis Anterior; ATP - Adenosine Triphosphate; CP - Creatine Phosphate.

$ - All metabolites are presented as mM/kg dry muscle weight.

** - $p < 0.01$ v ACUTE CONTROL.

6.3.2.1 <u>Body and organ weight.</u>

Body weight was 7% lighter in animals receiving the second administration of endotoxin at 24 hours compared to that of the CHRONIC CONTROL group ($p<0.05$, Table 6.4) and appeared to be the result of reduced food intake (64%, $p<0.05$, Table 6.4) since PAIR FED animals exhibited a similar reduction in body weight (8%, $p<0.05$, Table 6.4). Soleus and EDL muscle weight was not different between the three groups despite the reduced food intake in the ENDOTOXIN and PAIR FED animals ($p>0.05$, Table 6.4). No change in total protein content was discernible in the soleus and EDL muscles of these animals ($p>0.05$, Table 6.4). The TA muscle, however, exhibited a 9 and 8% lower mass in ENDOTOXIN and PAIR FED animals respectively compared to the CONTROL group ($p<0.05$, Table 6.4). This was not associated with a discernible change in protein content of the TA muscle ($p>0.05$, Table 6.4).

Liver weight was greater in the endotoxin–treated animals than in either the CONTROL or PAIR FED animals, this difference attained statistical significance when compared to the PAIR FED group (16%, $p<0.05$, Table 6.4). This reduced liver weight was associated with a 22% increase in total protein content of the liver, which did not attain statistical significance ($p>0.05$, Table 6.4).

6.3.2.2 <u>Contractile characteristics.</u>

6.3.2.2.i <u>Twitch characteristics.</u>

No differences in time to peak tension or half relaxation time was discernible in the muscles of animals treated twice with endotoxin compared to those receiving saline injections ($p>0.05$, Table 6.5). Animals pair fed the amount of food consumed by the CHRONIC ENDOTOXIN group also exhibited time to peak tension and half relaxation times which were not different to that of muscles from CHRONIC CONTROL animals ($p>0.05$, Table 6.5).

Peak twitch tension in animals treated twice with endotoxin was not different in any muscles from those of animals receiving saline when expressed in Newtons or relative to muscle weight ($p>0.05$, Table 6.5). Pair feeding animals the amount of food consumed by the CHRONIC ENDOTOXIN group did not alter the force generation of the twitch compared to that measured in muscles of the CHRONIC CONTROL group ($p>0.05$, Table 6.5). Force generation of the TA muscle of endotoxin treated animals was approximately 47% greater than that of PAIR FED animals when expressed in absolute terms or per unit muscle ($p<0.05$, Table 6.5), but was unaltered in the soleus and EDL muscles.

Table 6.4. Body and organ weight, organ protein content and food intake of wistar rats receiving normal saline (CHRONIC CONTROL, n=6), or receiving *E.coli* endotoxin twice in 32 hours (CHRONIC ENDOTOXIN) and those Pair Fed the amount of food consumed by the endotoxin group in the first 24 hours (PAIR FED, n=4, see text).

|  | CHRONIC CONTROL | | CHRONIC ENDOTOXIN | | CHRONIC PAIR FED | |
|---|---|---|---|---|---|---|
|  | mean | sem | mean | sem | mean | sem |
| Final Body Weight (g) | 179.2 | 2.9 | 165.8 | 4.3 | 164.0 | 3.3 |
| Food Consumption (g/24 hours) | 17.6 | 1.2 | 6.4 | 1.4 | 6.4 | 1.4 |
| Soleus Weight (mg) | 77.3 | 3.9 | 70.7 | 3.3 | 78.6 | 4.4 |
| EDL Weight (mg) | 89.3 | 7.2 | 75.2 | 3.3 * | 78.8 | 2.5 * |
| TA Weight (mg) | 339.7 | 8.8 | 309.9 | 10.4 | 312.5 | 7.9 |
| Liver Weight (g/kg body weight) | 39.9 | 0.9 | 43.6 | 1.8 | 37.7 | 1.3 † |
| TOTAL PROTEIN CONTENT | | | | | | |
| Soleus (mg) | 13.1 | 2.5 | 8.1 | 1.3 | 11.4 | 1.8 |
| EDL (mg) | 11.6 | 2.7 | 11.6 | 1.2 | 10.6 | 1.9 |
| TA (mg) | 51.6 | 4.0 | 41.2 | 2.7 | 47.4 | 8.9 |
| Liver (g) | 0.9 | 0.1 | 1.1 | 0.1 | 0.9 | 0.1 |

KEY : EDL - Extensor Digitorum Longus; TA - Tibialis Anterior;
* - p<0.05 v CONTROL; † - p <0.05 v ENDOTOXIN.

Table 6.5. The contractile characteristics of wistar rats following administration of saline (CHRONIC CONTROL) or *E.coli* endotoxin (CHRONIC ENDOTOXIN) twice in 32 hours and in rats fed the quantity of food consumed by the ENDOTOXIN animals in the first 24 hours (PAIR FED).

| | CHRONIC CONTROL n=6 | | CHRONIC ENDOTOXIN n=5 ! | | CHRONIC PAIR FED n=4 ! | |
|---|---|---|---|---|---|---|
| | mean | sem | mean | sem | mean | sem |
| **SOLEUS** | | | | | | |
| TPT (ms) | 32.8 | 1.5 | 31.4 | 2.5 | 34.3 | 1.7 |
| ½RT (ms) | 41.2 | 4.1 | 39.3 | 5.6 | 42.2 | 2.9 |
| PT (mN) | 1.1 | 0.1 | 1.4 | 0.2 | 0.9 | 0.1 |
| PT (mN/g muscle) | 12.8 | 1.9 | 15.9 | 3.5 | 12.4 | 2.3 |
| Fmax (mN) | 3.8 | 0.5 | 3.7 | 0.9 | 4.7 | 0.8 |
| Fmax (mN/g muscle) | 49.1 | 5.2 | 56.7 | 10.0 | 57.5 | 9.8 |
| **EDL** | | | | | | |
| TPT (ms) | 16.1 | 0.6 | 18.0 | 0.9 | 16.1 | 0.9 |
| ½RT (ms) | 13.9 | 0.8 | 13.7 | 1.0 | 12.8 | 2.1 |
| PT (mN) | 4.0 | 0.7 | 3.5 | 0.5 | 3.6 | 0.7 |
| PT (mN/g muscle) | 45.5 | 5.4 | 43.1 | 7.9 | 45.7 | 11.3 |
| Fmax (mN) | 11.9 | 1.0 | 14.2 | 1.0 | 10.6 | 1.1 |
| Fmax (mN/g muscle) | 148.1 | 12.9 | 176.6 | 17.4 | 110.7 | 25.1 |
| **TA** | | | | | | |
| TPT (ms) | 16.2 | 0.7 | 15.5 | 0.6 | 15.3 | 1.2 |
| ½RT (ms) | 14.2 | 1.6 | 12.9 | 0.8 | 10.9 | 0.9 |
| PT (mN) | 11.5 | 2.7 | 7.7 | 0.2 | 5.0 | 0.8 $ |
| PT (mN/g muscle) | 32.8 | 7.7 | 26.3 | 2.9 | 17.9 | 3.2 $ |
| Fmax (mN) | 31.1 | 6.0 | 28.2 | 5.4 | 21.7 | 9.4 |
| Fmax (mN/g muscle) | 118.7 | 9.1 | 98.5 | 11.7 | 154.7 | 21.8 |

KEY:KEY: TPT – Time to Peak Tension; ½RT – Half Relaxation Time;
PT – Peak Twitch Tension; Fmax – Maximal Tetanic Force.
! – n= 5 for TA of CHRONIC ENDOTOXIN as animal died during anaesthesia, n= 4 for PAIR FED group, see text;
$ – p<0.05 v CHRONIC ENDOTOXIN.

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6.3.2.2.ii <u>Tetanic responses.</u>

Maximal force generation of the soleus, EDL and TA muscles of animals receiving two injections of endotoxin was not different from that of animals receiving saline injections either in Newtons or when expressed per unit muscle (p>0.05, Table 6.5). Similarly, PAIR FED animals did not exhibit any discernible alteration in the maximal force generation of the soleus, EDL or TA muscles when compared to that measured in the CHRONIC CONTROL group (p>0.05, Table 6.5).

Neither endotoxin administration twice in 24 hours, nor pair feeding animals the amount of food consumed by the animals of the CHRONIC ENDOTOXIN group influenced the force–frequency relationship of the soleus, EDL or TA muscles compared to the CHRONIC CONTROL group (p>0.05, Figure 6.3). There was a trend for raised relative force generation in the EDL muscle of animals in the CHRONIC ENDOTOXIN and PAIR FED groups, but this difference did not attain statistical significance.

Fatigue, assessed by the calculation of the fatigue index, was not altered in the muscles of animals receiving two doses of endotoxin at a 24 hour interval (p>0.05, Figure 6.4). No difference in the fatigue index of muscles from animals in the PAIR FED group could be discerned from those of the CHRONIC CONTROL group (p>0.05, Figure 6.4). The range of values was large, but not different between the 3 groups of animals (Figure 6.4).

6.3.2.3 <u>Blood, serum and skeletal muscle metabolic profiles.</u>

No difference in the concentration of blood lactate was observed following the administration of endotoxin twice when compared to animals of the CHRONIC CONTROL group (p>0.05, Table 6.6). Blood lactate concentration was also unaltered in the animals pair fed the amount of food consumed by paired animals in the CHRONIC ENDOTOXIN group, compared to those of the CHRONIC CONTROL group (p>0.05, Table 6.6) and are lower than those measured in the ACUTE CONTROL and ACUTE ENDOTOXIN animals (Table 6.3).

Serum concentrations of albumin and zinc were 36% and 49% lower in animals receiving two administrations of endotoxin than in CHRONIC CONTROL (p<0.01, Table 6.6) and 29% and 47% lower than PAIR FED animals (p>0.05, Table 6.6).

Muscle ATP concentration was 22% greater in the TA of animals receiving endotoxin twice than CONTROL or PAIR FED animals (p<0.05, Table 6.6), but was unchanged in the soleus and EDL muscles. Total muscle creatine was not different in the soleus and TA muscles of the animals in the CHRONIC ENDOTOXIN group compared to those in the CHRONIC CONTROL group (p>0.05, Table 6.6) but was 27% lower in the EDL muscle (p<0.05, Table 6.6). This appeared to
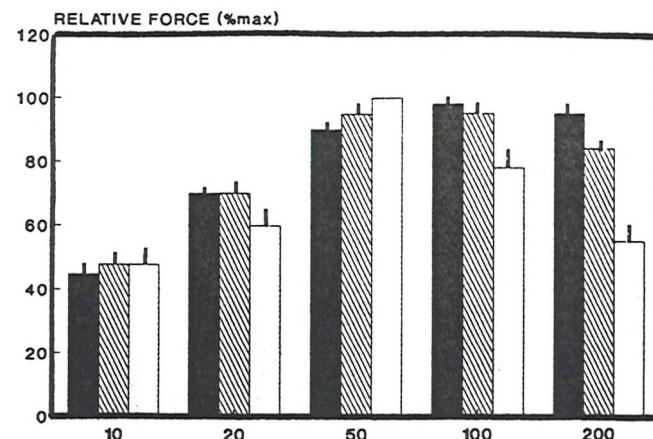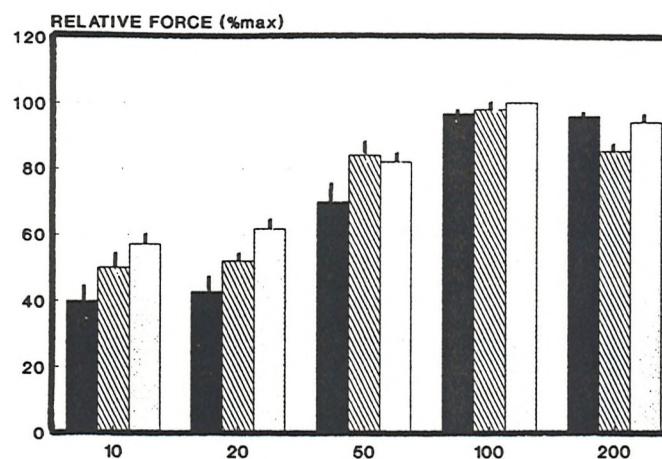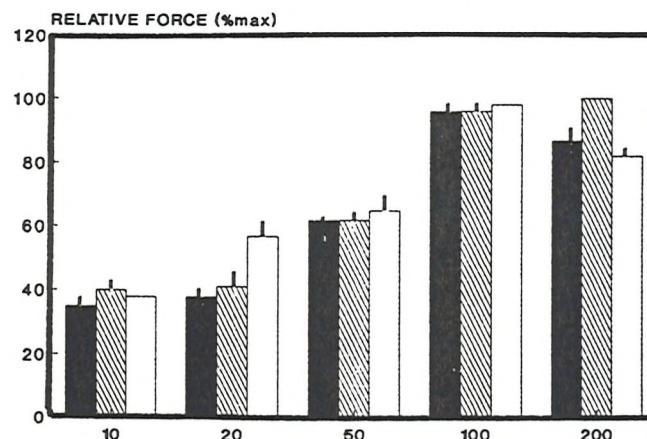
Figure 6.3. The force–frequency relationship of the soleus (A), extensor digitorum longus (EDL, B) and tibialis anterior (TA, C) muscles of CONTROL animals (CHRONIC CONTROL, ■, n=6) , 32 hours following administratio of *E.coli* endotoxin (CHRONIC ENDOTOXIN, ▨ , n=6) and following pair feeding to the quantity of food consumed by the CHRONIC ENDOTOXIN group during the first 24 hours of study (PAIR FED,□ n=4). Bars represent mean ± 1 sem.

FATIGUE INDEX
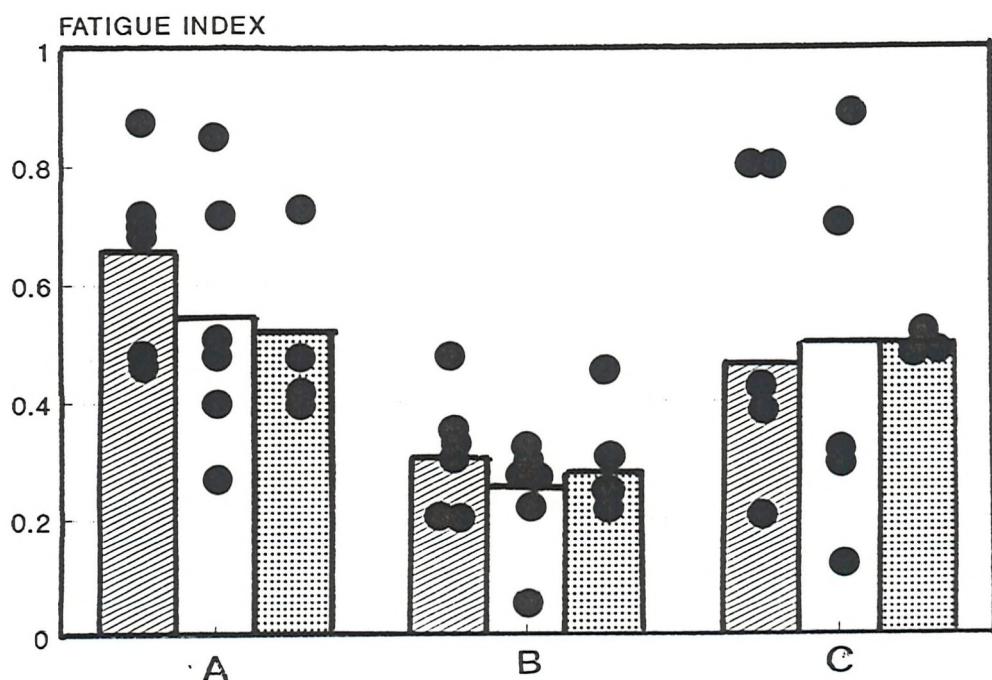
Figure 6.4. The fatigue characteristics of the soleus (A),
extensor digitorum longus (EDL, B) and tibialis anterior
(TA, C) muscles of CONTROL animals (CHRONIC CONTROL,☑,
n=6) , 32 hours following administratio of *E.coli* endotoxin
(CHRONIC ENDOTOXIN,□, n=5) and following pair feeding to
the quantity of food consumed by the CHRONIC ENDOTOXIN group
during the first 24 hours of study (PAIR FED,⊡, n=4).
Bars represent the mean value.

Table 6.6. Blood, serum and muscle metabolite profiles of wistar rats following administration of saline (CHRONIC CONTROL, n=6) or *E.coli* endotoxin (CHRONIC ENDOTOXIN, n=6) twice in 32 hours and in rats fed the quantity of food consumed by the ENDOTOXIN animals in the first 24 hours (PAIR FED, n=4).

| | CHRONIC CONTROL | | CHRONIC ENDOTOXIN | | CHRONIC PAIR FED | |
|---|---|---|---|---|---|---|
| | mean | sem | mean | sem | mean | sem |
| **Blood Lactate** | | | | | | |
| Concentration (mmoles/l) | 4.8 | 0.6 | 3.5 | 1.1 | 5.7 | 2.0 |
| **Serum Albumin** | | | | | | |
| Concentration (mg/ml) | 50.2 | 1.5 | 31.9 | 2.5 **\$\$ | 44.8 | 4.7 |
| **Serum Zinc** | | | | | | |
| Concentration (μg/ml) | 1.43 | 0.17 | 0.73 | 0.15 **\$\$ | 1.39 | 0.04 |
| | | | | | | |
| **MUSCLE METABOLITES**\$ | | | | | | |
| **SOLEUS** | | | | | | |
| ATP | 24.4 | 2.7 | 24.5 | 1.9 | 22.2 | 1.7 |
| CP | 47.7 | 9.3 | 47.4 | 5.3 | 50.8 | 4.9 |
| Free Creatine | 57.8 | 4.9 | 49.5 | 10.9 | 78.9 | 11.2 |
| Total Creatine | 104.8 | 9.3 | 94.5 | 17.8 | 129.6 | 8.7 |
| Lactate | 16.1 | 5.3 | 15.4 | 2.2 | 10.8 | 1.9 |
| **EDL** | | | | | | |
| ATP | 30.7 | 1.0 | 36.2 | 3.9 | 34.5 | 5.3 * |
| CP | 92.5 | 10.5 | 73.2 | 8.3 | 48.0 | 4.4 |
| Free Creatine | 59.9 | 8.4 | 38.6 | 10.0 * | 85.8 | 11.5 |
| Total Creatine | 154.6 | 8.8 | 112.2 | 10.6 | 146.2 | 15.8 |
| Lactate | 12.6 | 1.5 | 11.6 | 1.9 | 17.8 | 8.3 |
| **TA** | | | | | | |
| ATP | 35.5 | 1.7 | 43.3 | 2.8 *\$ | 30.3 | 7.5 |
| CP | 81.3 | 10.1 | 71.7 | 6.9 | 55.4 | 6.1 |
| Free Creatine | 59.4 | 4.2 | 43.8 | 12.9 | 75.2 | 21.6 |
| Total Creatine | 141.1 | 12.9 | 120.2 | 5.3 | 130.6 | 19.3 |
| Lactate | 11.9 | 2.7 | 16.9 | 1.0 | 16.5 | 7.5 |

KEY: EDL - Extensor Digitorum Longus; TA - Tibialis Anterior; ATP - Adenosine Triphosphate; CP - Creatine Phosphate;
*- $p<0.05$ v CHRONIC CONTROL; ** - $p<0.01$ v CHRONIC CONTROL;
\$- $p<0.05$ v PAIR FED; \$\$ - $p<0.01$ v CHRONIC PAIR FED;
\$ - All muscle metabolites presented as mM/kg dry muscle weight.

be the result of a 36% lower free creatine concentration in the EDL muscle of the CHRONIC ENDOTOXIN group rather than a change in creatine phosphate concentration. This difference in the concentration of free creatine did not, however, attain statistical significance. No difference was discerned in the muscle concentrations of creatine phosphate, free creatine or total creatine in the soleus and TA muscles of animals in the CHRONIC ENDOTOXIN group compared to CONTROLS ($p > 0.05$, Table 6.6). PAIR FED animals exhibited a 40% lower creatine phosphate concentration in the EDL muscle compared to the CHRONIC CONTROL group ($p < 0.05$, Table 6.6). This did not influence the EDL total creatine concentration as there was a coincident increase in the free creatine concentration of the EDL muscle of the PAIR FED group (Table 6.6).

Muscle ATP concentration in the PAIR FED animals was not different when compared to CHRONIC CONTROL animals for the soleus, EDL or TA ($p > 0.05$, Table 6.6). No discernible difference was detected in the concentration of creatine phosphate, free creatine or total creatine in the soleus, EDL or TA muscles of the PAIR FED group compared to the CONTROL animals ($p > 0.05$, Table 6.6).

Muscle lactate concentration was not different in the muscles of the CHRONIC ENDOTOXIN or PAIR FED groups compared to muscles of CHRONIC CONTROL animals ($p > 0.05$, Table 6.6).

### 6.3.2.4 Summary of changes following chronic endotoxin administration.

Following the second endotoxin challenge reduced serum concentrations of albumin and zinc, reduced body and TA muscle weight and increased liver weight was noted. These changes have been reported by previous workers following endotoxin administration (see Chapter 1). It should be noted that the soleus and EDL muscle mass was not different in endotoxin and non-endotoxin challenged animals, hence loss of muscle tissue is not as ubiquitous as suggested in some of the reviews (e.g. Beisel 1977). Despite the apparent production of endotoxemia following repeated administration of *E.coli* endotoxin over 24 hours no impairment of skeletal muscle function was noted, though a tendency for raised relative force generation in the EDL muscle was apparent. This may be related to the reduced food intake in these animals since it was also present in the PAIR FED animals. Generally, no discernible difference in the concentration of ATP, creatine or lactate were noted.

## 6.4 <u>DISCUSSION.</u>

The aim of this study was to investigate changes in skeletal muscle contractile characteristics associated with infectious insults. This used a model of infection resulting from *E.coli* endotoxin administration which is believed to be associated with the progress of many bacterial infections. In particular the effects of endotoxemia on the contractile characteristics of muscles selected to reflect the different muscle fiber types was specifically examined.

The development of endotoxemia in the rats of the present study was confirmed by the loss of body weight (7–8%), lower TA muscle weight (9%), raised relative liver weight (9%) and reduced serum concentrations of albumin (36%) and zinc (49%). Blood lactate concentration, soleus and EDL weight and protein concentration of all three muscles studied was not discernibly different from that of CONTROL animals. Twitch characteristics of the soleus, EDL and TA muscles were not impaired by endotoxin administration (either following single or repeated administration), or pair feeding animals at the level of intake of the endotoxin administered animals. No alteration in tetanic force or fatigue susceptibility was detected. A tendency for fast–twitch muscles to exhibit a shift to the left in the force–frequency relationship was apparent but did not attain statistical significance. No significant difference in the muscle concentrations of ATP, creatine or lactate was observed.

### 6.4.1 <u>Body and organ weight.</u>

Body weight of animals 8 hours following endotoxin administration was unaltered, but loss of body mass was greater at 32 hours, following a second challenge with *E.coli* endotoxin (i.e. CHRONIC ENDOTOXIN). This loss of body weight at 32 hours following endotoxin administration appears to be the result of reduced food intake since the PAIR FED animals exhibited a similar loss of body mass at 32 hours. This therefore agrees with reports from the literature of both reduced food intake and body weight although it should be noted that different studies have used different endotoxin doses, routes of administration and exposure periods (Long *et al* 1982; Baracos *et al* 1983; Ruff & Secrist 1984; Jepson *et al* 1988; Wan & Grimble 1986). The lower body weight of 7% in the CHRONIC ENDOTOXIN group is less than that which was observed following 1 day fasting (9%, data not presented), but greater than that observed following 1 day of food restriction at 25% of normal consumption (4%, data not presented). This suggests that the metabolic response to infection is greater than the response to food restriction at the same time point and less than that due to fasting.

Contrary to reports in the literature, however, muscle weight generally appeared unchanged following endotoxin treatment. The TA muscle exhibited a 8–9% lower mass at 32 hours compared to the CONTROL animals. This appears to be the result of a reduced food intake

since PAIR FED animals exhibited a comparable decrease in muscle mass. It is important however, to note that the reduced food intake in fasted animals did not result in loss of TA muscle tissue (Chapters 4). This suggests that the difference was present in animals limited in food intake but remained undetected or alternatively, the animals in the endotoxin study were more susceptible to reduced food intake than in previous studies. As there would appear to be a large inherent inter–animal variation in muscle mass it is possible that alteration in muscle mass may go undetected. This is unlikely, however, due to the large mass of the TA muscle a small variation of which should be readily detectable. As the soleus and EDL muscles did not atrophy, the reported loss of muscle tissue in endotoxin–treated animals is not as ubiquitous as some reviews would suggest (Beisel 1977). This is supported by the lack of atrophy of the EDL muscle in the study of Moldawer and associates (Moldawer, Svaginger, Gelin & Ludholm 1987).

Liver weight was not different 8 hours post endotoxin administration than that of CONTROL animals, but was greater in animals receiving the second endotoxin challenge. Since PAIR FED animals did not exhibit this elevated liver weight it can be concluded that this was a response to endotoxin administration. This may be associated with the initiation of the acute phase response which would be expected to have commenced by this time (see Beisel 1977 and Chapter 1).

Despite lower TA muscle weight and raised liver weight in the animals receiving two endotoxin challenges no statistically significant changes in total muscle and liver protein content was discernible. Thus, for the TA muscle, atrophy must be associated with loss of non–protein components, although from this study the identity of such components remains unclear. In the case of the liver, the increased synthesis and release of some acute phase reactants and reduced synthesis/release of other reactants coupled to sequestration of micronutrients (see Chapter 1) might explain these observations.

6.4.2 Contractile characteristics.

Force generation in the soleus, EDL and TA muscles of rats receiving single or repeated challenge with endotoxin was not different to that of CONTROL animals. Similarly animals of the PAIR FED group exhibited twitch and tetanic forces of the same magnitude as CHRONIC ENDOTOXIN (32 hours) and CHRONIC CONTROL animals. This preservation of force generation was observed following limitation of both quantity (Chapter 4) and quality (Chapter 5) of food supplied. Thus even for the TA muscle of animals receiving two challenges of endotoxin, where muscle mass was reduced, the ability of muscle to generate force is maintained. This therefore appears to be a universal response to reduced food intake.

The preservation of force generation in the TA muscle of animals receiving two doses of

endotoxin could be explained by the apparent preservation of total muscle protein content. Thus it could be suggested that while the mass of the TA muscle was lower in the endotoxin treated animals this was not the result of a loss of contractile proteins. Thus the number of crossbridges which can attach remains unaltered and the force generation is apparently preserved in the face of atrophy. No evidence is available from the data collected in this study to confirm the preservation of contractile elements in the face of endotoxin–mediated reductions in skeletal muscle mass.

Other twitch characteristics were also preserved in the animals administered endotoxin and in the PAIR FED group. This would therefore tend to agree with the data presented earlier under conditions of limiting food intake (Chapter 4), suggesting that a simple reduction in food intake is not responsible for changes in the variables regulating the contractile characteristics of skeletal muscles. This has important implications for the interpretation of clinical data (e.g. Lopes *et al* 1982; Russell *et al* 1983a,b) and is discussed further in the general discussion (see Chapter 8). This finding, however, is not consistent with the report of Drew and colleagues (Drew *et al* 1988). In their study of hamsters subjected to up to 12 weeks of infection with *L.donavani*, shifts in the force–frequency relationship and reduced maximal force generation was noted. This was also apparent in paired animals exhibiting a 50% reduction in body mass. It would therefore appear that the observations of Drew *et al* (1988) are the result of the excessive loss of weight of these animals which is more comparable to that seen in the animals limited in the composition of food (Chapter 5) than those limited in amount of food consumed (Chapter 4). It is important to stress that considerable differences in the animal models exist, particularly that a 12 week parasitic infection is likely to place a greater demand upon the body than the 32 hours of endotoxin administration of the present study. It is therefore possible that the alteration noted in these hamster muscles may be dependent upon factor(s) other than endotoxin.

No alteration in the force–frequency relationship of the soleus muscle was observed in endotoxin–challenged animals. The fast–twitch muscles, however, did exhibit a tendency for a shift in this relationship. Eight hours following endotoxin challenge the EDL muscle exhibited a shift to the right at all frequencies which, at present, remains ill–explained. The TA muscle at this time point exhibited a tendency for raised force generation at low frequencies of stimulation (i.e. a shift to the left) although this did not attain statistical significance. Following 32 hours of endotoxin administration (i.e. 8 hours after the second endotoxin challenge) the EDL and TA muscles exhibited a trend for raised relative force generation, which was apparent in the EDL of PAIR FED animals. This would agree with data presented earlier concerning food restriction where a similar trend was observed in the EDL muscle (see Chapter 4). It would appear that a reduction in food intake initiates a susceptibility to changes in the manner in which the muscle contracts at intermediate frequencies of stimulation, without altering the twitch force or maximal achievable force generation. Thus, as previously suggested, this shift in the force–frequency

relationship may reflect the abnormal frequencies over which the force—frequency curve is constructed rather than a functional change within the skeletal muscle.

In none of the muscles studied was there evidence of increased fatigue susceptibility. This again appears to agree with the data presented for food restriction (Chapter 5) where a tendency for a shift in the force—frequency relationship was observed with no change fatigue susceptibility. This further implies that the lethargy that human subjects associate with infectious insults (e.g. that associated with influenza) is unlikely to lie at the peripheral level, i.e. within the muscle itself, but is more likely to be at the central (i.e. motivational) level (see Chapter 1).

### 6.4.3 Skeletal muscle metabolites.

In general, no difference in the muscle concentration of ATP, creatine or lactate was discernible in the ENDOTOXIN or PAIR FED animals. However, the ATP concentration of the TA muscle of animals challenged twice with endotoxin was raised, total creatine of the EDL muscle reduced and creatine phosphate of the EDL muscle of PAIR FED animals low, compared to CONTROL concentrations. It is unclear what these changes reflect in the absence of changes in other metabolites. For example, the creatine phosphate and free creatine concentrations of the EDL muscle of the animals receiving repeated challenge with endotoxin are within the normal range, yet total muscle creatine is reduced. Food restricted animals in the previous studies (Chapter 4), however, did not exhibit this response and does not aid in the analysis of the data. The soleus muscle of the ACUTE CONTROL animals exhibited raised lactate concentrations which may be the result of impaired tissue perfusion. This high lactate concentration for the ACUTE CONTROL group would appear to be biased by some animals only, as demonstrated by the large sem for this group of animals. The lower values noted in the ACUTE ENDOTOXIN group are more likely to reflect more normal levels.

Despite the apparent reductions in creatine and creatine phosphate concentrations for the EDL and possibly other muscles of the PAIR FED and CHRONIC ENDOTOXIN groups there did not appear to be a relationship between these resting metabolite concentrations and skeletal muscle contractile characteristics.

## 6.5 SUMMARY.

No impairment of skeletal muscle function was observed in muscles selected to reflect the different muscle fiber types despite the inference of the production of endotoxemia suggested by the loss of body and muscle weight, the lower serum concentrations of albumin and zinc and the raised liver weight, either early or late in the response to endotoxin. Changes in muscle function reported during infection (e.g. Drew *et al* 1988) would therefore appear to be the result of unidentified factor(s) either alone or in conjunction with endotoxin (IL−1/TNF) exhibiting a facilitative action on normal body and muscle function.

## CHAPTER 7.

# THE COMBINED INFLUENCE OF MALNUTRITION AND INFECTION ON SKELETAL MUSCLE CONTRACTILE CHARACTERISTICS.

## 7.1 <u>INTRODUCTION.</u>

The lower physical working capacity of malnourished individuals in developing countries reported by many workers (see Spurr 1987) could result in a downward spiral for the well being of the individual and his/her immediate dependents. The reduced working capacity results in lower wage generation, thus less food can be purchased resulting in further lowering the physical working capacity. The physical working capacity of individuals is not however, solely dependent upon the ability of skeletal muscle to function. Changes may also occur at the central and cardiovascular levels (see Chapter 1). The assessment of skeletal muscle function independently of changes in these other systems by the development of a model of stunting and wasting in the rat by the use of low protein diets has suggested a degree of impairment at the peripheral level particularly associated with fatigue susceptibility ( Chapter 5).

Malnutrition in developing countries is often associated with infection. This may ultimately result in death. The physical working capacity of these malnourished, infected individuals might therefore be further reduced due to the additional demands associated with mounting an immune response. An animal model of infection, utilizing endotoxin administration in rats, has been presented in Chapter 6. Endotoxin administration was not associated with impairment of skeletal muscle function.

Animals therefore appear to accommodate a single stress with varying degrees. Food deprivation and restriction (Chapter 4) and endotoxin administration (Chapter 6) were not associated with impairment of skeletal muscle function while the consumption of low protein diets resulted in slowing of the twitch time of the soleus muscle, shifts in the force–frequency relationship of the fast–twitch muscles and a tendency for greater fatigue (Chapter 5). No evidence is available as to the ability of the body to accommodate metabolic stress associated with the combination of malnutrition and infection. Studies have indicated that immunocompetence in malnourished subjects is lowered (Smythe *et al* 1971; Powanda, Wannemacher & Cockerell 1972; Scrimshaw 1975; Tomkins *et al* 1983; Moldawer *et al* 1985; Long *et al* 1982) or unaltered (Young *et al* 1968; Drabik *et al* 1987). The differences in the findings of these studies may reflect the severity of malnourishment (see Section 1.2.2.4). In the light of such reports it may be expected that infection in the stunted and wasted animals consuming low protein diets (Chapter 5) would result in greater changes in skeletal muscle mass

and function than that observed following consumption of low protein diets alone.

This study therefore investigated the contractile characteristics of skeletal muscles of rats consuming low protein diets following an additional insult, that of endotoxin administration. As administration of endotoxin either once or twice did not further influence skeletal muscle contractile characteristics over and above that seen at 8 hours (Chapter 6), the present study investigated skeletal muscle function only at the 8 hour time point. This study investigated the function of muscles specifically selected to reflect the different muscle fiber types soleus (type I), extensor digitorum longus (EDL, type IIB) and the tibialis anterior (TA, type IIA/B).

## 7.2 METHODS.

The data quoted for non−endotoxin treated animals in this study are those previously presented in Chapter 5 since both experiments were conducted at the same time, thus all animals in this chapter are littermates to those reported in Chapter 5 and assessment of function was conducted together. Thus, in addition to the animals previously reported in Chapter 5, eighteen male Wistar rats (mean initial body weight 97 ± 1.9 g) were allocated to one of three dietary groups:

A) FED — consuming a 15% protein diet containing 580 mg sodium/Kg diet and 2930 mg potassium/kg diet for 21 days (n=6);

B) Low Protein, Low Electrolyte (LPLE)
— consuming a 0.5% protein diet containing 580 mg sodium/Kg diet and 2930 mg potassium/kg diet for 21 days (n=6);

and C) Low Protein, High Electrolyte (LPHE)
−consuming a 0.5% protein diet containing 4900 mg of both sodium and potassium/kg diet for 21 days (n=6).

The composition of these diets was presented in Table 2.2. All animals were maintained individually in a room maintained at 23°C with a 12 hour light−dark cycle and free access to water. Food was withdrawn for the eight hours immediately preceding assessment of skeletal muscle function. Body weight and food intake was monitored at the same point in each light period.

The animals received intraperitoneal *E.coli* endotoxin (0.5mg/kg body weight) following 21 days on these diets and assessment of skeletal muscle contractile characteristics was conducted eight hours later. It should be noted that this dose of endotoxin is approximately half that previously used (Chapter 6). This lower dose of endotoxin was necessary as the animals consuming the low protein diets were unable to tolerate the larger dose of endotoxin, resulting in

the death of two animals, one from the low protein, low electrolyte group and one form the low protein, high electrolyte group, when anaesthetic was administered.

On the day of study animals were anaesthetised using 0.44 mls Sagatal/kg body weight and prepared for measurement as described in Section 2.1. Assessment of contractile characteristics was conducted as reported in Section 2.2 thus the time to peak tension (TPT), half relaxation time (½RT) & peak twitch tension (PT) of the twitch and the maximal tetanic force (Fmax) & force frequency relationship were examined.

Following assessment of contractile characteristics the muscles of both hindlimbs were rapidly excised, weighed and stored as previously described (see Section 2.3). Full carcus analysis was completed with assessment of serum albumin and zinc concentrations (see Section 2.4).

The metabolite profile of muscles from some of the animals in the LOW PROTEIN, HIGH ELECTROLYTE + ENDOTOXIN group were assessed. Since these were not different from the metabolite profile of FED and CONTROL animals this data has not been presented for two reasons. Firstly, previous chapters have identified that resting muscle metabolite concentrations did not correlate with skeletal muscle contractile characteristics. Secondly, had the data been presented it would not have been complete.

## 7.3 RESULTS.

Before discussing the results of the additional influence of endotoxin administration in malnourished rats on skeletal muscle contractile characteristics, it may be convenient to remind the reader of the findings relating to those animals consuming normal or low protein diets not receiving endotoxin, previously described in Chapter 5.

Animals in the FED group, consuming a 15% (normal) protein diet exhibited a normal growth patter resulting in an increase in body and muscle weight of 2-2.5 fold. This increased muscle weight was probably responsible for the raised force generation in Newtons measured in the soleus, EDL and TA muscles. No difference in the force—frequency relationship or fatigue susceptibility was discernible in any muscles compared to those of CONTROL rats.

Little difference was noted between the characteristics of animals consuming the low protein, low electrolyte and low protein, high electrolyte diets. Thus, these animals were stunted and wasted to a similar degree when compared to the FED animals. Force generation in Newtons was preserved despite a lower muscle mass, but the force generating capacity was increased when expressed per unit muscle in comparison to CONTROL and FED animals. Time to peak tension and half relaxation time of the soleus muscle of animals consuming both low protein diets was prolonged. The force—frequency relationship of the soleus muscle was not different to

that of CONTROL animals. The force—frequency relationship of the fast—twitch muscles tended to exhibit raised relative force generation at 50 Hz, attaining statistical significance in the EDL of animals consuming the low protein, low electrolyte diet and the TA of animals consuming the low protein, high electrolyte diet. Fatigue susceptibility tended to be increased following consumption of low protein diets, although muscles of some animals exhibited fatigue characteristics similar to that of CONTROL animals.

### 7.3.1 The effect of endotoxin administration to animals consuming a normal protein diet on skeletal muscle contractile characteristics.

This study describes the administration of endotoxin to animals consuming the 15% protein diet for a period of 21 days prior to the administration of endotoxin. The data for the non—endotoxin group has previously been presented in Chapter 5, although the endotoxin studies were conducted at the same time as those presented earlier. For further discussion of normal animals receiving endotoxin the reader is referred to the discussion (Chapters 6 & 8).

Eight hours following endotoxin administration body weight was slightly lower than that of animals not receiving endotoxin, but this difference did not attain statistical significance (5%, $p > 0.05$, Table 7.1). Similarly, snout—to—anus length, body mass index and liver weight were not different between both groups ($p > 0.05$, Table 7.1). Soleus, EDL and TA muscle weight was approximately 34, 16 and 12% lower in animals receiving endotoxin challenge respectively ($p < 0.05$ v FED, Table 7.1). This was not associated with statistically significant changes in total protein content of the soleus and EDL ($p > 0.05$) while the TA muscle exhibited a 19% lower total protein content than non—endotoxin treated FED animals ($p < 0.05$, Table 7.1) Serum concentrations of albumin were not different following endotoxin administration, but serum zinc concentrations were 48% greater than that of animals not challenged with endotoxin ($p < 0.05$, Table 7.1).

No statistically significant differences in the time to peak tension, half relaxation time, peak twitch tension or fatigue susceptibility were observed in the animals consuming a normal protein diet and receiving endotoxin when compared to the non—endotoxin treated FED group (Table 7.2 and Figure 7.1). Peak twitch tension was 32% lower in the soleus muscle of endotoxin—treated animals ($p > 0.05$), 70% lower in the TA ($p < 0.05$) and 15% greater in the EDL ($p > 0.05$). When expressed per unit muscle weight no statistically significant differences between the muscles of endotoxin—treated and control animals could be discerned ($p > 0.050$. Maximal force generation was lower in all three muscles of endotoxin treated animals, attaining statistical significance for the soleus (56%, $p < 0.05$) and TA (76%, $p < 0.01$) muscles. When expressed per unit muscle the difference in force generation capacity attained statistical significance only for the TA muscle (77%, $p < 0.01$, Table 7.2). There was no alteration in the force—frequency relationship of soleus,

Table 7.1. The influence of endotoxin administration on body variables of animals consuming a diet of normal (15%) protein content.

| | FED$ n=6 | | FED + ENDOTOXIN n=6 | |
|---|---|---|---|---|
| | mean | se | mean | se |
| Body Weight (g) | 230.0 | 5.6 | 219.0 | 4.0 |
| Snout to Anus Length (mm) | 200.0 | 2.9 | 199.7 | 3.1 |
| Body Mass Index! | 57.3 | 1.4 | 55.2 | 1.6 |
| | | | | |
| Liver Weight (g) | 9.9 | 0.3 | 9.7 | 0.3 |
| Soleus muscle Weight (mg) | 117.4 | 7.7 | 77.2 | 5.3 * |
| EDL muscle Weight (mg) | 100.6 | 4.9 | 84.6 | 5.9 * |
| TA muscle weight (mg) | 437.4 | 4.9 | 384.7 | 12.6 * |
| | | | | |
| Liver Protein Content (g) | 1.80 | 0.13 | 1.49 | 0.05 |
| Soleus Protein Content (mg) | 21.8 | 3.9 | 25.9 | 3.7 |
| EDL protein Content (mg) | 25.1 | 3.3 | 19.2 | 1.4 |
| TA protein Content (mg) | 137.8 | 2.8 | 111.5 | 10.6 * |
| | | | | |
| Serum Albumin Concentration (mg/ml) | 33.7 | 3.8 | 34.7 | 1.5 |
| Serum Zinc Concentration($\mu$g/ml) | 2.1 | 0.2 | 3.1 | 0.5 ** |

KEY: EDL - Extensor Digitorum Longus; TA - Tibialis Anterior;

$ - see Chapter 5;

! - Body Mass Index = body weight/ (snout-to-anus length)$^2$;

* - p<0.05 v FED by students t-test;** - p<0.01 v FED.
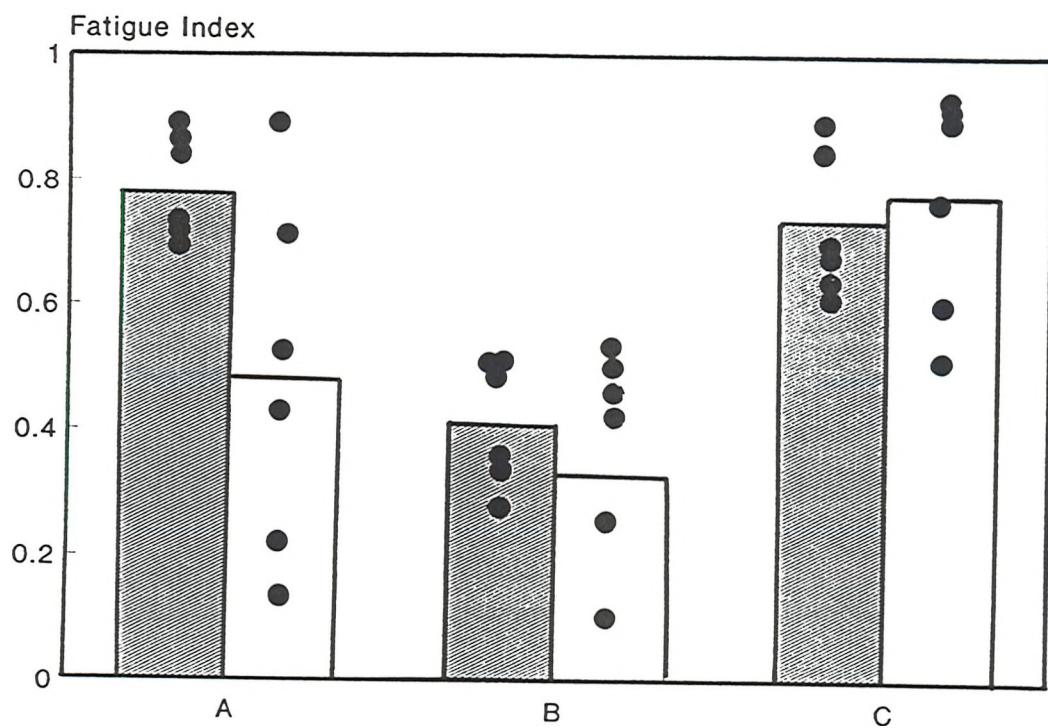
Figure 7.1. The fatigue characteristics of the soleus,
extensor digitorum longus and tibialis anterior musles
of animals consuming diets of normal protein content in
the presence (FED+E,☐, n=6) or absence (FED,☑, n=6)
of endotoxin administration. Bars represent the mean value.

Table 7.2. The effect of endotoxin administration on the contractile characteristics of rats consuming a diet of normal protein content (FED).

| | FED$^\$$ n=6 | | FED + ENDOTOXIN n=6 | |
|---|---|---|---|---|
| | mean | se | mean | se |
| **Soleus** | | | | |
| Time to Peak Tension (ms) | 32.2 | 1.9 | 33.0 | 2.6 |
| Half Relaxation Time (ms) | 33.8 | 5.5 | 41.9 | 5.1 |
| Peak Twitch Tension (mN) | 1.9 | 0.4 | 1.3 | 0.3 |
| Peak Twitch Tension ! | 15.9 | 3.4 | 16.1 | 2.5 * |
| Maximal Tetanic Tension (mN) | 11.2 | 1.2 | 4.9 | 1.2 |
| Maximal Tetanic Tension ! | 71.9 | 18.0 | 59.6 | 12.2 |
| Fatigue Index | 0.73 | 0.09 | 0.51 | 0.14 |
| **EDL** | | | | |
| Time to Peak Tension (ms) | 15.6 | 0.8 | 18.3 | 1.4 |
| Half Relaxation Time (ms) | 12.7 | 1.6 | 13.9 | 2.6 |
| Peak Twitch Tension (mN) | 2.0 | 0.5 | 2.3 | 0.3 |
| Peak Twitch Tension ! | 24.7 | 6.7 | 28.2 | 6.0 |
| Maximal Tetanic Tension ! (mN) | 14.8 | 4.3 | 9.7 | 1.9 |
| Maximal Tetanic Tension ! | 171.6 | 40.6 | 118.6 | 35.7 |
| Fatigue Index | 0.44 | 0.10 | 0.31 | 0.08 |
| **TA** | | | | |
| Time to Peak Tension (ms) | 12.7 | 1.0 | 13.8 | 0.6 |
| Half Relaxation Time (ms) | 10.9 | 1.3 | 13.3 | 0.3 ** |
| Peak Twitch Tension ! (mN) | 13.0 | 1.0 | 3.9 | 1.1 |
| Peak Twitch Tension ! | 20.5 | 4.2 | 10.5 | 3.0 * |
| Maximal Tetanic Tension ! (mN) | 40.3 | 7.5 | 9.6 | 1.9 ** |
| Maximal Tetanic Tension ! | 108.8 | 6.7 | 25.1 | 5.1 |
| Fatigue Index | 0.80 | 0.08 | 0.79 | 0.33 |

KEY: EDL – Extensor Digitorum Longus; TA – Tibialis Anterior; $ – See Chapter 5; ! – Force in mN/g muscle; * – $p < 0.05$ v FED; ** – $p < 0.01$ v FED.

EDL and TA muscles of animals challenged with endotoxin (Figure 7.2).

### 7.3.2 The influence of endotoxin administration following consumption of diets low in protein and low in electrolyte content on skeletal muscle contractile characteristics.

This section compares the contractile characteristics of the animals receiving endotoxin following consumption of low protein, low electrolyte diets for 21 days with those of animals consuming the low protein diet alone. The data for the non−endotoxin administered animals has previously been presented in Chapter 5.

Body weight, snout−to−anus length, organ weight and protein content of animals receiving endotoxin challenge following consumption of the low protein, low electrolyte diet was slightly lower than that of animals consuming the low protein diet alone (Table 7.3), but the difference did not attain statistical significance. Total liver protein content was 25% higher in the animals treated with endotoxin compared to those consuming the low protein, high electrolyte diet alone (p>0.05, Table 7.3). Both groups consuming the low protein, low electrolyte diet however, were smaller than animals consuming a diet of normal protein content (FED, compare Tables 7.1 and 7.3). Serum albumin and zinc concentrations were not statistically lower than that of animals not challenged with endotoxin (Table 7.3).

Time to peak tension, half relaxation time and peak twitch tension were not statistically different in the EDL and TA muscles of the animals receiving the endotoxin from those muscles of non−endotoxin treated animals consuming the low protein, low electrolyte diet. The time to peak tension and half relaxation time of the soleus muscle was 37% and 32% slower respectively than CONTROL (p<0.05 and p>0.05, Table 7.4). Maximal tetanic force per unit muscle tended to be lower in the endotoxin−treated animals than those not challenged with endotoxin. This difference attained statistical significance for the soleus muscle (58%, p<0.05, Table 7.4). All muscles of the endotoxin−challenged animals tended to fatigue more than those from animals not receiving endotoxin and the greater fatigue index attained statistical significance in the soleus muscle (63%, p<0.05, Table 7.4 and Figure 7.3). It should be noted, however that the tendency for increased fatigue in the animals receiving endotoxin resulted in a greater difference when compared to CONTROL or FED animals than seen for the non−endotoxin treated animals (compare with Chapter 5).

No difference in the force−frequency relationship of the soleus and EDL muscles was noted, but the TA muscle exhibited a greater relative force generation at 20 and 50 Hz compared to animals not receiving endotoxin (p<0.05, Figure 7.4).
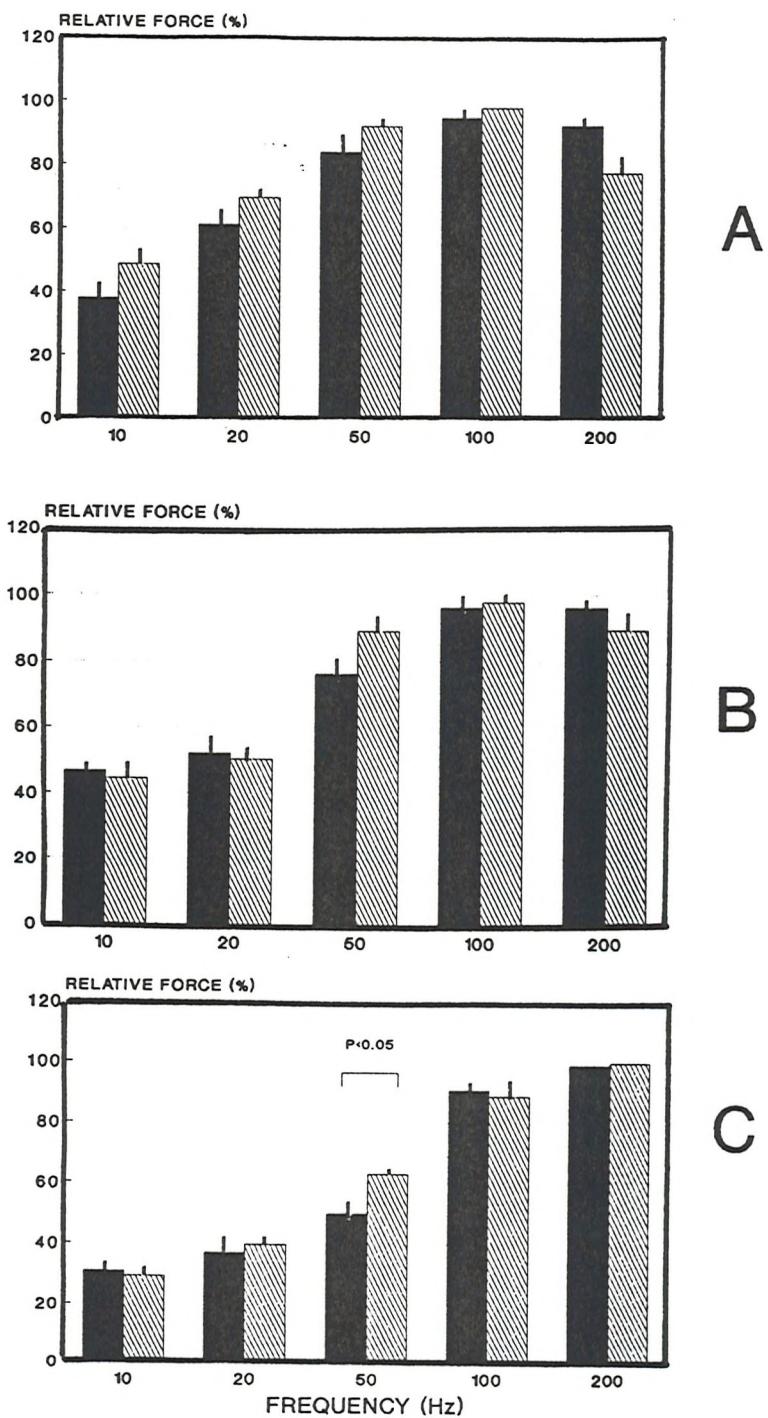
Figure 7.2. The force−frequency realtionship of the soleus
(a), extensor digitorum longus (b) and tibialis anterior (c)
muscles of animals  consuming diets of normal protein content
in the presence (FED+E,◺, n=6) or absence (FED,■, n=6)
of endotoxin administration. Bars represent mean ± 1 sem.

Table 7.3. The effect of endotoxin administration on body variables
of animals consuming a low protein, low electrolyte diet.

|  | LOW PROTEIN $ LOW ELECTROLYTE | | LOW PROTEIN LOW ELECTROLYTE + ENDOTOXIN | |
|---|---|---|---|---|
|  | mean | sem | mean | sem |
|  | n=6 | | n=5 | |
| Body Weight (g) | 73.0 | 2.9 | 71.6 | 2.7 |
| Snout to Anus Length (mm) | 144.8 | 1.2 | 132.0 | 7.8 |
| Body Mass Index ! | 34.7 | 1.0 | 44.7 | 7.9 |
| Liver Weight (g) | 2.80 | 0.15 | 2.46 | 0.09 |
| Soleus muscle Weight (mg) | 37.7 | 0.4 | 30.4 | 1.8 |
| EDL muscle Weight (mg) | 35.4 | 2.8 | 31.3 | 1.3 |
| TA muscle weight (mg) | 160.9 | 6.0 | 130.9 | 5.4 |
| Liver Protein Content (g) | 0.57 | 0.04 | 0.71 * | 0.04 |
| Soleus Protein Content (mg) | 6.4 | 1.0 | 4.1 | 0.6 |
| EDL protein Content (mg) | 7.2 | 1.3 | 4.7 | 0.6 |
| TA protein Content (mg) | 33.8 | 1.7 | 28.9 | 4.3 |
| Serum albumin concentration (mg/ml) | 22.2 | 0.9 | 26.30 | 2.10 |
| Serum Zinc Concentration ($\mu$g/ml) | 1.2 | 0.25 | 0.98 | 0.38 |

KEY: EDL - Extensor Digitorum Longus; TA - Tibialis Anterior;

$-  See Chapter 5; * - $p<0.05$ v Low protein, low electrolyte.

! - Body Mass Index = body weight/ (snout-to-anus length)$^2$.

Table 7.4. The effect of endotoxin administration on the contractile characteristics of the soleus, extensor digitorum longus (EDL) and tibialis anterior (TA) muscles of animals consuming a low protein, low electrolyte diet.

|  | LOW PROTEIN LOW ELECTROLYTE$ n=6 | | LOW PROTEIN LOW ELECTROLYTE + ENDOTOXIN n=5 | |
|---|---|---|---|---|
|  | mean | sem | mean | sem |
| **Soleus** | | | | |
| Time to Peak Tension (ms) | 46.6 | 5.2 | 29.2 | 7.1 * |
| Half Relaxation Time (ms) | 46.8 | 3.3 | 31.7 | 10.9 |
| Peak Twitch Tension (mN) | 1.0 | 0.2 | 0.4 | 0.1 |
| Peak Twitch Tension ! | 25.1 | 7.0 | 13.8 | 3.8 * |
| Maximal Tetanic Tension (mN) | 5.0 | 0.4 | 2.1 | 0.44 ** |
| Maximal Tetanic Tension ! | 139.4 | 4.7 | 67.0 | 10.6 * |
| Fatigue Index | 0.64 | 0.15 | 0.24 | 0.06 |
| **EDL** | | | | |
| Time to Peak Tension (ms) | 15.8 | 0.5 | 14.9 | 0.5 |
| Half Relaxation Time (ms) | 10.4 | 0.8 | 11.9 | 1.6 |
| Peak Twitch Tension (mN) | 2.6 | 0.4 | 2.9 | 0.3 |
| Peak Twitch Tension ! | 72.4 | 8.5 | 88.5 | 6.2 |
| Maximal Tetanic Tension (mN) | 8.2 | 1.6 | 7.6 | 0.9 |
| Maximal Tetanic Tension ! | 233.7 | 37.9 | 239.3 | 40.4 |
| Fatigue Index | 0.22 | 0.09 | 0.11 | 0.03 |
| **TA** | | | | |
| Time to Peak Tension (ms) | 14.3 | 0.5 | 15.6 | 0.3 |
| Half Relaxation Time (ms) | 12.7 | 0.6 | 14.5 | 2.2 |
| Peak Twitch Tension (mN) | 6.5 | 0.5 | 3.5 | 0.4 |
| Peak Twitch Tension ! | 35.1 | 4.5 | 25.2 | 2.5 |
| Maximal Tetanic Tension (mN) | 21.8 | 3.3 | 16.7 | 6.4 |
| Maximal Tetanic Tension ! | 117.2 | 23.7 | 125.8 | 46.5 |
| Fatigue Index | 0.50 | 0.14 | 0.29 | 0.09 |

KEY : * – $p < 0.05$, ** – $p < 0.01$ v LOW PROTEIN, LOW ELECTROLYTE.
! – force in N/ g muscle; $ – See Chapter 5.
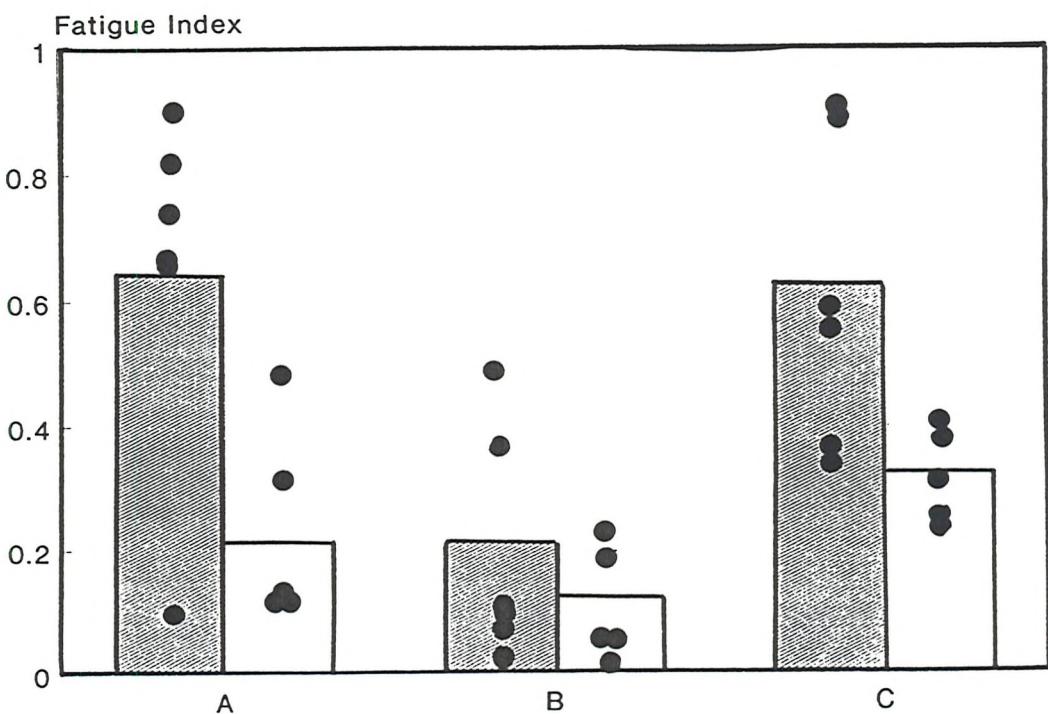
Figure 7.3. The fatigue characteristics of the soleus, extensor digitorum longus and tibialis anterior muscles of animals consuming a low protein, low electrolyte diet in the presence (LPLE+E,□,n=5 and absence (LPLE☑, n=6) of endotoxin administration.
Bars represent the mean value.

### 7.3.3 The influence of endotoxin administration following consumption of diets low in protein and high in electrolyte content on skeletal muscle contractile characteristics.

This section will discuss the contractile characteristics of animals receiving endotoxin following consumption of the low protein, high electrolyte diet. The results will be compared to those of animals not receiving endotoxin, previously reported in Chapter 5.

Body weight, snout–to anus–length and organ weights of animals receiving endotoxin challenge following consumption of the low protein, high electrolyte diet were slightly lower than those of animals not given endotoxin, but this difference did not attain statistical significance (Table 7.5). Organ protein content was not different in animals consuming low protein, high electrolyte diet receiving endotoxin than that measured in animals consuming the low protein diet alone (Table 7.5). Body, muscle and liver weights were lower than those of animals consuming a diet of normal protein content (compare v FED Tables 7.1 and 7.5). Serum albumin concentration was not lower in the endotoxin–treated animals compared to the untreated group, but serum zinc was 77% lower (p<0.05, Table 7.5).

Time to peak tension and half relaxation time were not statistically different in the animals challenged with endotoxin than in those consuming the low protein high electrolyte diet for the soleus and TA muscles. However, a 18% slowing of the EDL muscle time to peak tension was observed (p<0.05) and a 28% slowing of half relaxation time (p<0.05, Table 7.6). Peak twitch tension was lower in all three muscles of endotoxin treated animals, attaining statistical significance for the soleus muscle (77%, p<0.05). Maximal tetanic tension was similarly reduced, being 57% lower in the soleus muscle of endotoxin treated animals than those consuming the low protein, high electrolyte diet alone (p<0.05), 44% lower in the EDL (p<0.05) and 42% lower in the TA (p>0.05). When expressed per unit muscle, the capacity to generate force was lower in all three muscles of the endotoxin treated group, but this difference did not attain statistical significance (p>0.05, Table 7.6). Fatigue susceptibility was greater in muscles of animals challenged with endotoxin following consumption of the low protein, high electrolyte diet when compared to the animals consuming the low protein, high electrolyte diet alone, attaining significance for the soleus muscle (69%, p<0.05, Table 7.6 and Figure 7.5). The difference in fatigue susceptibility however, was large in comparison to those animals of the FED and CONTROL groups (compare Chapter 5).

The soleus muscle of endotoxin challenged animals exhibited enhanced relative force generation at 10 Hz compared to those consuming the low protein, high electrolyte diet (p<0.05, Figure 7.6), but no alteration was not apparent in the EDL or TA muscles (Figure 7.6).
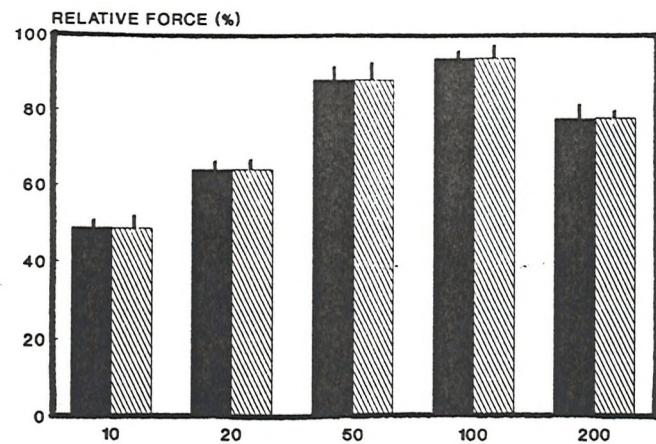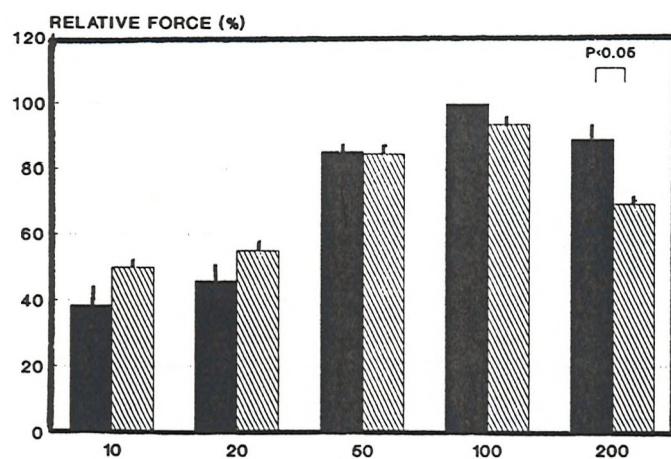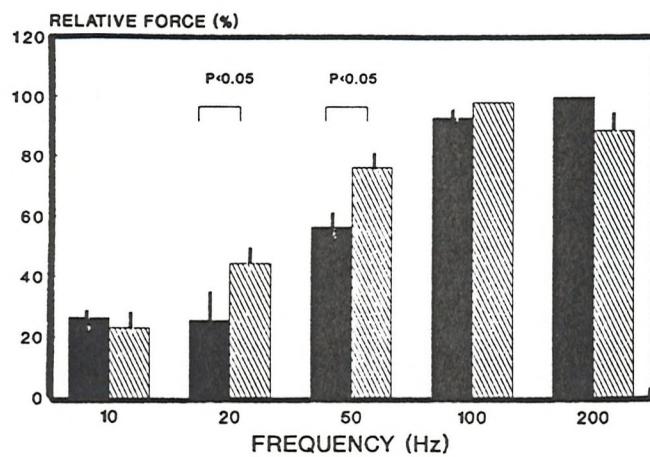
Figure 7.4. The force—frequency relationship of the soleus
(A), extensor digitorum longus (EDL, B) and tibialis anterior
(TA, C) muscles of animals consuming a low protein, low
electrolyte diet in the presence (LPLE+F,▨,n=5 and absence
(LPLE,■, n=6) of endotoxin administration.
Bars represent mean ± 1 sem.

Table 7.5. The influence of endotoxin administration on body variables of rats consuming a low protein, high electrolyte diet.

| | LOW PROTEIN HIGH ELECTROLYTE $ | | LOW PROTEIN HIGH ELECTROLYTE + ENDOTOXIN | |
|---|---|---|---|---|
| | n=6 | | n=5 | |
| | mean | se | mean | se |
| Body Weight (g) | 71.7 | 0.9 | 68.1 | 2.9 |
| Snout to Anus Length (mm) | 145.7 | 1.5 | 137.6 | 1.7 |
| Body Mass Index ! | 33.7 | 0.6 | 36.0 | 1.5 |
| Liver Weight (g) | 2.50 | 0.06 | 2.27 | 0.03 |
| Soleus muscle Weight (mg) | 32.8 | 1.9 | 30.4 | 1.8 |
| EDL muscle Weight (mg) | 36.6 | 2.7 | 31.3 | 1.7 |
| TA muscle weight (mg) | 153.9 | 9.0 | 121.2 | 4.2 |
| Liver Protein Content (g) | 0.37 | 0.02 | 0.34 | 0.01 |
| Soleus Protein Content (mg) | 9.7 | 1.2 | 7.4 | 1.9 |
| EDL protein Content (mg) | 6.9 | 1.2 | 8.3 | 1.3 |
| TA protein Content (mg) | 42.8 | 8.2 | 45.3 | 9.5 |
| Serum Albumin Concentration (mg/ml) | 24.9 | 2.6 | 22.8 | 0.02 |
| Serum Zinc Concentration ($\mu$g/ml) | 1.8 | 0.45 | 0.42 * | 0.06 |

KEY: EDL -Extensor digitorum Longus; TA - Tibialis Anterior;

$ - See Chapter 5;

! - Body Mass Index = body weight /(snout-to-=anus length)$^2$

* - p<0.05 v LOW PROTEIN, HIGH ELECTROLYTE.

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Table 7.6. The influence of endotoxin administration on the contractile characteristics of the soleus, extensor digitorum longus (EDL) and tibialis anterior (TA) muscles of animals consuming a low protein, high electrolyte diet.

| | LOW PROTEIN HIGH ELECTROLYTE $ | | LOW PROTEIN HIGH ELECTROLYTE + ENDOTOXIN | |
|---|---|---|---|---|
| | n=6 | | n=5 | |
| | mean | se | mean | se |
| **Soleus** | | | | |
| Time to Peak Tension (ms) | 40.1 | 2.1 | 38.4 | 5.2 |
| Half Relaxation Time (ms) | 44.8 | 6.9 | 39.2 | 7.2 |
| Peak Twitch Tension (mN) | 0.9 | 0.2 | 0.3 | 0.08 * |
| Peak Twitch Tension ! | 26.0 | 6.7 | 8.8 | 2.3 * |
| Maximal Tetannic Tension (mN) | 5.6 | 1.4 | 2.4 | 0.4 |
| Maximal Tetanic Tension ! | 164.9 | 42.3 | 73.9 | 13.3 * |
| Fatigue Index | 0.73 | 0.10 | 0.23 | 0.09 * |
| **EDL** | | | | |
| Time to Peak Tension (ms) | 16.8 | 0.4 | 13.8 | 0.6 ** |
| Half Relaxation Time (ms) | 12.9 | 0.9 | 9.2 | 1.2 ** |
| Peak Twitch Tension (mN) | 2.4 | 0.4 | 1.3 | 0.2 * |
| Peak Twitch Tension ! | 75.5 | 11.2 | 39.8 | 6.5 |
| Maximal Tetannic Tension (mN) | 8.1 | 1.1 | 4.5 | 0.8 * |
| Maximal Tetanic Tension ! | 218.6 | 33.3 | 140.4 | 2.7 |
| Fatigue Index | 0.31 | 0.08 | 0.12 | 0.22 |
| **TA** | | | | |
| Time to Peak Tension (ms) | 15.9 | 2.2 | 14.0 | 1.5 |
| Half Relaxation Time (ms) | 13.2 | 2.3 | 11.7 | 1.1 |
| Peak Twitch Tension (mN) | 7.7 | 1.0 | 3.4 | 0.9 |
| Peak Twitch Tension ! | 44.3 | 4.3 | 28.7 | 7.8 |
| Maximal Tetannic Tension (mN) | 18.9 | 2.8 | 11.0 | 1.8 |
| Maximal Tetanic Tension ! | 106.6 | 1.8 | 89.7 | 6.7 |
| Fatigue Index | 0.42 | 0.14 | 0.27 | 0.09 |

KEY : *- p,0.05, ** - p<0.01 v Low Protein, High Electrolyte.
   $- See Chapter 5; ! - force in mN/g muscle.
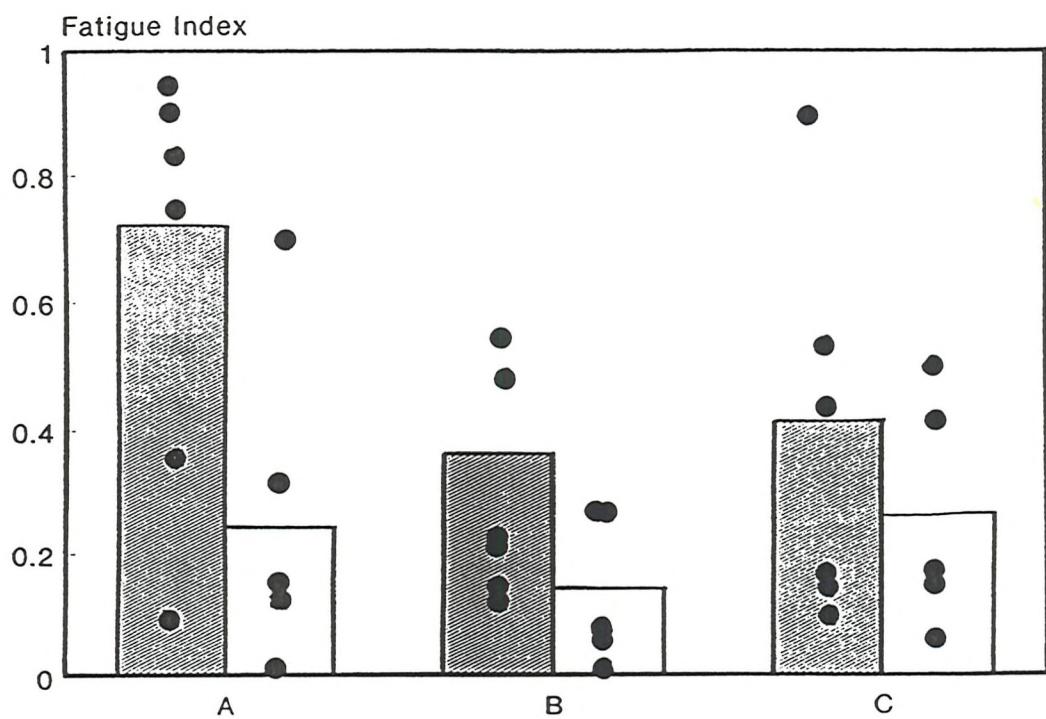
Figure 7.5. The fatigue characteristics of the soleus (A), extensor digitorum longus (EDL, B) and the tibialis anterior (TA, C) muscles of animals consuming a low protein, high electrolyte diet in the presence (□ , n=5) or absence (☑ , n=6) of endotoxin administration. Bars represent the mean value.
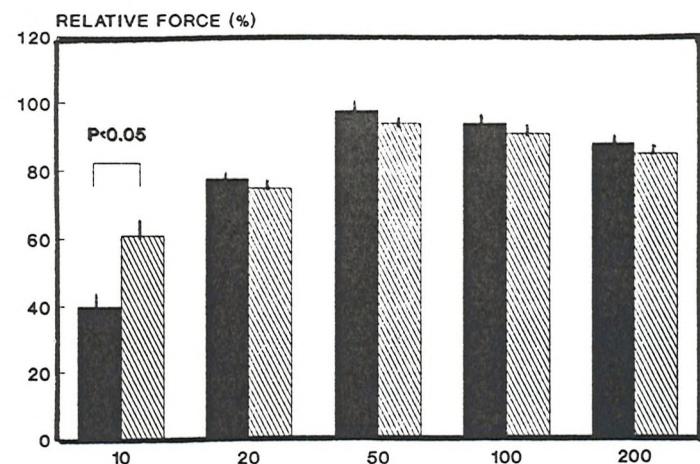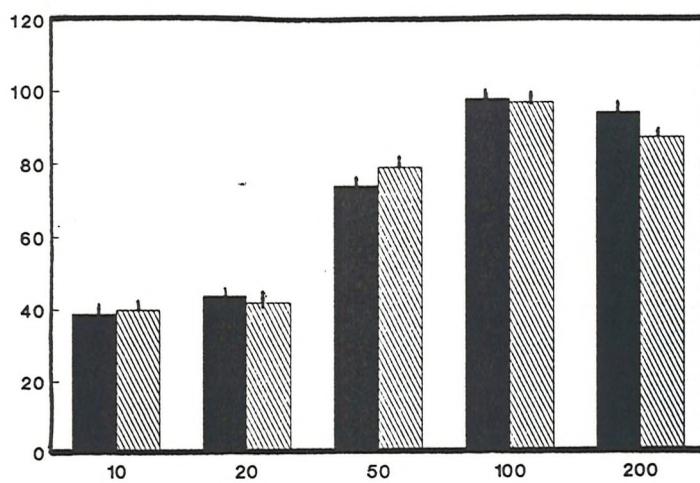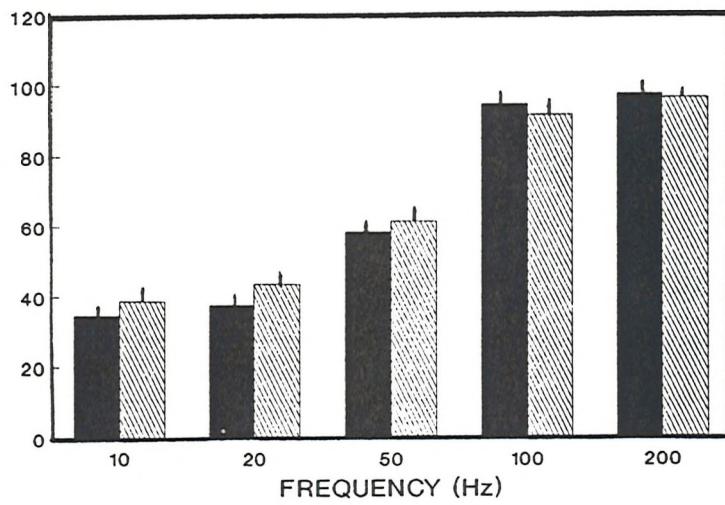
Figure 7.6. The force–frequency relationship of the soleus (A), extensor digitorum longus (EDL, B) and the tibialis anterior (TA, C) muscles of animals consuming a low protein, high electrolyte diet in the presence ( ☑ , n=5) or absence ( ◼ , n=6) of endotoxin administration. Bars represent the mean value ± 1 sem.

### 7.3.4 <u>Summary.</u>

Generally, the animals treated with endotoxin following consumption of low protein diets exhibited body and muscle mass changes of a similar magnitude to that of animals consuming the low protein diets alone. Thus, animals of the FED group exhibited normal growth pattern while animals consuming both the low protein, low electrolyte and high electrolyte diet were stunted and wasted compared to the FED group. There was a tendency for animals in the endotoxin group to have lower body weight and organ weight than the non−endotoxin control animals and snout−to anus−length was shorter.

The contractile characteristics of the FED animals receiving endotoxin were not different from that of untreated animals. There was, however, a tendency for maximal tetanic force to be lower in the endotoxin treated animals.

The contractile characteristics of animals in the low protein, low electrolyte group were also not different from the endotoxin−treated and untreated animals. Thus, the soleus muscle of both groups exhibited prolongation of the twitch time in comparison to that of FED or FED + ENDOTOXIN animals. The fatigue index of muscles tended to be lower in the endotoxin treated group. The TA muscle of endotoxin treated animals also exhibited raised relative force generation at 20 and 50 Hz compared to the non−treated group.

Similar trends were noted in the animals consuming the low protein, high electrolyte diet. The twitch characteristics of endotoxin−treated animals was not different from untreated animals, thus the soleus muscle exhibited a prolongation of twitch time compared to the FED groups. Fatigue susceptibility was generally increased, but again was only significant for the soleus muscle. Despite this, however, all three muscles exhibited a fatigue index which was greatly reduced in comparison to the FED and CONTROL groups (see Chapter 5). Thus the fatigue index of the soleus, EDL and TA muscles was 68%, 65% and 66% lower than FED animals respectively. In comparison the fatigue index of the non−endotoxin treated animals consuming the low protein diet was not different in the soleus compared to CONTROL/FED groups (Chapter 5), 20% lower in the EDL and 47% lower in the TA. There was a tendency for a shift to the left in the force−frequency curve, in this case of the soleus muscles, at 10 Hz.

Thus, the additional insult of endotoxin administration did not significantly increase the changes in whole body variables or twitch characteristics. Force generation was maintained although a tendency for slightly lower force production was noted. Fatigue susceptibility increased further and shifts in the force−frequency relationship became apparent − at 10 Hz for the slow twitch soleus muscle and at 20−50 Hz for the fast twitch muscles.

7.4 <u>DISCUSSION.</u>

The aim of this investigation was to assess the combined effects of malnutrition due to consumption of low protein diets and endotoxin on skeletal muscle function in rats. As such this represents the first investigation of this area.

In general the body and organ weight of the animals receiving the additional insult of endotoxin administration was similar to that of the animals consuming either a diet of normal or low protein content respectively. There was a tendency for animals receiving endotoxin challenge to exhibit a lower muscle mass. No change in the twitch characteristics were noted when compared to the animals not challenged with endotoxin, but for both low protein diets this represented prolongation of the twitch time of the soleus muscle compared tp CONTROL/FED animals previously described (Chapter 5). Both groups consuming low protein diets and additionally receiving endotoxin exhibited a greater tendency to fatigue than those animals untreated with endotoxin. Thus, the fatigue susceptibility of the animals was further increased when compared to the FED groups. This resulted in a difference in fatigue susceptibility of between 60 and 70% compared to the FED/CONTROL animals not receiving endotoxin. Changes in the force—frequency relationship were apparent in the soleus muscle of animals in the low protein, low electrolyte group receiving endotoxin and the TA muscle of equivalent animals on the low protein, high electrolyte diet. Thus it would appear that the additional insult of endotoxin administration has further reduced the ability of skeletal muscle to maintain its functional capacity.

7.4.1 <u>Body variables.</u>

Generally, body size and muscle mass of animals consuming the normal (15%) protein and low (0.5%) protein diets receiving endotoxin were similar to those animals consuming the normal (15% protein) or low (0.5%) protein diets respectively. Thus all animals consuming the low protein diets were both stunted and wasted compared to the FED animals. Endotoxin, when administered alone (chapter 5) did not significantly effect these variables with the exception of TA muscle weight following two challenges over 32 hours. Thus the greater degree of muscle loss observed in the FED animals receiving endotoxin in comparison to that previously reported would indicate that these animals may be more susceptible to the effects of endotoxin than previously noted. This is particularly important when the difference in the dose of endotoxin administered is noted. It would however appear that one possible explanation of this enhanced endotoxin mediated event is the larger size of the animals in the FED group. Larger animals are believed to produce greater responses to endotoxin administration, particularly with respect to the febrile response (Bibby & Grimble, personal communication). Hence, mounting of response to the administered endotoxin in the larger animals may increase the energy demands on body

stores and result in an increased loss of muscle tissue. It is interesting to note that in the studies of endotoxin administration on skeletal muscle, presented in Chapter 6 atrophy was only detected in the TA muscle, yet in these larger animals all the muscles studied were effected. Thus the growth of the animal may play an important role in the ability of the animal to respond and accommodate infectious insults. This may be of particular importance in human studies where malnutrition may arise later in life followed by infectious insults compared with the malnourishment model considered in this study.

The mounting of an immune response may be limited in malnourished individuals as has been previously described (Chapter 1). The immune response may be absent due to thymic atrophy for example that associated with kwashiorkor (Vint 1937 – see Alleyne *et al* 1977; Smythe *et al* 1971) The malnourished subjects of these studies also exhibited delayed and reduced hypersensitivity, impaired lymphocyte transformation in response to phytohaemaglutinin and a lower than expected spleen weight. Reduced antibody production in malnourished children has been reported by several workers (see Scrimshaw 1975 for review). The production of an acute phase response to various bacterial infections in children and animals was reduced or absent (Young *et al* 1968; Powanda *et al* 1975; Long *et al* 1982; Tomkins *et al* 1983; Drabik *et al* 1987). It is unclear from these studies if these infection mediated changes were associated with changes in skeletal muscle function. The findings of this study however, demonstrate that the acute phase response, changes in circulating serum zinc and albumin can occur in the presence of malnutrition with a suggestion of impaired skeletal muscle function. The response to endotoxin is rapid relative to the more clinical conditions reported in the studies described above and it may be that this response would not be evident had the infection been more long term. Whether the body can accommodate such reductions in infection mediated responses without impairing skeletal muscle function therefore remains unanswered.

The animals consuming the low protein diets did not exhibit any greater loss of skeletal muscle mass following endotoxin administration. This may reflect a mechanism which requires a period greater than 8 hours to have effect or may simply reflect the lack of change in muscle weight which has previously been described at this time (Chapter 6). The animals consuming the low protein, low electrolyte diet did not exhibit further reductions in serum concentrations of albumin and zinc compared to the untreated animals while those of consuming the high electrolyte diet exhibited a further fall in the serum concentration of zinc. This reduction in serum zinc concentration is greater than that reported by Wan following repeated administration of endotoxin over 3 days (Wan 1979), but consistent with that reported by Bibby 24 hours after a single endotoxin challenge (Bibby 1989). The serum albumin concentration did not become further lowered and may therefore have reached a point at which the body needed to maintain its serum concentration for other purposes (for example to maintain blood volume). The

mechanism by which animals stunted and wasted (i.e those consuming the low protein, high electrolyte diet) appear to exhibit an increased inflammatory response remains unexplained. The increased inflammatory response may result in additional demands upon body energy and substrate stores which cannot be afforded. This increased demand upon the body may play a crucial role in the etiology of further complications.

It is important to remind the reader that the dose of endotoxin administered in this series of experiments was lower than that used in Chapter 6. The reason for this lowered dose of endotoxin was the inability of the malnourished animals to tolerate the higher dose. Hence while no additional effect on whole body variables was observed in the animals consuming the low protein diet this is not directly comparable to the data of Chapter 6, but illustrates just how "brittle" these animals were. In addition, it should be noted that these results give further support to the suggestion that larger animals are more responsive to endotoxin than smaller animals. Thus the loss of skeletal muscle observed in the FED group following endotoxin administration was greater and present in all muscles studied in the larger animals receiving half the dose of endotoxin than that seen in smaller well−nourished animals of Chapter 6.

### 7.4.2 Contractile characteristics.

The administration of endotoxin to animals in all three dietary groups did not result in greater changes in function than that seen by the consumption of the diets alone, except for force generation in the animals consuming the high electrolyte diet receiving endotoxin. Hence in the FED group, administration of endotoxin did not result in changes in contractile characteristics. The tendency for slightly lower force generation in those animals treated with endotoxin is likely to reflect the lower muscle mass observed in these animals.

Animals consuming both low protein diets and receiving endotoxin exhibited contractile characteristics similar to those of the animals not receiving endotoxin. Hence there would not appear to be an additional response to endotoxin administration with respect to time to peak tension, half relaxation time. Force generation was generally lower in the animals consuming the low protein diets, particularly the high electrolyte diet. This probably reflects the maintenance of skeletal muscle mass at the size of animals consuming the low protein diet.

Force generation in animals consuming the low protein diets were greater than that in FED animals when expressed per unit muscle weight. Animals consuming low protein diets receiving endotoxin exhibited a lowering of this raised relative maximal tetanic tension. This difference, of up to 50%, did not attain statistical significance due to the great variations amongst the animals. It is interesting, however, to note that the force generating capacity of the muscles was approximately that observed in FED animals not receiving endotoxin. Previously, it was

suggested that this raised relative force generation (per unit muscle weight) was the result of loss of substances from the muscle, but not loss of protein since protein content of the muscles of protein deficient animals was unchanged. For endotoxin to have lowered this raised relative force generation to that of FED animals would therefore suggest a re-influx of the substance(s) of unknown identity into skeletal muscle, again with no alteration in muscle mass or protein content. Alternatively, there is a rapid change in the manner in which muscle generates force. It is unclear from the measurements made in this series of studies how this reduction in capacity to generate force occurred and the mechanism(s) by which it was achieved.

The fatigue susceptibility appears to have increased in all muscles of animals consuming the low protein diets receiving endotoxin challenge. Deficits in function may therefore become apparent despite apparently normal twitch and tetanic responses. In both groups of endotoxin treated animals, the most obvious changes were seen in the soleus muscle. This may reflect the slow contraction and relaxation time noted in these animals, but clearly an additional change must be present since this was not observed in the animals consuming the low protein diets alone. Thus there is an indication of an interaction between the two insults, although the site of interaction remains unclear.

This would not appear to involve the turnover of protein since raising the potassium intake by additional electrolytes in the diet (High Electrolyte group) which would be expected to increase protein turnover (Cannon *et al* 1952; Leach *et al* 1959) did not further exacerbate changes in skeletal muscle contractile characteristics. It should be noted, however, that the muscles of some animals consuming the low protein diet and receiving endotoxin were similar to those of animals consuming the low protein diets alone. Hence some animals still appear to be able to function in a normal manner and would therefore appear better able to adapt or tolerate the endotoxin challenge in the malnourished state.

While the responses at the twitch, tetanic and fatigue level have been similar in the animals consuming the low electrolyte and high electrolyte diets, the force-frequency relationship of these animals is not as uniform. Animals consuming the high electrolyte diet exhibited a further shift in the force-frequency relationship at 10 Hz in the soleus muscle, without any additional effects observed in the EDL or TA. It should be noted, however, that these animals would still exhibit a tendency for a shift in the force-frequency relationship of fast-twitch muscles in comparison to FED animals. All three types of muscle in the malnourished animals administered endotoxin now appear to be effected with shifts in their force-frequency relationship. It should be noted that the changes the relationship occur over different frequency ranges in the fast- and slow- twitch muscles. The slow twitch muscles exhibit a shift at low frequencies while the fast-twitch muscles exhibit this shift at intermediate frequencies of the range investigated. This is likely to reflect the frequency at which the muscles would normally

receive neural drive and would agree with the suggestions made in Chapter 5, where similar responses were discussed for animals consuming low protein diets.

In the animals consuming the low protein, high electrolyte diet, the force–frequency relationship of the soleus and EDL muscles of endotoxin–treated animals did not change compared to unchallenged animals. The relationship of the TA, however exhibited a shift over the 20 –50 Hz frequency range compared to the animals made protein deficient alone, resulting in a greater shift in this relationship when compared to the FED animals. Thus in the animals consuming the high electrolyte diet receiving an additional insult, the soleus muscle did not exhibit a shift in its force–frequency relationship compared to well–nourished animals, while the fast–twitch muscle exhibit either a trend (EDL) or a significant shift in the force–frequency relationship at intermediate frequencies. Since the function of the soleus muscle appears to be preserved in the animals consuming this high electrolyte diet it would suggest that the shift in the force–frequency relationship observed in animals consuming the low protein, low electrolyte group challenged with endotoxin may be related to changes in protein turnover, or other responses related to potassium deficiency. The reader is reminded however, that the animals consuming the low protein, low electrolyte diet have not been shown to be deficient in potassium in this study, but at present this is being inferred.

### 7.4.3 Summary.

The ability of skeletal muscle to maintain its contractile characteristics following consumption of low protein diets is altered following the additional insult of endotoxin administration. Thus while some variables of function such as twitch time appear independent of additional insults those such as fatigue may become exaggerated. Thus the fatigue susceptibility increases and shifts in the force–frequency relationship occur. These alterations in the force–frequency relationship appear to depend upon electrolyte intake –with animals consuming a low electrolyte diet exhibiting shifts in the relationship of the soleus muscle while high electrolyte diets results in shifts for fast–twitch muscles. The mechanisms underlying these changes remain ill defined.

The most important observation appears to be the lower force generation and an increased susceptibility to fatigue. Thus the malnourished individual who is also infected is unlikely to be able to maintain his/her physical work capacity without behavioural changes occurring simultaneously. This is further discussed in the general discussion (Chapter 8).

# CHAPTER 8.

## GENERAL DISCUSSION.

Physical activity is an integral part of human behaviour involving both socioeconomic and cultural components including both work and leisure pursuits. Reductions in physical activity following malnutrition therefore have important implications for the well being of individuals, their dependents and on society in general. The inability to maintain physical working capacity following malnutrition does not lie solely in skeletal muscle but, in part is mediated through central and cardiovascular mechanisms. The capacity of skeletal muscle to function may therefore be overridden by changes in the central and cardiovascular systems such that an individuals' physical working capacity may be lowered in order to accommodate other requirements of the body.

Assessment of physical working capacity by measuring task completion (e.g. tons of sugar cane cut and loaded in a given time) therefore does not necessarily indicate the functional capacity of skeletal muscle. The techniques developed in this thesis are unique in their ability to assess skeletal muscle contractile characteristics in the absence of central and cardiovascular influences. As such, the findings of this thesis represent the first attempt at evaluating the influence of malnutrition and infection on skeletal muscle in a consistent fashion. In addition, the techniques allow both cross–sectional and longitudinal comparisons to be made, a novel approach to the complex investigation of skeletal muscle function in malnutrition and infection.

It has been suggested that both fasting and food restriction result in impaired skeletal muscle function in both humans (Lopes *et al* 1982; Russell *et al* 1983a,b) and in the rat (Russell *et al* 1984). Furthermore, similar impairments were also apparent in infected hamsters (Drew *et al* 1988). It has therefore been suggested that these impairments may be due to selective atrophy of type II fibers or due to impaired calcium handling (Russell *et al* 1984).

At the commencement of the present study little was known as to the effect these conditions may have on skeletal muscle function, independent of central and cardiovascular influences (see Table 1.6.

The series of experiments reported in this thesis investigated skeletal muscle contractile characteristics under conditions of limitation of the amount or composition of food and the further influence of infectious insults. Models have been developed for acute food deprivation (fasting for 5 days), chronic food restriction (consumption of 25% normal intake for 21 days), consumption of low protein diets (0.5% v 15%) and the influence of endotoxin administration in the rat. In particular the effects of changes in food intake and infection were studied in skeletal muscles selected to reflect the different muscle fiber types thereby overcoming the limitations implicit in the studies of other workers. The main body of data is summarised in Table 8.1. results obtained from these experiments have made it possible to fill important gaps in the literature (compare Tables 1.6 and 8.1). It should be noted that there was a large between animal variation. As such small changes in skeletal muscle function may go undetected because they come to lie within a normal range for the group of animals. Thus it may not be possible to catagorically refute change in contractile characteristics but trends may be evident within the measurements.

## 8.1 PERIPHERAL ADAPTATION.

An overriding conclusion from the foregoing chapters is the remarkable preservation of skeletal muscle function in the face of nutritional and infectious insults. Preservation of skeletal muscle function occurs in the face of a variety of metabolic stresses. This point has not been widely appreciated. Skeletal muscle function is preserved in association with a simple reduction in food intake (acute food deprivation or chronic food restriction) but may become disordered in association with unbalanced changes in the components of the food consumed. From this it is concluded that reduction in food intake resulting in the consumption of a balance of nutrients enables an adaptive response to occur despite a lower energy intake, maintaining skeletal muscle function. The consumption of diets with unbalanced nutrient content does not allow this adaptive response to develop and functional alterations become apparent. This suggests that the limiting factor is not energy alone but that the protein:energy ratio may play an important role. Thus, animals consuming the low protein diets (Chapter 5) exhibited a lower energy and protein intake than the animals restricted in food intake (Chapter 4). Surprisingly, though, complete cessation of food intake (Chapter 4) did not produce a change in the functional

capacity of skeletal muscles. This may indicate a rapid adaptation to changes in food intake which is effective in maintaining skeletal muscle function. A primary mechanism of this adaptive process is the immediate cessation of growth. This would be expected to result in a reduction in body size which itself results in a reduced demand for energy due to a lower BMR. It is unclear from the observations made during acute food deprivation and chronic food restriction from the present series of studies what, if any, behavioural changes occurred in these animals limited in food supply. The work of Duffy and colleagues suggests that food restricted rats spend less time in physical movement than animals with free access to food, but that this results in alterations in the type of activity undertaken, for example a greater number of feeding and drinking episodes were noted in the food restricted rats (Duffy *et al* 1989). These alterations at the level of the central and cardiovascular systems would appear sufficient to maintain skeletal muscle contractile characteristics since little alteration in the twitch, tetanic and fatiguability of the soleus, EDL and TA muscles were observed in the data presented earlier (Chapter 4). These findings would appear to be contrary to the report of Russell and colleagues where alterations in both the tetanic characteristics and fatiguability responses of the rat gastrocnemius muscle following both acute food deprivation and chronic food restriction were reported (Russell *et al* 1984). The findings from the studies of food deprivation and restriction suggest that cessation of growth and the smaller body size of animals following limitation of food supply plays a vital role in the adaptation of young animals to undernutrition.

With alteration of diet composition, such as that imposed by the low protein diets, alteration in skeletal muscle contractile characteristics became apparent. In the protein deficient animals complete cessation of growth was observed as in the studies of limitation of food supply. However, it would appear that the limitation of food composition places greater demands upon the body resulting in significantly greater reductions in body and organ mass. Hence, atrophy of muscle was apparent over and above the cessation of growth and development.

It is the difference in the ability of the body to maintain body functions by the cessation of growth alone which may result in adaptation at the peripheral level (i.e. within the muscle itself) in order to maintain skeletal muscle function.

Table 8.1. The effects of fasting (FAST), food restriction (FR), endotoxin administration (ENDO) and consumption of low protein (0.5%) diets (LP) in the presence (+E) and absence of endotoxin administration on skeletal muscle contractile characteristics of the rat.

| | FAST | FR | ENDO | LP | LP+E |
|---|---|---|---|---|---|
| TPT | = | = | = | =/↓sol | =/↓sol |
| ½RT | = | = | = | =/↓sol | =/↓sol |
| PT | = | = | = | = | = |
| PT /mg | = | ↑ | =/↑ | ↑ | ↑ |
| Fmax | = | = | = | = | = |
| Fmax /mg | = | ↑ | =/↑ | ↑ | ↑ |
| Shift in FFC | no | trend | no | EDL/TA | SOL/EDL/TA |
| Fatigue | not measured | = | = | ^ trend | ↑ |

KEY: TPT – Time to peak Tension; ½RT – Half Relaxation Time; PT – Peak Twitch Tension; Fmax – Maximal Tetanic Force; = - no change; ↑ - increase; ↓ - decrease; sol - effect on soleus muscle.

The mechanism(s) of this loss of skeletal muscle tissue were not examined in this series of studies, but may represent a common response to reduced food intake since this was also apparent following endotoxin administration. That the reduced skeletal muscle mass was the response to the reduction in food intake in these endotoxin-treated animals was suggested by a similar loss of muscle weight in animals pair fed the quantity of food consumed by the animals administered endotoxin (Chapter 6). This loss of muscle tissue has previously been suggested to be the result of selective atrophy of type II muscle fibers (Heymsfield *et al* 1982; Russell *et al* 1984). Yet Sieck and colleagues were unable to observe atrophy of a significant number of fibers in the diaphragm of fasted rats to account for such changes (Sieck *et al* 1989). Similarly, it is difficult to envisage the selective loss of a significant number of type II fibers in the present series of experiments since the muscle protein content remained unchanged following nutritional or infectious insults. From these results it would appear that the lower muscle mass and protein content reported in studies in the literature reflects a lack of growth rather than an atrophy of skeletal muscle. Muscle atrophy of a greater magnitude might become apparent, as in those animals of the present investigation consuming low protein diets (Chapter 5), but this may be small relative to that previously assumed because comparisons have been drawn to inappropriate control groups. Several investigators reported the difference in muscle function between animals with a limited food intake compared to well nourished animals. The findings of this thesis however, indicate that under conditions of food deprivation and restriction skeletal muscle mass remains close to that of animals at the commencement of the study. This represents a true control group. Thus food deprivation and restriction do not result in a large loss of muscle tissue but reflect a lack of further development. Previous workers may therefore have misinterpreted their results by not taking into account changes in cross-sectional area of muscle and age-related developmental activity.

Despite the wasting of skeletal muscle, force generation was generally preserved following the limitation of the amount or composition of food consumed compared to weight matched animals. While the absolute force generation was lower than that of age-matched, well-nourished animals this represents an enhanced capacity to generate force (i.e. per unit muscle). Thus it would appear that skeletal muscles retain a remarkable ability to accommodate nutritional stress. The conservation of force generation could be made possible by preservation of the

proteins which constitute the contractile machinery while loss of other less essential proteins occurs, although there is no direct evidence for such a mechanism being in operation. The selective atrophy of type II muscle fibers reported in both clinical (Heymsfield *et al* 1982; Russell *et al* 1983) and experimental (Russell *et al* 1984) conditions would appear to argue that such a system is involved in adaptive processes. However, the type II fibers tend to generate larger forces than type I fibers and selective atrophy would be expected to result in lower muscle force generation. Lower force generation was not apparent in the studies reported earlier. This would argue that selective fiber atrophy may not be playing a major role. It is a possibility that due to the selection of muscles to reflect the different muscle fiber types the extent of selective fiber atrophy has been reduced. Since histological investigation of muscles from the present studies have not been made this point remains open. Another possible explanation for the preservation of force generation could be that the smaller muscles size allows a closer packing of the contractile proteins resulting in greater interaction per impulse thus maintaining force generation. Again there is no evidence that such a mechanism(s) is involved in the process of adaptation.

The soleus, EDL and TA muscles of animals following food deprivation or following endotoxin administration did not exhibit changes in the twitch time to peak tension or half relaxation time. Food restricted animals exhibited a slowing of twitch time which was further prolonged following consumption of low protein diets. No effect was discernible for the EDL or TA muscles. This prolongation of the soleus twitch time was not further enhanced when animals consuming low protein diets were treated with endotoxin. It may be that when animals are limited in the amount of food available the body initiates mechanisms to maintain the function of the body, including that of skeletal muscle. However, the provision of a diet whose composition is unbalanced does not allow this mechanism to function adequately, perhaps due to the processes involved in the utilization of those nutrients provided.

Since the soleus muscle was effected specifically this would appear to be an effect on type I fibers. The mechanism(s) responsible for such prolongation of twitch time was not studied in this investigation but, since both contraction and relaxation time are dependent upon ATP— and calcium— mediated processes it may be that these processes are specifically effected. Slower

twitch time was apparent only in animals consuming the low protein diets and may reflect either the lower energy or protein intakes of animals in this group. Alternatively, this may be a result of reduced protein turnover due to insufficient potassium intake. It is known that protein turnover is dependent upon potassium intake (Cannon *et al* 1952 & Leach *et al* 1959) which was reduced in the animals consuming both low protein diets.

The slower contraction time of the soleus muscle, may result from reduced release of calcium either due to alteration in the membrane potential— resulting in a prolongation of the time before which an action potential may be elicited in the muscle membrane, slower propogation of the action potential via the transverse tubules, reduced calcium release per impulse or a combination of these effects. Prolonged relaxation may result from a reduced availability of ATP — due to reduced dietary nutrient intake or reduced endogenous production, or increased intracellular calcium concentrations —due to reduced sequestration of calcium at the end of the twitch, both initiating further slowing of calcium sequestration over the sarcoplasmic reticulum. These processes, however, would be expected to be effected in both fast— and slow— type of muscle fibers, hence it should be expected that slower contraction and relaxation would be noted in all three types of muscle studied. This was generally not observed, but the EDL muslce of food restricted animals was 16% slower to contract than that of control animals. It remains a possibility that the effect is present in all three muscles but becomes more obvious in the soleus muscle which already possesses lower ATPase activity (hence slower contraction —see Chapter 1) and less calcium—ATPase in the sarcoplasmic reticulum (hence slower relaxation— see Chapter 1). Thus, the slowing of these energy related processes became more detectable in the slow—twitch muscle. However, since the computer system used in these studies enables a sampling rate of 4000 Hz, i.e. a time discrimination of 0.25ms, it is unlikely that such a slowing of fast—twitch time, if present, would go unnoticed.

The effect of fasting on fatigue were minimal in all types of muscle studied. Again it would appear that the complete cessation of food intake allows for the maintenance of skeletal muscle function. Theoretically this is an important adaptation since it would allow life—saving work activity such as food searching or gathering to continue. Endotoxin administration was also not associated with changes in fatigue susceptibility.

The consumption of low protein diets however, increased the fatigue susceptibility of all muscles, particularly that of the TA muscle. Some animals appeared to be able to maintain the fatigue characteristics of their muscles. Thus, it appears that when the severity of insult is small, muscles are able to preserve their functional capacities by the cessation of growth. As the severity of insult increases muscle weight declines and changes occur in the capacity of the muscles to function. Some animals appear better able to maintain work performance in the face of these insults than others. The manner in which this was achieved, however, remains illusive.

Since this increased fatigue susceptibility was further enhanced when the animals consuming low protein diets received endotoxin it would appear to suggest that the capacity of the adaptive mechanisms responsible for the conservation of function have been exceeded. This may reflect the assumed additional energy requirements for mounting an immune response, although several studies have indicated that protein deficient individuals are not always capable of mounting a full response to infection (see Chapter 1). Skeletal muscle may be important in the immune function by the provision of carbon and/or nitrogen, the supply of specific amino acids associated with muscle proteins required in the synthesis of acute phase proteins or other compounds associated with muscle protein (see Chapter 1).

It is clear that the mechanisms underlying this susceptibility to fatigue is not fiber type specific since all three muscles studied exhibited similar trends. This may be related to changes in the conduction velocity of neurones, changes in the excitation−contraction coupling or energy depletion. Consumption of diets typical of the north east region of Brazil resulted in slowing of the rat sciatic nerve conduction velocity (daSilva, Costa, Costa, Teodosio, Cabral−Filho & Guedes 1988). Thus force generation over time may be limited by the number of impulses arriving, which may decline due to the inability to propagate action potentials. Severely ill patients are reported to exhibit reduced muscle membrane potential (Cunningham *et al* 1971) therefore it may seem more likely that slowing of conduction velocity may be important, but the animals receiving endotoxin in the data presented in this thesis did not exhibit alteration in fatigue characteristics. The lack of agreement between these reports may reflect the longer time course associated with severe illness compared to the more acute response to administered endotoxin. The depletion of energy, expected in animals fed deficient diets would also be expected to limit the $Na^+K^+-$, and $Ca^{2+},Mg^{2+}-ATPase$ activity resulting in slower contraction and relaxation, as observed in the soleus muscle of protein−deficient animals. Reduced concentrations of circulating $T_3$ may also be implicated in these malnourished individuals since euthyroid monkeys exhibit lower myocardial ATPase activity (Tulp *et al* 1979), although it is not clear if this is also apparent in skeletal muscle tissue. In the present studies the fatigue susceptibility was similar in animals consuming 15% protein diets (i.e. the FED group, Chapters 5 & 7) and in animals at the commencement of the study (i.e. CONTROL animals, Chapter 5), thus these changes in fatigue susceptibility were not a developmental effect, hence they represent an impaired function of skeletal muscles. The nature of this process is unclear.

The manner in which skeletal muscle produces force over a range of frequencies is believed to give an appreciation of its functional ability. Shifts in the force−frequency relationship to the left, i.e. increased relative force generation at low frequencies of stimulation, have been taken to reflect an impairment in function (Russell *et al* 1984). Such changes in the force−frequency relationship may be explained by slowing of maximal relaxation rate and reduced maximal tetanic tensions (Russell *et al* 1984). This has been reported for the rat gastrocnemius muscle

following fasting and food restriction (Russell *et al* 1984) and is indicated in hamsters infected with *Leishmania donovai* (Drew *et al* 1988). However, shifts in the force−frequency relationship were not apparent in the fasted rat for the diaphragm or medial gastrocnemius muscle reported by Sieck and associates (Sieck *et al* 1989).

It is difficult to interpret these observations due to the complex nature of the gastrocnemius muscle. Assessment of function in the soleus, EDL and TA muscles in the series of experiments comprising this thesis was expected to differentiate the contribution to such changes made by the different muscle fiber types. This was particularly important since many of the explanations for this shift in the force−frequency relationship presume atrophy of type II fibers (Russell *et al* 1984).

In the series of experiments presented in this thesis, no alteration in the force−frequency curve was apparent following limitation of the amount of food available or following administration of endotoxin. A tendency for a shift in the force−frequency curve was apparent in fast−twitch muscles but this did not attain statistical significance. Such a tendency, even though not statistically significant, would agree with reports of preservation of slow−twitch muscle protein synthesis following undernutrition to a greater extent than that of fast−twitch muscles. Since slow−twitch muscles tend to serve a postural role this would appear to be an essential adaptation to malnutrition. Such a tendency would also support the suggestion of type II fiber atrophy, since there would be a greater proportion of slow fibers resulting in slower relaxation and hence an earlier production of tetani. However, it is difficult to envisage sufficient atrophy of type II fibers in the absence of a significant change in muscle mass and protein content to account for a shift in the force−frequency relationship.

In animals subjected to more severe nutritional insults, i.e. consumption of low protein diets, shifts in the force−frequency relationship were apparent. Thus the EDL and TA exhibited a shift over 20 −50 Hz. It is tempting to suggest a mechanism by which muscle function is maintained when animals are subjected to nutritional insults which can be accommodated by cessation of growth alone (e.g. fasting, food restriction, endotoxin−administration). Imbalance imposed by the consumption of unbalanced diets (e.g. protein−deficient) initiates mechanisms which prepares skeletal muscle to alter the manner of force generation. This may not represent an impairment of function, but may be a preemptive action to maintain function prior to changes within the muscle being manifest. Alternatively, it may be of little functional significance, but may represent the abnormal frequency range over which the force−frequency relationship is constructed.

It seems unlikely that this shift in the force−frequency curve is due to atrophy of type II fibers since the muscles of animals following limitation in the amount or composition of food intake

exhibited preservation of maximal tetanic force in comparison to muscles of control animals. Thus type II fiber atrophy would be expected to reduce muscle mass and force generation.

This reduced force generation may be expected to be considerable since type II fibers are believed to produce greater force than type I fibers (Burke *et al* 1973; Sieck *et al* 1989).

Thus it can be concluded that alteration of the contractile characteristics of skeletal muscle does occur during adaptation to nutritional insults when the level of insult is greater than that which can be accommodated simply by cessation of growth.

## 8.2 THE INFLUENCE OF ADDITIONAL INSULTS.

In the clinical situation, malnourishment appears to present coincident with infection (Waterlow 1986). Thus the investigation of work capacity in malnourished humans may be influenced greatly by concomitant infection, which in some cases may initiate the observed undernutrition. In order to investigate this further animals consuming the low protein diets were administered *E.coli* endotoxin to simulate secondary infection (Chapter 7). Previous assessment of the effects of endotoxin administration alone on skeletal muscle contractile characteristics suggested that rats were able to maintain their functional capacity despite the endotoxin insult (Chapter 6). An additional endotoxin insult in the animals fed low protein diets however, resulted in significant alteration in the contractile characteristics of skeletal muscles. In these animals the relative force production in the fast–twitch EDL and TA muscles was elevated at intermediate frequencies of stimulation (20 & 50 Hz see chapter 6) and fatiguability of the muscles increased. This increase in the relative force production at intermediate frequencies of stimulation was not apparent in the animals consuming the low protein, high electrolyte diet suggesting that the dysadaptation which had occurred in these animals represented a limit to the adaptability of the animal and its skeletal muscle function. The soleus muscle also exhibited a shift in the force–frequency relationship, at 10 Hz. Since the frequency at which a shift is observed was different for the different types of muscle there would appear to be a fiber specific effect. It is important to note that slow twitch muscles generally receive its input at around 20 Hz while that of fast twitch muscles is 40 Hz (Adrian & Bronk 1929). From these results it would appear that the shift in force–frequency relationship is related to the frequency range over which the muscle would be expected to receive input.

Despite the force generation in protein deficient animals receiving endotoxin being unaltered compared to those consuming the low protein diets alone, the capacity to generate force (i.e. per unit muscle) was reduced. Force generating capacity was similar to that of animals at the commencement of the study (CONTROL, Chapter 5) and animals consuming the 15% protein diet (FED, Chapters 5 & 7) in endotoxin–treated animals consuming low protein diets. The

implications of this lower, but normalised, capacity to generate force is unclear, but would suggest a lower ability to achieve muscular work.

The animals consuming a 15% protein diet receiving endotoxin also exhibited some alteration in the relative force generation at intermediate frequencies of stimulation and exhibited greater loss of muscle mass than had been experienced in the earlier study of endotoxin administration alone (Chapter 6 & 7). Since the only difference which could be noted between the two studies was that of fat content (3% v 10%) this would suggest that the fat content of the diet plays a significant role in the expression of endotoxemia. This would agree with previous studies where increased fat content of the diet resulted in the enhancement of the response to endotoxin and TNF (Wan 1987; Bibby 1989).

The enhanced response to endotoxin is likely to be due to the increased incorporation of arachidonic acid into the cell membranes of animals on the high fat diet, although this is generally considered to take approximately four weeks. Greater arachidonic acid content of the cell membranes may result in increased endotoxin−mediated prostaglandin production and possibly to enhanced proteolytic activity. In considering the protein deficient model of malnutrition the fat content of the diet may also be involved in the adaptive process such that following administration of endotoxin, enhanced endotoxin−mediated effects may have been produced. How these may interrelate with the underlying adaptation to protein deficiency is unclear and requires further investigation. That the response to endotoxin in the animals consuming the low protein, high electrolyte diet was not different to the non−endotoxin treated animals implies that there may be a limit to the magnitude of the adaptive process which is achievable at the peripheral (i.e within the muscle) level.

## 8.3 WHAT DOES THIS MEAN AT THE WHOLE BODY LEVEL?

The discussion so far has concentrated on the ability of electrically−stimulated muscle to generate force, the time constraints of this activity, the manner in which force develops with increasing frequency of stimulation and the decline of force on repeated stimulation. A remarkable preservation of function was noted in the face of limitations of food intake but not when food composition was limited. However, even under conditions of limitation of food composition force generation was preserved at the control level despite wasting of skeletal muscle tissue. Clearly, this force represents that which the muscle is capable of generating but may not be the force which would be generated by volition. Such responses are not easily measured.

It is important to note that this preservation of force generation only applies in comparison to the animals assessed at the commencement of experimentation. As such this may be taken to

represent a reduced physical work capacity of animals compared to age-matched, heavier, well-nourished animals. This would agree with the clinical data. Spurr and co-workers (summarised in Spurr 1987) for example, have indicated a reduced physical work capacity in terms of tons of sugar cane cut and loaded in malnourished Columbian individuals. However, in ambulatory humans the peripheral (i.e.muscle) phenomenon following a period of malnourishment are profoundly influenced by central (i.e.motivational) and cardiovascular factors.

Thus, the Columbian cane cutter can maintain productivity by adaptation at all three levels. At the peripheral level muscle force generation is reduced in absolute terms resulting in a reduced load carrying capacity. This results in an alteration at the cardiovascular level to lower energy expenditure. But in order to maintain productivity (and thus wages) there must be a behavioural adaptation at the central level resulting in a lengthening of work time (i.e. the working day) thus total work output has remained constant. This behavioural change results in less activity being expended outside the working environment (Ferro-Luzzi 1990).

The results of the series of studies of this thesis therefore argues that the muscle of such malnourished individuals is functioning normally, with respect to force generation, but that due to a lack of growth and development, depending upon the time of undernutrition, the absolute force generated would be lower than the well developed muscles of larger, well-nourished individuals.

The ability to maintain work performance has been reported to be lower in malnourished agricultural workers (Viteri 1971), Columbian sugar cane cutters (Spurr *et al* 1984) and other workers (see Spurr 1977 for review) than well-nourished workers. These individuals fatigued more rapidly in agreement with the work of Russell and colleagues in fasted and food restricted rats (Russell *et al* 1984), undernourished animals exhibiting a greater loss of force during sustained contractions than well-nourished animals. However, the ability to maintain a sustained contraction is an unphysiological activity. The studies presented in this thesis utilized the generation of force over intermittent stimulation at a tetanic frequency to investigate fatigue. Although this has previously only been used in the investigation of single motor units (Burke *et al* 1973), this procedure was appropriate in that the fatigue profiles obtained agreed with the known composition of the different types of muscle and EMG activity monitored simultaneously was not diminished during the stimulation sequence (see Chapter 2).

That animals which were most stunted and wasted (i.e. those consuming low protein diets, Chapter 5) exhibited greater fatigue compared to control animals suggests that fatigue noted in malnourished humans is the result of impaired peripheral function. As noted above, however, this increased fatigue susceptibility may become more pronounced as the result of other

behavioural changes (see Ferro–Luzzi 1990).

As fatigue susceptibility following the administration of endotoxin was not increased, this would suggest that the lethargy associated with infection in man does not lie at the peripheral level. However, a peripheral component may be apparent in malnourished infected individuals since endotoxin administration to animals following consumption of low protein diets exacerbated the increases in fatigue susceptibility. Malnourished workers with concomitant infections might be expected, therefore, to perform less well than well–nourished or malnourished individuals without infection. To date attempts to evaluate such responses have not been conducted.

It is unclear how, or why, the fatiguability of skeletal muscles of some animals consuming the low protein diets was unaltered while the muscles of others became more fatiguable (Chapter 5). This may be indicative of a genetic basis, although no epidemiological data of such a link is available. Clearly, though, this increased fatigue susceptibility in the muscles of some animals consuming low protein diets would limit their work performance. As such this would agree with the observations of reduced physical working capacity in malnourished workers (see Spurr 1977).

From this discussion it would appear that the functional deficits reported in malnourished humans can, in part, be explained by the peripheral changes as assessed in the rat model. However, it is unclear the extent to which the alterations in skeletal muscle function described in the rat model are applicable to the human situation. Wootton and colleagues reported reduced maximal force generation and maximal relaxation rate of the electrically stimulated adductor pollicis muscle following fasting in human volunteers (Wootton *et al* 1987). The impaired function was no longer apparent 6 hours following refeeding (Wootton *et al* 1987). Skeletal muscle contractile characteristics in rats were not changed following 5 days fasting (Chapter 4). This suggests that the rat has a more efficient mechanism for maintaining skeletal muscle function than that which is present or functional in humans. The rat would appear to be a robust animal, since 5 days fasting had no influence on the skeletal muscle contractile characteristics, yet had the experiments continued for a further 2 days the animals would be expected to be dead (Home Office, personal communication). While no data has been collected in fasting humans over a comparable period the impairment of skeletal muscle function within 48 hours (Wootton *et al* 1987) would suggest that there are species differences. Changes in skeletal muscle function following starvation have been cited in the substantiation of the use of such measurements in the assessment of nutritional status (Pichard & Jeejeebhoy 1988). Much of the evidence for this use of skeletal muscle assessment has arisen from animal studies (Russell *et al* 1984) or from the assessment of obese subjects on reducing diets (Russell *et al* 1983a,1984b). It is unclear however, how skeletal muscle function is influenced by obesity, hence any alteration in skeletal muscle function of obese individuals following reduced food intake may not indicate a change

from "normality". Since muscle function returns to normal 6 hours after refeeding in 48 hour starved individuals, (Wootton *et al* 1987) it is unlikely that skeletal muscle function could be used reliably as an index of nutritional status. The difference in susceptibility of skeletal muscle to changes in function following reductions in food intake may also be related to the muscle which has been studied. This thesis has clearly shown that different muscles respond differently to changes in food intake. Thus it is possible that the adductor pollicis, for unknown reasons, is more susceptible to changes in food intake than the muscles selected for study in the rat (i.e. the soleus, EDL and TA muscles). The muscle fiber composition of the adductor pollicis is nearer that reported for the gastrocnemius muscle than of the muscles studied in this thesis, hence it is possible that impairments in skeletal muscle function occur rapidly in this muscle as reported by Jeejeebhoy and associates (Lopes *et al* 1982; Russell *et al* 1983). No data is available for functional characteristics of human muscles in the malnourished state other than the adductor pollicis.

## 8.4 IMPLICATIONS FOR THE MEASUREMENT OF PHYSICAL WORKING CAPACITY.

It is clear that the assessment of physical working capacity by determination of the tons of sugar cane cut, miles of road dug or similar activities does not provide information as to the nature or site of impairment of functional capacity. Assessment of stimulated function also limits the information obtained such that it does not necessarily relate to the physical working capacity. Assessment of stimulated function simultaneous with muscle work performance has not been completed. Yet it would appear that such a research programme is required.

It is therefore necessary to determine the maximal voluntary force, the maximal stimulated force and determine how these change relative to each other in the malnourished state. Similarly, the assessment of fatigue would be important to both stimulation and volition. Such studies should more fully characterise the ability of the individual to perform mechanical work than either assessment alone. The development of a unique system of measuring the contractile characteristics of skeletal muscle described in this thesis would therefore represent a major step in the capacity to further the current understanding in the area of muscle function during and following malnutrition. Further investigations are required to develop this novel approach for the assessment of skeletal muscle function in human malnourished individuals. The assessment of stimulated twitch characteristics will be of minor use in the determination of functional impairments.

These studies however are not simple to complete. In the malnourished individual it is difficult to quantify the nature, cause and longevity of the undernutrition. Compounding this the assessment of stimulated function relies upon that of the adductor pollicis muscle which cannot be repeatedly biopsied for biochemical analysis. It may also be important to determine whether

the contractile characteristics of the adductor pollicis muscle is representative of the musculature as a whole.

Conducting such studies in animal models would also be difficult. Firstly it is still unclear whether the rat represents the most appropriate model for human undernourishment. Secondly in order to assess non–stimulated function it would be necessary to train rats to perform tasks which could be measured. Such training could preselect animals which are better at the task and hence make the detection of impairments in function more difficult. It would also be difficult to assess and ensure the motivation of rats while the task was completed. Despite these reservations, the utilization of the rat as a model for human malnutrition will suggest those areas of human muscle function worth pursuing.

In assessing physical working capacity it is also important to remember that the results from this thesis suggest that the muscles of malnourished individuals may not be impaired in their function, but may, in fact, be functioning normally. Thus the muscles can generate force but are limited in the magnitude of that force produced by the size of the muscle. Growth and development of individuals results in differences in physical working capacity which are not directly related to present nutritional status. While maximal force production may be important in the daily work–related activity of manual labourers it does not represent impaired muscle function in that the contractile characteristics of the muscle reflect those of size matched muscles. Changes in fatigue may occur in some individuals which may have a genetic basis but appear to become apparent only in severe conditions.

## 8.5 FURTHER FUTURE DIRECTIONS.

From the data provided on the adaptation of skeletal muscle to malnutrition several questions remain unanswered. For example the manner in which force generation in the malnourished animals was conserved despite the loss of muscle tissue, the mechanisms underlying the trends of increased fatigue susceptibility of the fast–twitch muscles and the surprising slowing of the twitch characteristics of the soleus muscle following consumption of low protein diets.

The manner by which force generation is preserved may be studied further by investigation of the histochemical and morphological characteristics of the muscles selected for study. The knowledge as to the contribution of selective fiber atrophy as suggested by some workers to the loss of muscle mass described in the animals following limitation of food supply and content would be of use in assessing the mechanisms of adaptation. Furthermore, it would be useful to investigate the loss of protein during and following malnutrition to determine the relative contribution of preservation of the proteins which constitute the contractile apparatus. It would be possible therefore, to assess the myosin or actin content of skeletal muscles before and

following malnourishment and determine if the contractile machinery is maintained to a greater extent than other proteins. The effect of selective fiber atrophy could also be investigated by examining the cross sectional area of fibers within skeletal muscles of malnourished rats, since the relative contribution of the fiber types to the cross sectional area of the muscle could be related to the changes in force generation noted.

The mechanisms underlying the fatiguability of the muscles could be investigated further by examination of the metabolic profile of skeletal muscle once they have achieved the same task. The ability of the muscle to achieve work can be assessed with reference to the changes inherent with a given task rather than to a given stimulation sequence. The ability of the enzyme systems which provide energy for contraction of skeletal muscle have not been addressed in the present investigation and it is likely that alterations in the glycolytic and oxidative capacities of malnourished muscle occurs.

The mechanisms by which the slowing of the soleus muscle twitch time was brought about could be investigated. Since both contraction and relaxation are energy dependent, calcium mediated processes it would be interesting to investigate the intracellular calcium concentration of skeletal muscles following malnutrition and to discover the calcium which is released per impulse.

Clearly, from the data collected more information is required as to the interaction of the components of the diet during and following malnutrition with specific reference to the protein— energy ratio and potassium intake and possibly the modifying activity of higher fat content.

The assessment of physical working capacity by measuring the amount of sugar cane cut or miles of road constructed is unlikely to enlighten us as to the nature and degree of impairment of skeletal muscles in the malnourished state. By far the largest area which requires further investigation is that of the importance of electrically—stimulated functional measurements similar to those described in the studies of this thesis with respect to the physical working capacity of malnourished individuals. This will require assessment of both electrically—stimulated and self motivated contractions under carefully controlled conditions.

In addition, the techniques developed for the assessment of skeletal muscle contractile characteristics following limitation of quantity or quality of food and infection will prove useful in the investigation of other conditions. Further studies may include a better understanding of the role of skeletal muscle in specific nutrient deficiencies (e.g. vitamins and minerals), growth deficits (e.g. lack of growth hormone production or unresponsiveness) and other disease states (e.g. obesity, diabetes and cancer).

APPENDIX I


a users guide to the assessment of skeletal
muscle function using computerised systems.

## OVERVIEW

The programmes used in the assessment of skeletal muscle function provide a unique standardised method for the collection and analysis of electrically stimulated muscle contraction using an IBM compatible computer system.

The computer software for the collection of data, written in Turbo Pascal, allows the creation of stimulation sequences suited to the trial requirements which can be stored for subsequent use. Force development is determined utilising a 16 channel A/D 12 bit collector (e.g. Amplicon Liveline, Brighton, England) up to a rate of 4000 data points per second.

The data analysis programme, written in Turbo Basic, provides both a visual display of force development with time and a numerical display of the extent of force generation at pre-set points.

The A/D digital data is saved to a named file which can be stored for future reference. During the collection procedures, a hard copy of the force generation elicited to electrical stimulation can be simultaneously obtained using a pen-recorder.

This guide is designed to enable easy access to the collection and analysis programmes used in conjunction with an IBM computer. For the duration of this guide it will be assumed that the files required to run the programme are resident in a directory called **ATEST**

## SETTING UP THE SYSTEM

The manner in which the components of the system are connected is shown in the Figure 1.1 below. Note that if a hard copy of the force generation be required a pen-recorder should be connected in parallel to the IBM computer.

The files required to elicit stimuli, collect and analyze data are:

ANN.BAS
ANN.EXE
DEFAULT.MDA
DEFAULT.MSE
EDT.EXE
IPC-26.PAS
LAST.BSE
M4000.BAK
M4000.COM
M500.BAK
M500.COM
MUSCLE.MFA
MUSCLE.PAS
PC-28.PAS
TEST.MDA

Additionally, the following files contain set stimulation sequences as used in the assessment of skeletal muscle function in the Department of Human Nutrition, University of Southampton (see Levy 1990):

MUSCLE.MSE
FFC.COM
FFC.MSE
BURKE.COM
BURKE.MSE
HUMANFFC.COM
HUMANFFC.MSE
HUMANBUR.COM
HUMANBUR.MSE

FIGURE 1.1 Diagram to illustrate the arrangement of equipment for assessment of muscle function.



key:
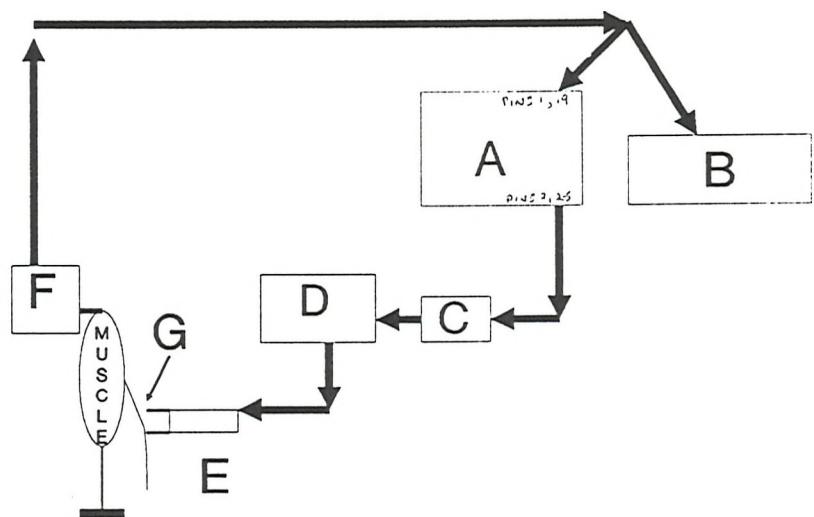
A: IBM computer
C: Optically isolated switch
E: Stimulating electrodes
G: Nerve

B: Pen recorder
D: Stimulator
F: Force transducer

## 1. GETTING STARTED

1. Locate the directory in which the software is resident (ATEST).
2. Two programmes are available to initiate the programme, these enable collection of data at different rates:

    M500 - allows collection of data at 2.5 ms intervals.
    M4000- allows collection of data at 0.25 ms intervals.
3. Having selected the appropriate programme the screen will display the main menu options (see Figure 1.2). At this time only options 1, 3 and 7 are accessible via the main menu.
4. Select option by typing an integer between 1 and 7.
5. Note that the data displayed will only be correct if the transducer is correctly calibrated (see TRANSDUCER CALIBRATION).

FIGURE 1.2.

```
1. DISPLAY/EDIT STIMULATION SEQUENCE

3. COLLECT DATA




7. RETURN TO DOS


                    Select option Number ?_____
```

## 2. TRANSDUCER CALIBRATION

In order to obtain correct data the transducer needs to be calibrated correctly and regularly checked.

The simplest method of calibration consists of collecting data to a file having hung weights on the transducer.  The A/D decimal value can be viewed and the slope of calibration determined by plotting the A/D value against the force.

## 2.1 Calibration of the Harvard Bioscience Transducer

The slope of calibration of the Harvard transducer (model ) was calculated over the 0-50 g and 0-500 g range.  The A/D decimal value was plotted against the force and Figures 2.1 and 2.2 obtained.  Note that this data represents the average of numerous calibration tests and may change with the conditions.  It is essential therefore that the calibration be checked before assessment of function is attempted.

For this transducer
  128000 Units = 1 Newtons over the 0-50 g range,
   14400 Units = 1 Newtons over the 0-500 g range.

FIGURE 2.1. Calibration of the Harvard Bioscience Force
Transducer over the 0-50 g range.
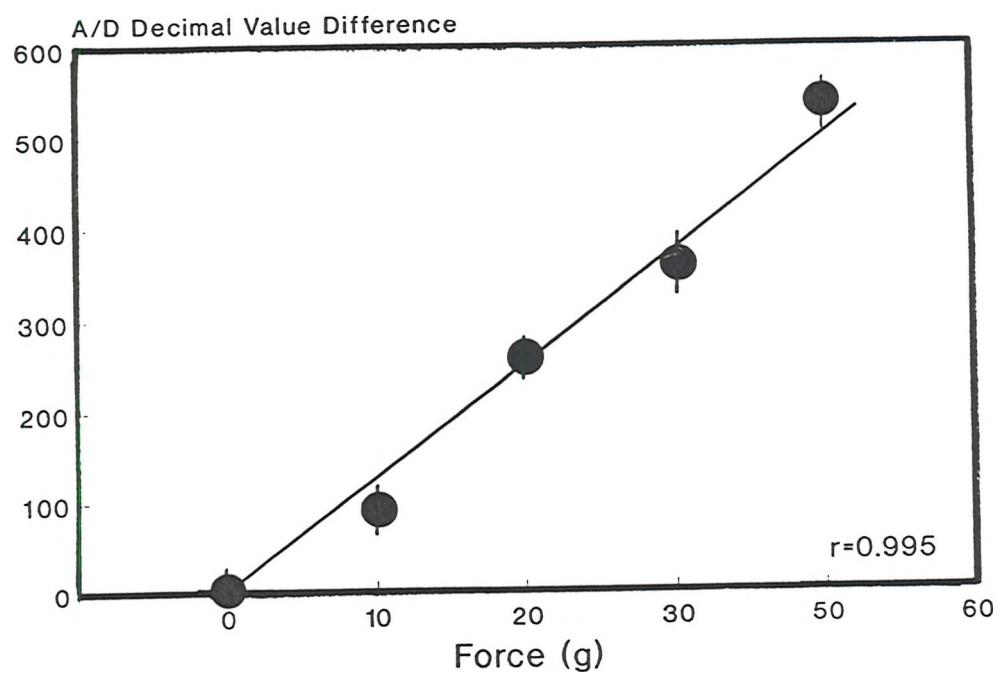
FIGURE 2.2. Calibration of the Harvard Bioscience Force Transducer over the 0-500 g range.
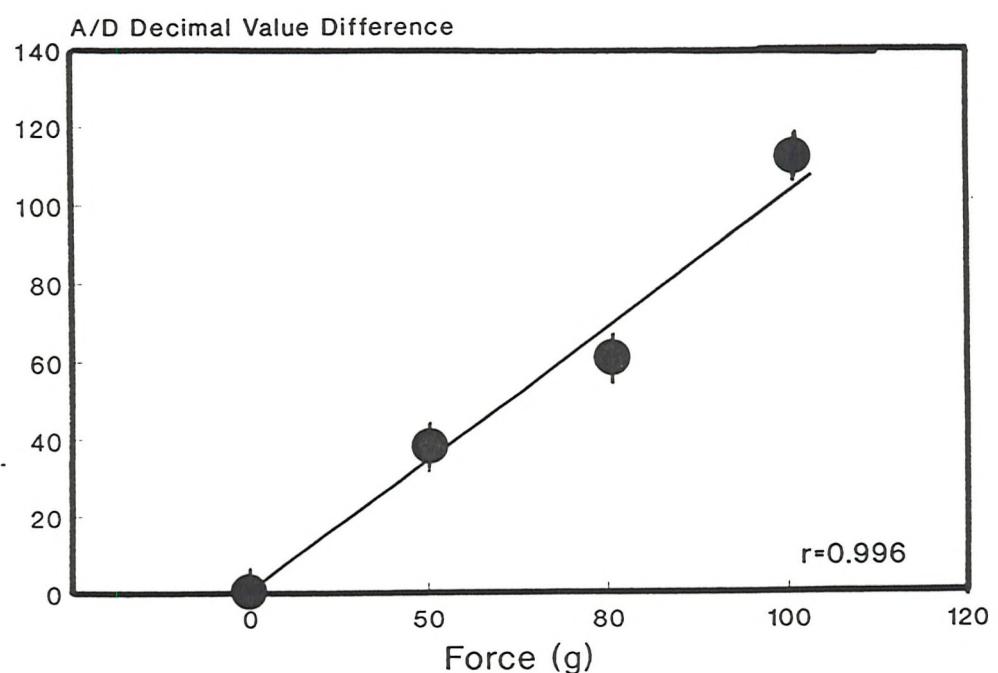
## DISPLAY/EDIT STIMULUS SEQUENCE

This option allows the creation of a new stimulus sequence or changes to be made to an existing sequence.

Having selected this option, the screen display will be similar to that seen in Figure 3.1.

FIGURE 3.1.

```
┌─────────────────────────────────────────────────────────────┐
│                                                              │
│     FILE NAME A                  SUB-SEQUENCE NUMBER B        │
│                                                              │
│                                                              │
│                                                              │
│                                                              │
│     STIMULATION FREQUENCY (HZ)      : C                      │
│                                                              │
│                                                              │
│     PULSE DURATION (secs)           : D                      │
│                                                              │
│                                                              │
│                                                              │
│     NUMBER OF PULSES (0 =duration): E                        │
│                                                              │
│                                                              │
│                                                              │
│     SUB-SEQUENCE DELAY (secs)       : F                      │
│                                                              │
│                                                              │
│                                                              │
│                                                              │
│    [F8] LOAD FILE;  [F9] FILE DATA; [F10] ACCEPT DATA        │
│                                                              │
│    [F7] SUB-SEQUENCE; [ENTER] NEXT FIELD; [ESC] EXIT         │
│                                                              │
└─────────────────────────────────────────────────────────────┘
```

**A FILENAME:** This shows the name of the current file (up to 8 characters) the stimulation sequence is stored. It should be a **.MSE** file.

**B SUB-SEQUENCE NUMBER:**
The programme allows up to 20 sub-sequences in a given file. This number shows which sub-sequence is currently displayed.

**C STIMULATION FREQUENCY :**
Displays the frequency at which the stimulation is set. The actual frequency of stimulation may not be this value but will be that frequency nearest the frequency set which the computer can achieve. This value should be entered as an integer.

**D PULSE DURATION :**
Displays the length of each stimulation period in seconds.

**E NUMBER OF PULSES :**
Displays the number of pulses to be elicited during the stimulus. A value of 0 indicates that the pulses should be delivered for the duration of the stimulation period.

**F SUB-SEQUENCE DELAY:**
Displays the time in seconds between successive sub-sequences. A negative value indicates the end of the sequence.

[F8]        : Function key F8 allows loading of another stimulation sequence file.

[F9]        : Function key F9 files the stimulation sequence data once a new stimulus sequence has been created.

[F10]    : Allows changes to a current stimulus sequence to be accepted.

[F7]        : Calls up another sub-sequence within the file displayed. This is achieved by typing the appropriate number from 1-20.

[ENTER] : Allows progression from one field of the stimulation sequence to another.

[Esc]    : Exits the DISPLAY/EDIT mode.

## 3.1 Creation/alteration of a stimulation sequence.

When selected the screen display is similar to Figure 3.1, with the cursor flashing at **STIMULATION FREQUENCY**. The file selected will be that stimulation sequence last used.

1. Type in new stimulation frequency in Hz, or press enter if unaltered. Press [ENTER] to move to next field.

2. Type new duration in seconds, followed by [ENTER].

3. Type in new number of pulses, followed by [ENTER].

4. Type in new sub-sequence delay in seconds, followed by [ENTER]. Note that a negative number will terminate stimulation sequence after this sub-sequence.

5. If the sequence has several sub-sequences press [F7] to go to next sub-sequence.

A message will be displayed at the top left corner of the screen : **"next sub-sequence"**

A message will be displayed at the bottom left corner of the screen : **"sub-sequence number ?_"** ⸱

Type sub-sequence number desired and press [ENTER].

6. Repeat steps 1-5 until all changes have been completed.

7. Go to last sub-sequence and check that a negative value has been entered in the "SUB-SEQUENCE DELAY".

8. If you have changed a current file press [F10] to accept new sequence.

A message will be flashed at the top left corner of the screen :**"data accepted"**.

If you have created a new sub-sequence press [F9] to file sequence under a new file name.

A message will appear at the top left of the screen : **"file data"**
A message will appear at the bottom left of the screen **"filename?_____"**

Enter new filename (up to 8 characters) and press [ENTER].

9. Having completed and saved changes press [Esc] to leave DISPLAY/EDIT mode.

## 3.2 To load existing files.

Having selected DISPLAY/EDIT mode the stimulation sequence of the most recently displayed file appears.  To call up another file :

1. Press [F8] to load new file.

A message appears at the top left corner of the screen "load file"
A message appears at the bottom left corner of the screen :"filename?___"

Type name of required stimulation sequence, followed by [ENTER].

2. This loads the file for use or allows any changes to be made (see section 3.1).

3. Exit programme by pressing [Esc].


## 4. COLLECT DATA

This programme collects data using the sequence loaded in DISPLAY/EDIT STIMULUS SEQUENCE (See Section 3).

When selected the screen displays the stimulation protocol as in Figure 4.1.


FIGURE 4.1.

```
┌─────────────────────────────────────────────────────┐
│                                                       │
│ FILENAME :A                      CLOCK FREQUENCY :B   │
│                                                       │
│                                                       │
│                                                       │
│ STIMULUS FREQUENCY   STIMULUS DURATION   PULSE NUMBER   DELAY │
│        C        ·           D              E          F │
│                                                       │
│                                                       │
│                                                       │
└─────────────────────────────────────────────────────┘
```

**A** :FILENAME - Displays the name (up to 8 characters) to which the collected A/D decimal values will be stored.

**B** :CLOCK FREQUENCY -Displays the frequency at which the computer clock is working.

**C** :STIMULATION FREQUENCY - Displays the frequency in Hz of stimulation as set by the sequence stored when in the DISPLAY/EDIT mode.

**D** :STIMULATION DURATION - Displays the duration of the stimulation in seconds as set by the sequence stored when in the DISPLAY/EDIT mode.

**E** :NUMBER OF PULSES - Displays the number of pulses to be elicited during stimulation as set by the sequence stored when in the DISPLAY/EDIT mode.

**F** : SUB-SEQUENCE DELAY - Displays the delay between sub-sequences in seconds as set by the sequence stored when in the DISPLAY/EDIT mode.

[F10] : Initiates the collection of data according to the sequence displayed on the screen  During collection a flashing cursor is displayed next to the sequence number (far left) eliciting stimuli.

[ENTER] : Allows the user to move between the filename and clock frequency fields.

[ESC] : quits programme.


## 4.1. Initiation of data collection.

1. Type in name of file to which data should be filed, followed by [ENTER].

2. Type in clock frequency (in Hz) if required, followed by [ENTER].

3. Check stimulation sequence displayed on screen.
If correct go to 4.  If incorrect go to 6.

4. Press [F10] to collect data.
A message appears at top left of screen :"collecting data".
Flashing cursor highlights the sub-sequence eliciting stimuli.

5. Test can be repeated by following steps 1-4 or changed (go to 6).

6. Press [Esc] to leave data collection and go to main menu.

## 5. RETURN TO DOS.

Selection of this option quits the collection programme and returns the user to DOS.

## 6.COMPILING FILES

To change the frequency at which data is collected via the A/D board a new file needs to be compiled using commercially available Turbo Pascal files.

To compile a new file:
1. Call file "**muscle .pas**" into a word processing package.

2. Edit the frequency statement as appropriate.

3. Save file as "{filename}.pas".

4. Exit word processing.

5. Using Turbo Pascal commands select **TURBO**

6. Workfile :   {filename}.pas
   Options   :   Compile

7. Quit to main menu.

8. Compile.

9. Compilation complete run programme using ATEST commands.

## 7. ANALYSIS

The analysis programme is also resident in **ATEST,** but cannot be accessed directly via the main menu of the data collection programme.

The analysis programme is accessed by typing **ANN** after the **C:\ATEST>** prompt, followed by [ENTER].

The screen should now look like Figure 7.1.

```
FIGURE 7.1




    next filename (TYPE end TO QUIT)?

    TYPE * FOR DIRECTORY, return to stay in presenT file.
```

## 7.1 To load a file.

1. If name of file known type name followed by [ENTER].

2. If name of file not known type * for directory.

3. The [ENTER] key allows the current file to be edited once quit.

4.When completed type END to return to DOS.

## 7.2 Analysis of twitches.

1. Type filename (see 7.1), followed by return.

2. The screen will prompt :"enter 1 for twitch analysis, 2 for FFC analysis".

Type 1 followed by return.

3. Screen will now show data being analyzed, e.g.

**data set 1     5 Hz    800 data points** .

when analysis complete the line will end with **ok.**

next data set will be analyzed until the sequence is complete.

4. The data in the file will be displayed on the screen as shown for example in Figure 7.2.  The scale of the box is adjusted automatically such that the force generated and time scale completely fill the display area.   It is therefore important to note the time scale bar situated at the top left of the display box (**A**).

FIGURE 7.2. A typical twitch response.



Screen layout:

```
FILE :                    A

STIMULUS #:

FREQUENCY:

STATUS:

SMOOTHING:


CALIBRATION:


MAXIMAL FORCE:

        at    ms

RISE RATE:

 0-100%  in      ms

RELAXATION:

100-50%  in     ms

 95-50%  in     ms


              OPTIONS:Next Last Reject Edit Calibration Print Finish
```
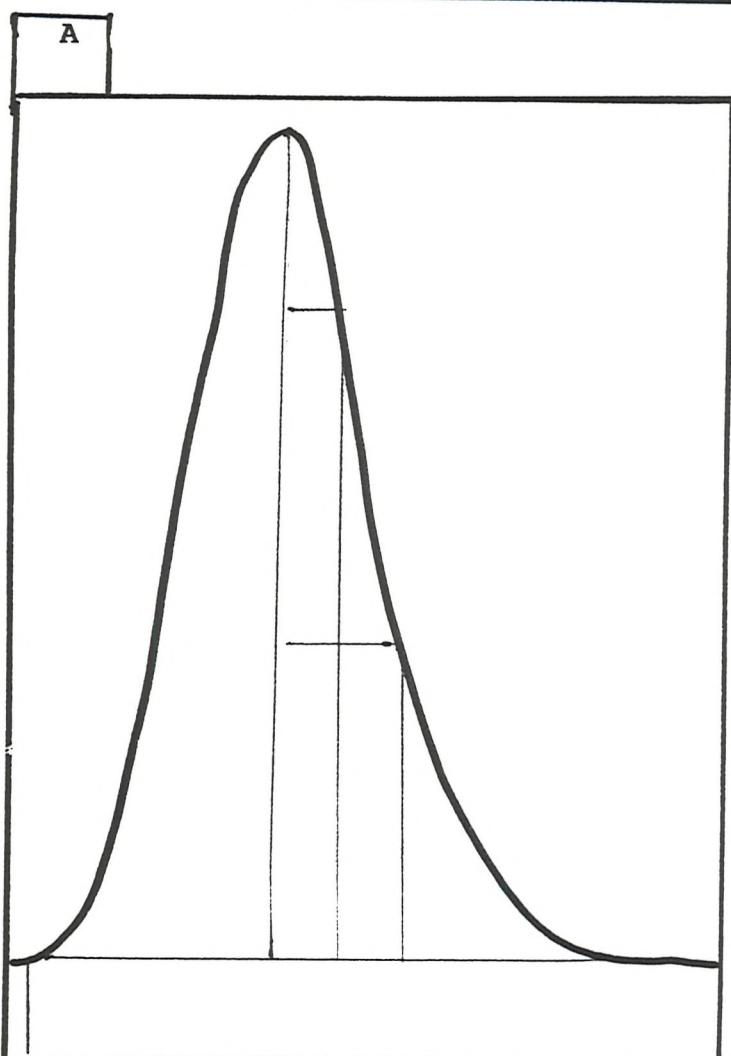
KEY:

**A:** This shows the time scale for the display box, such that the force generation and the time is maximal i.e. the displayed response fills the total available screen area.

**FILE** : This shows the file currently being displayed. To change file see **FINISH**.

**STIMULUS #** : This shows the number of the stimulus, equivalent to the subsequence number, several may be elicited up to a maximum of 20 as set in the DISPLAY/EDIT mode.

**FREQUENCY** : Shows the frequency at which the displayed force was elicited, as set in the sequence stimulation in the DISPLAY/EDIT mode.

**STATUS** : This should read **ok**. However, should the force generation be judged to be unrepresentative or not complete in some aspect it can be rejected such that the data is not taken into the analysis mode when calculations are conducted. Status will read either **ok** or **rejected**. See **reject** below.

**SMOOTHING** :Shows the current smoothing details.  0 = no smoothing, i.e. represent each data point collected.  There are up to 4 smoothing options.  See **SMOOTHING** below.

**CALIBRATION** : Allows the units per Newton to be defined according to the calibration of the transducer.  See **TRANSDUCER CALIBRATION** above.

**MAXIMUM FORCE** : Shows the force in newtons at the peak force and the time in ms after the **RISE** at which this occurs.

**RISE RATE** : Shows the time, in ms, for the force to reach maximum after the **RISE**.  This therefore is equivalent to the time to peak tension.

**RELAXATION RATE** : Shows the time taken for the force generated to decline : Displays both 100-50% (i.e. half relaxation time) and 95-50%.

**OPTIONS:** the selection of the following options allows the default settings for the analysis to be altered:
**Next** : displays the next stimulus in the sequence.
**Last** : displays the last stimulus in the sequence.
**Reject** : changes the stimulus status from **ok** to **rejected** or vice versa.
**Edit** : allows alteration of
**Smoothing** : can be set for one of four smoothing options. 0 = raw data display. 1 = average over 1 point. 2= average the difference between 2 data points ..4 average over 4 data points. Changing the smoothing option may be

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important in the analysis of the data. 0 = raw data, therefore smoothing may alter both the time and height of the maximal response as data points become lost. This ultimately is at the discretion of the user.

**Baseline** : This option sets the left then the right decimal value of the baseline and indicates the height of the current baseline which can be left unaltered (press [enter]), set automatically (type -1) or can be set by typing an integer followed by return.
**Max** : enlarges the initial part of the response to allow setting of the peak force generation. Press [enter] to complete peak averaging.
**Rise** : enlarges the initial part of the response such that the point at which contraction occurs can be defined.
**[enter]** = no change; -1 = manual setting using the left/right cursor keys; -2 = sets delay to 0.
**Calibration** : allows a new calibration slope to be entered. See TRANSDUCER CALIBRATION above.
**Print:** prints summary analysis to screen, giving mean values = one standard deviation. Rejected sequences are eliminated from analysis. Press space bar to return to force display.

**Finish** :  quits current file and allows another to be analyzed.


## 7.3 FORCE-FREQUENCY ANALYSIS.

To analyze a file containing data relating to a force-frequency curve:

1. Enter name of file to load (see section 7 above), followed by [enter].

2. Type 2 for force-frequency analysis, followed by return.

3. Screen will now show data analysis occurring e.g.:

   Data set 1   1 Hz   1500 data points.

   When acquisition complete **ok** will be displayed. This will continue until all sub-sequences have been collated.

4. Screen will look similar to Figure 7.2.

FIGURE 7.2. A typical force response at high frequencies of stimulation.
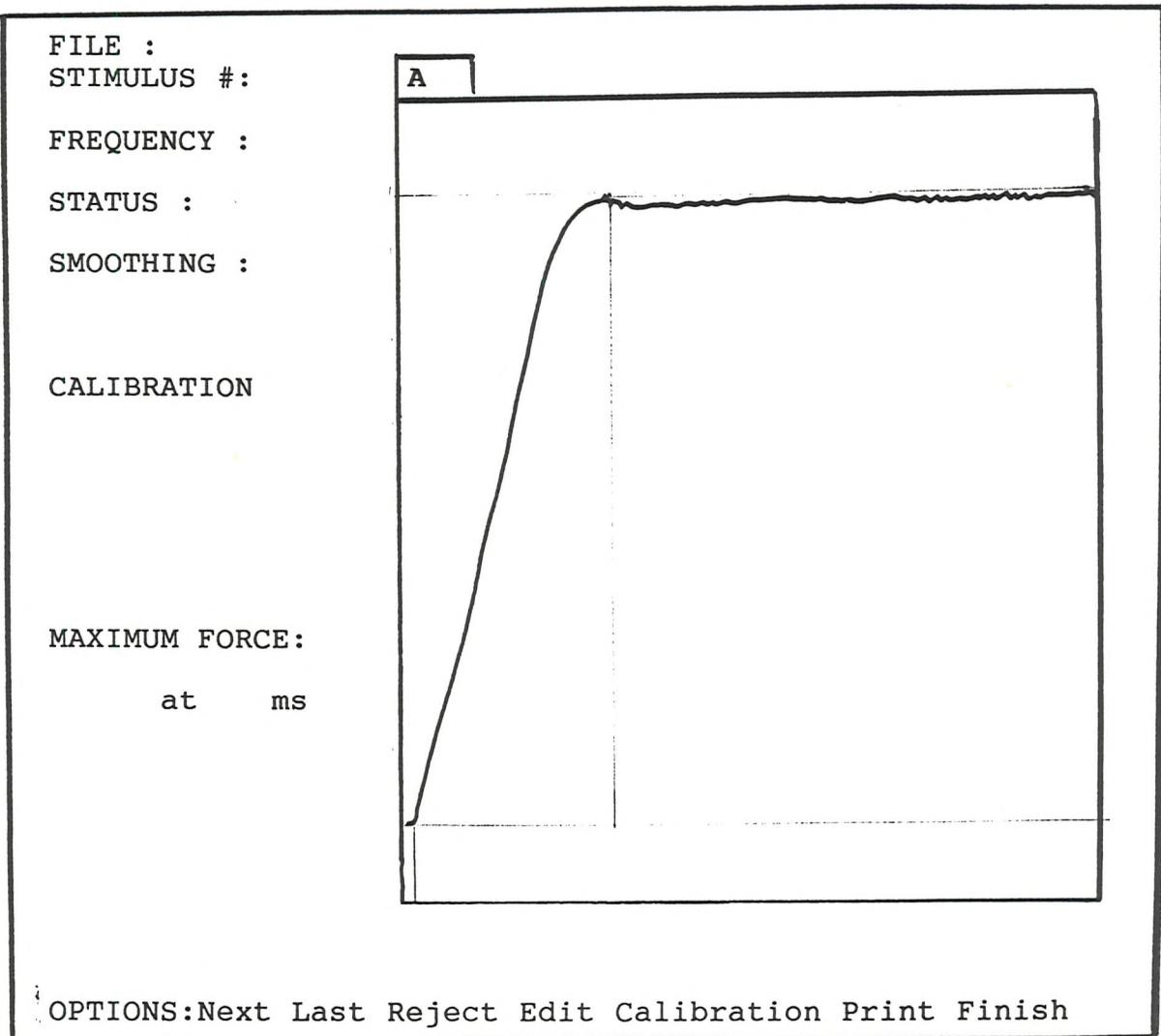
```
FILE :
STIMULUS #:          ┌─A─┐

FREQUENCY :

STATUS :

SMOOTHING :


CALIBRATION




MAXIMUM FORCE:

    at    ms




OPTIONS:Next Last Reject Edit Calibration Print Finish
```

KEY:

A: This shows the time scale for the display box, such that the force generation and the time is maximal.

FILE : This shows the file currently being displayed. To change file see **FINISH**.

**STIMULUS #** : This shows the number of the stimulus, equivalent to the subsequence number, several may be elicited up to a maximum of 20 as set in the DISPLAY/EDIT mode.

**FREQUENCY** : Shows the frequency at which the displayed force was elicited, as set in the sequence stimulation in the DISPLAY/EDIT mode.

**STATUS** : This should read **ok**.  However, should the force generation be judged to be unrepresentative or not complete in some aspect it can be rejected such that the data is not taken into the analysis mode when calculations are conducted. Status will read either **ok** or **rejected**. See **reject** below.

**SMOOTHING** :Shows the current smoothing details.  0 = no smoothing, i.e. represent each data point collected.  There are up to 4 smoothing options.  See **SMOOTHING** below.

**CALIBRATION** : Allows the units per Newton to be defined according to the calibration of the transducer.  See **TRANSDUCER CALIBRATION** above.

**MAXIMUM FORCE** : Shows the force in newtons at the peak force and the time in ms after the **RISE** at which this occurs.

**OPTIONS:** the selection of the following options allows the default settings for the analysis to be altered:
**Next** : displays the next stimulus in the sequence.
**Last** : displays the last stimulus in the sequence.
**Reject** : changes the stimulus status from **ok** to **rejected** or vice versa.
**Edit** : allows alteration of
**Smoothing** : can be set for one of four smoothing options. 0 = raw data display. 1 = average over  1 data point. 2= average the difference between 2 data points 4 = average over 4 data points.
**Baseline** : This option sets the left then the right decimal value of the baseline and indicates the height of the current baseline which can be left unaltered (press [enter]), set automatically (type -1) or can be set by typing an integer followed by return.  When analyzing FFC responses the baseline may be lost during smoothing.  The user should be alert for such losses.
**Max** : enlarges the initial part of the response to allow setting of the peak force generation.  Type 0 for automatic setting of maximum; type 1 to find calculate average of peak values having set the threshold using the up/down cursors; type **2** to set maximum manually using the up/down cursors; type **m** to allow analysis of part of the time period only, using the left/right cursors.
**Calibration** : allows a new calibration slope to be entered. See TRANSDUCER CALIBRATION above.

Print: prints summary analysis to screen, giving mean values = one standard deviation. Rejected sequences are eliminated from analysis. Press space bar to return to force display.
**Finish** : quits current file and allows another to be analyzed. Type END to return to DOS.

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