BIOCHEMICAL AND PHYSIOLOGICAL MECHANISMS OF CREATINE KINASE RELEASE FROM AVIAN SKELETAL MUSCLE DURING ACUTE STRESS

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List of Abbreviations

ADP	Adenosine di-phosphate
AOV	Analysis of variance
AST	Aspartate amino-transferase
ATP	Adenosine tri-phosphate
CK	Creatine kinase
CO_2	Carbon dioxide
2, 4 DNP	2, 4 di-nitrophenol
Dpm	Radio-active disintegrations per minute
EC-coupling	Excitation/contraction coupling
LDH	Lactate dehydrogenase
M199	Medium 199
N_2	Nitrogen
O_2	Oxygen
PLA_2	Phospholipase A_2
%RH	Percentage relative humidity
RyR	Ryanodine receptor
S.D.	Standard deviation
SR	Sarcoplasmic reticulum
TG	Thapsigargin

Declaration

This thesis is of my own composition and the work presented is entirely my own.
Any contribution by others to any part of the thesis has been acknowledged

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Abstract

Marked changes in the plasma activities of the intracellular enzyme creatine kinase (CK) have been observed in broiler chickens subjected to a range of stressful stimuli. Using a novel anion exchange chromatography technique to separate the various CK isoenzymes, it was demonstrated that the skeletal muscle form of CK (MM-CK) was predominant in plasma (96.8%) and that its activity increased 65.4% ($p<0.05$) in response to an episode of acute heat stress (2 h, 32°C/80% relative humidity). An investigation of mechanisms that might mediate the release of CK from this tissue was undertaken using a novel validated isolated *in vitro* chicken skeletal muscle preparation (*m. tensor patigialis*) and incubation system. CK loss from the muscle preparation under optimised control incubation conditions (150 min at 41.5°C, pH 7.4) was negligible, constituting less than 0.3% of total muscle CK content. Incubation at 45°C had no effect on the rate of CK loss above that observed under control conditions. Incubation under conditions of reduced O_2 availability (anoxia) was without effect on CK loss from the preparation. Limiting ATP availability by impairing muscle mitochondrial oxidative metabolism induced a 4-fold increase ($p<0.05$) in the rate of loss of CK. Promoting external ionic calcium (Ca^{2+}) entry into the muscle cells using 4 Br-A23187 calcium ionophore (25μM) caused a 60-fold increase ($p<0.001$) in the release rate of CK. Incubation with the ionophore induced a significant increase in Ca^{2+} accumulation (79.7%; $p<0.05$) as measured by the uptake of radio-labelled ^{45}calcium and was associated with a 8.6-fold greater total loss of CK. The results suggest that the entry of external Ca^{2+} into muscles represents an important initial event in the development of muscle membrane damage and subsequent CK loss. It is proposed that CK loss occurs as a consequence of Ca^{2+} mediated alterations in muscle cell membrane permeability, possibly caused by Ca^{2+}-activated phospholipase A_2 (PLA_2). In support of this suggestion, incubation with 25 μM Ro-31-4493/001 (an inhibitor of PLA_2 activity) significantly decreased CK loss by 44.6% ($p<0.05$) in muscle incubated with 4Br-A23187 (5 μM). The role of elevated external sodium (Na^+) entry into skeletal muscle was investigated as a potential mechanism for mediating trans-membrane changes in Ca^{2+} flux *in vivo*. Concentration-dependent

increases in ^{45}calcium uptake and total CK release were observed in muscle incubated with monensin Na^+ ionophore. The largest responses occurring at monensin concentrations of 100 μmol or greater, corresponding to a 49.0% increase ($p<0.05$) in ^{45}calcium uptake and a 2.4-fold increase ($p<0.001$) in total CK release. The incubation of muscles in medium containing an elevated external Na^+ concentration, and the prevention of Na^+ extrusion significantly augmented increases in ^{45}calcium uptake and CK loss in Na^+ loaded muscle cells, possibly through the action of Na^+/Ca^{2+} exchange. Direct inhibition of the Na^+/Ca^{2+} exchanger using amiloride (1 mM) had no effect on muscle ^{45}calcium uptake or CK efflux where active Na^+ extrusion via Na^+/K^+ exchange had been prevented. However, reducing external Ca^{2+} availability for Na^+/Ca^{2+} exchange in monensin treated muscles significantly reduced ^{45}calcium uptake (37.8%, $p<0.05$) but paradoxically produced a 84.5% increase ($p<0.001$) in total CK release, thus implicating the possible involvement of Ca^{2+} from some intracellular source, such as the sarcoplasmic reticulum (SR). Mobilisation and redistribution of Ca^{2+} from intracellular stores produced a significant increase in CK release (2.7-fold, $p<0.001$) in the absence of external Ca^{2+} entry. Inhibition of the release of Ca^{2+} from the SR through ryanodine-sensitive Ca^{2+} channels using dantrolene (25 μM) significantly reduced the release of CK (32.2%, $p<0.05$) in monensin treated muscles.

In complementary *in vivo* studies, birds treated with dantrolene (2.5 mg/kg) demonstrated no increase in plasma CK activity following exposure to acute heat stress. This contrasted with a 70.8% increase in plasma CK activity exhibited in vehicle treated controls exposed to the same conditions. It is therefore proposed that acute heat stress induces Ca^{2+} release from the SR in chicken skeletal muscle through the ryanodine-sensitive Ca^{2+} channels which in combination with the entry of external Ca^{2+} possibly through Na^+/Ca^{2+} exchange increases PLA_2 mediated alterations in sarcolemmal permeability which facilitates the intracellular loss of CK.

Chapter 1

Introduction

Currently, in excess of 680 million broiler chickens are produced per annum in the UK alone (Poultry World, 1997). The rearing of these animals on large numbers of geographically dispersed sites necessitates their transportation to centralised processing for slaughter. In transit the birds may be exposed to a variety of potential stressors including the thermal demands of the transport micro-environment, motion, acceleration vibration, impact, fasting withdrawal of water, social disruption and noise. The adverse effects of these factors and their combinations may range from discomfort and mild aversion to death. Mortalities in transit are generally 0.4% or less but this may represent approximately 2 million birds per annum in the UK. It is estimated that up to 40% of the mortalities observed at the processing plant are a consequence of stress.

Through extensive research undertaken at the Roslin Institute (Edinburgh) in collaboration with Silsoe Research Institute (Bedfordshire) it has been established that the thermal environment is the major potential source of stress during transportation (Kettlewell, 1989, Mitchell *et al.*, 1992; Kettlewell *et al.*, 1993; Kettlewell and Mitchell, 1993; Mitchell and Kettlewell, 1996). Adverse thermal environments develop as a direct result of metabolic heat production by the birds with an associated inadequacy in the removal of this heat. The average chicken at slaughter age (6 weeks) can produce between 10-15 W of heat. On a vehicle carrying a typical load of 6500 birds, the total heat produced may be nearly 100 kW (Mitchell and Kettlewell, 1996). It is evident that if this thermal load is not removed then the effective result at bird level will be an increase in temperature which will at least compromise welfare and in extreme cases may be fatal. These effects are also heavily influenced by the prevailing ambient conditions (Kettlewell and Mitchell, 1993).

In order to determine the extent of stress imposed upon birds in transit there have been several experimental studies performed by the research group at Roslin employing a wide range of temperature-humidity combinations representative of those encountered in commercial practice. Laboratory simulation studies have assessed the degree of physiological stress imposed and have determined the combinations of temperatures and humidities producing equivalent biological effects. The birds' responses have been accurately quantified in simulation experiments using commercial transport containers in controlled climate chambers. The physiological measurements employed include deep body temperature, changes in blood chemistry such as acid-base status (e.g. pH and pCO_2), electrolytes (e.g. sodium, potassium and calcium)

and metabolites (e.g. glucose, triglyceride, non-esterified fatty acids and lactate), differential white blood cell (leucocyte) profiles (e.g. heterophil-lymphocyte ratios) as well as recognised stress-related hormones (e.g. corticosterone and glucagon). In addition, the plasma activities of several "tissue-specific" enzymes have also routinely been measured as indicators of stress-induced tissue dysfunction or damage. These enzymes include creatine kinase (CK), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alkaline phosphatase (ALP). These studies have attempted to correlate changes in the physiological variables measured with the degree of heat stress imposed. Several relationships have been established between increasing heat load and the associated degree of hyperthermia, hypocapnia and alkalosis. Similarly, the magnitude of the increase in the heterophil-lymphocyte ratio (indicative of corticosterone secretion) and the extent of the apparent efflux of intracellular enzymes have also been shown to be related to the extent of the thermal stress imposed. In addition, these studies have also shown depletion of energy reserves resulting in fatigue and dehydration (as reflected by changes in plasma metabolites and blood chemistry) are additional factors related to the intensity of the heat load imposed and that the duration of exposure further compromises the animals adaptive or homeostatic capacity and thus exert detrimental effects upon welfare and productivity. The current project was therefore designed in this context as part of a multi-disciplinary approach in to understanding the mechanisms of the physiological stress responses in broiler chickens during transportation, placing emphasis on those responses caused by the thermal component of "transportation stress".

Stress-induced skeletal muscle damage ?

The reports of elevations in plasma creatine kinase (CK) activity in broiler chickens following transportation and in the transport simulation studies are believed to be of relative importance to the poultry industry not only from an animal welfare perspective but also from a commercial stand point. Historically, increases in plasma CK activity are usually indicative of damage to skeletal muscle which in the broiler chicken is the marketable product. Whilst it is assumed that the stress-induced increases in plasma CK activity are specific to skeletal muscle, its loss from other tissues is recognised. Therefore, identification of the origin of CK is imperative if we are to able to understand why under varying stress situations, the stress response is specific to that tissue. This study was undertaken to elucidate the fundamental biochemical and physiological mechanisms involved in the release of creatine kinase in the broiler chicken in response to acute stress.

Outline of Thesis

Chapter 1 sets the context in which the current Ph.D. project was conceived and designed, in relation to the on-going research programme examining the impact of stress in livestock during transportation and exposure to extreme climatic conditions. Included in this chapter is a review of the current knowledge of the effects of, and physiological responses to stress in domestic fowl, specifically in relation to acute heat stress. In addition to this there is a comprehensive overview of avian skeletal muscle, its morphology, biochemistry and physiological function. Following on from this is a detailed synopsis of the intracellular enzyme creatine kinase (CK), its isoforms, tissue distribution and role as a diagnostic marker of tissue damage or dysfunction. **Chapter 2** is a general materials and method section which describes the subjects used in the Ph.D. study together with detailed descriptions of the various protocols and procedures used as well as the statistical analyses employed. **Chapter 3** describes the development of a solid phase chromatographic technique for the separation of the CK isoenzymes from chicken tissue extracts and plasma to identify the origin of the isoenzyme/s responsible for basal CK activity in plasma of non-stressed broiler chickens, and then subsequently establish the origin of the isoenzymes responsible for the increase in total plasma CK activity in these birds following acute heat stress exposure. **Chapter 4** describes the development and validation of a novel isolated *in vitro* chicken skeletal muscle preparation and incubation system for examining the biochemical and physiological mechanisms of CK release under acute stress conditions. The experiments presented this chapter characterise the "baseline" efflux of CK from the muscle preparation under optimised control incubation conditions. **Chapter 5** examines the effect of elevated incubation temperature on CK release from the isolated skeletal muscle preparation (comparable with deep body temperatures achieved during heat stress). **Chapter 6** characterises the profile of CK release from the isolated skeletal muscle preparation under conditions of impaired oxidative energy metabolism. **Chapter 7** investigates the role of external calcium (Ca^{2+}) entry into skeletal muscle as a potential key determinant in the development of cellular damage. The results of the experiments presented in this chapter strongly support this hypothesis. In light of these findings, the work undertaken and presented in **Chapter 8** investigates the potential role of Ca^{2+}-mediated phospholipase A_2 activity in producing alterations in muscle cell membrane integrity and subsequent CK release. **Chapter 9** examines the possible role of external sodium ions (Na^+) as a mediator of trans-membrane Ca^{2+} flux and subsequent CK release from skeletal muscle during acute stress, using the Na^+ ionophore, monensin. The outcome of

work presented in this chapter suggesting that increases in intracellular Na^+ might mediate CK loss by increasing in external calcium uptake, but could also increase intracellular Ca^{2+} concentration by stimulating the release of Ca^{2+} from an intracellular source. **Chapter 10** investigates the effects on CK release of the mobilisation and re-distribution of Ca^{2+} from muscle sarcoplasmic reticulum (SR) stores and the effect of the inhibition of SR Ca^{2+} release through ryanodine-sensitive Ca^{2+} channels using dantrolene sodium. **Chapter 11** describes the myopathic consequences of monensin Na^+ ionophore *in vivo* as a corollary to experiments undertaken and presented in **Chapter 9**. Similarly, **Chapter 12** describes the *in vivo* effects of the inhibition of skeletal muscle Ca^{2+} release via the ryanodine-sensitive Ca^{2+} channels using dantrolene sodium in broiler chickens exposed to acute heat stress. Summary of the major findings and conclusions as well as a discussion of the possible applications and direction of future research relating to the findings presented in the thesis are put forward in **Chapter 13**.

Literature Review

STRESS

Definition of stress

The term "stress" still eludes a satisfactory definition as its meaning can change from situation to situation and from user to user. In a biological dimension stress is seen to cause a combination of physiological and behavioural adaptations which constitute what is regarded as a "stress response" to aversive stimulus or stimuli which present a challenge (Freeman, 1987). Stress physiology has been described by Yousef (1985) as the study of an animal's physiological, biochemical and behavioural responses to various factors of the physical, chemical and biological environment. Generally, stress denotes the magnitude of a force or forces external to the bodily system which can displace that system from its resting or ground state. In this context stress can seen to induce compensatory changes and/or adjustments either at subcellular or whole animal level which help reduce the aversive effects imposed upon it thus allowing the animal to cope better with its environment.

Types of stressors

A stress response can occur when an animal is presented with or exposed to a wide variety of stimuli which place some kind of demand upon the animal. These stimuli are often defined as "stressors" and can be conveniently categorised in simple terms as either environmental, physiological or psychological stressors. It is recognised however that with some stressors there is some degree of overlap and that under certain conditions several stressors may be imposed simultaneously. Examples of each type of stressor are shown in **Table 1.1**.

Table 1.1 Categories and examples of stressors

Category of stressor	Stimuli
Environmental	Exposure to cold, heat, noise, radiation, wind, air pressure pollutants, hunger and thirst.
Physiological	Surgery, trauma and blood loss, disease, infection, toxicosis, anaesthesia, exercise and reproduction
Psychological	Perceived predation threat, competition or potential conflict, novelty, anticipation, fear, unpredictability and change

Heat stress

The detrimental effects of exposure to high environmental temperatures and humidities (thermal load) on animal health and productivity have long been recognised and have been extensively investigated on many species including broiler chickens (Hillman *et al.*, 1985). Exposure to elevated thermal loads is more commonly referred to as heat stress (Ostrowski-Meissner, 1981; Mitchell *et al.*, 1992) which, dependent on duration, may be defined as acute (short term) or chronic (long term). Acute heat stress can be defined as short term exposure to an elevated thermal load which causes an rise in deep body temperature (hyperthermia) sufficient to illicit a compensatory "thermoregulatory response".

Responses to heat stress

Avians, like mammals, have evolved a thermoregulatory system designed to maintain deep body temperature within a narrow range, with the minimal expenditure of energy (Freeman, 1983). The sum of the heat gained from metabolism, muscular activity, heat increment of feed and heat loading from the environment is balanced by heat losses via radiation, conduction, convection and evaporation. At higher temperatures evaporation becomes the most important route for effective heat loss (Van Kampen, 1971). This is primarily achieved through increased respiratory evaporation which is accelerated by panting, and cardiovascular changes. Some of the most important physiological mechanisms used by birds to dissipate heat are discussed below.

Respiratory evaporative heat loss

Respiratory evaporative heat loss is the major route of heat loss in avians and other panting species under heat stress conditions (Yousef, 1985). The control of the respiratory system is greatly influenced by high environmental temperatures and has evolved to promote heat loss and the prevention of hyperthermia through a process of moisture evaporation during respiration (Thompson, 1985). Heat loss occurs during the respiratory process due to evaporation of water (latent heat of vaporisation = 2.45 MJ kg^{-1}) from the surfaces of the upper respiratory tract. Air passing over the wet surfaces of the respiratory tract, becomes saturated at near body temperature. Provided the inspired air is neither saturated nor equal to body temperature the expired air will contain a high proportion of heat and moisture. The rate at which moisture is evaporated from the respiratory tract is considerably increased by panting, as the rapid replacement of moist air over the evaporatory surfaces with fresh dry air creates a large gradient of vapour pressure between surface and air. Some cutaneous evaporative

heat loss has been shown to occur due to the passive diffusion of water through the skin (Siegel, 1976). In comparison to the relative amount of heat dissipated by respiratory evaporation under conditions of heat stress, the effects of cutaneous evaporative are minimal.

Cardiovascular changes

During heat stress, the unfeathered extremities on the head and legs of birds are normally vasodilated and cardiac output can be increased by nearly 30% (Richards, 1971). This increase in cardiac output is associated with an increase in heat rate which is believed to be a response to the effects of blood flow redistribution to the extremities and the tissues of the respiratory surfaces (Whittow *et al.*, 1964). The precise role of an increase in heart rate in response to heat stress is still unclear. However it has been suggested by Calder and King (1974) that the heart rate response during heat stress may also be influenced by other non-thermal stresses imposed on the birds during experimentation.

Exposure to heat stress leads to the redistribution of cardiac output to tissues essential for heat dissipation. Wolfenson *et al.* (1981) reported a 4-fold increase in capillary blood flow in the skin, nasobuccal tissues and expiratory muscles in heat stress exposed laying hens. In the same study, blood flow to bone and the digestive and reproductive tracts was shown to be reduced by between 50 and 80% of the control level. The increase in blood flow distribution to the skin and nasobuccal tissues increases heat loss and in the respiratory muscles help supply and sustain the metabolic demands of panting. Blood flow to the vital organs such as heart and brain under heat stress conditions is maintained by a reduced perfusion of non-vital tissues such as the abdominal viscera and non-respiratory skeletal muscle. Blood flow redistribution is achieved through an increase in flow rate through arteriovenous anastamoses (AVAs) and is increased from 2% under thermoneutral conditions to between 9 and 11% under acute heat stress conditions.

Behavioural thermoregulation

In situations where birds are unable to avoid the effects of exposure to heat stress (e.g. during confinement or transportation) they can still influence the rate of heat loss by changes in posture (Richards, 1975). These behavioural changes primarily involve the holding of the limbs away from the body to increase the effective area for convective heat loss (15-20%) from the whole body surface.

Endocrine responses

Short term exposure to heat stress increases plasma cortisol in mammals and corticosterone in avians (Yousef, 1985; Mitchell *et al.*, 1992). However, it is recognised that the consequent rise in glucocorticoid production may be as a result of a general stress reaction and may not necessarily be specific to the effects of thermal exposure *per se*. In addition, thermally induced changes in water and mineral metabolism have been shown in part to be hormonally controlled by the mineralocorticoid, aldosterone and anti-diuretic hormone (ADH) in many species including poultry and play a major role in the homeostasis of body fluid volume and concentration (Kechil *et al.*, 1981).

Metabolic consequences of heat stress

Disturbance in blood gas concentrations

Prolonged exposure to high or low environmental temperatures leads to disturbances in blood gas concentration and respiration pattern. During exposure to high environmental temperatures when panting begins there is an increase in respiratory rate coupled with a reduction in tidal volume (El Hadi and Sykes, 1982). The former outweighs the latter and the net effect is an increase in respiratory minute volume leading to rapid shallow panting. The observed reductions in tidal volume determine the extent to which the extrapulmonary ventilation is confined to the dead space of the respiratory passages where over-ventilation produces changes in pulmonary gas exchange and alkalosis. In severely hot environments, when body temperature rises continuously, rapid shallow panting is superseded by a second phase of slower deeper panting in which tidal volume increases to near normal but respiration rate remains above normal. The result of this is a respiratory minute volume which is even greater than it is during rapid shallow panting and is not confined to the respiratory dead space. Alveolar ventilation increases to 4 or 5 times the control level causing a marked respiratory alkalosis in which the partial pressure of arterial blood carbon dioxide (pCO_2) can fall below 10 mmHg and pH can exceed pH 7.7.

Respiratory Alkalosis

When birds are exposed to sufficiently high ambient temperatures to induce panting they are vulnerable to respiratory alkalosis. Increasing respiration rate lowers the partial pressure of carbon dioxide (pCO_2) in the lungs, which in turn lowers the concentration of blood bicarbonate (HCO_3^-) and raises blood pH (El Hadi and Sykes, 1982).

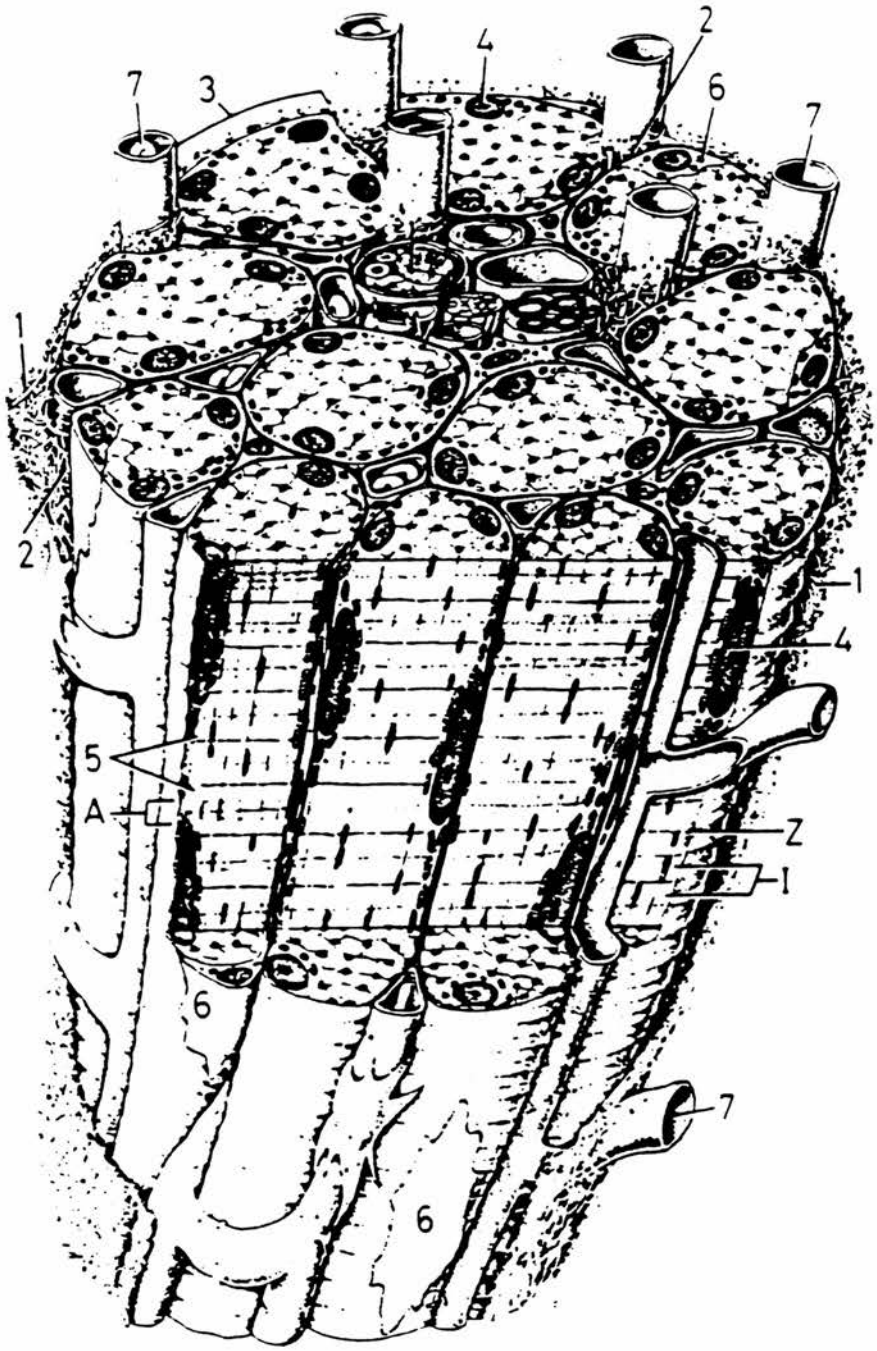

Figure 1.1 A diagrammatic representation of a fascicle of mammalian skeletal muscle cells (myofibres). The fascicle of myofibres is bounded externally by the perimysium (**1**), a feltwork of collagen fibrils which is continuous with a mesh of finer collagen fibrils, the endomysium (**2**), between the individual myofibres (**3**). The myofibres contain elongated nuclei (**4**) and contractile myofibrils (**5**); small myosatellite cells (**6**) are situated in close apposition to the myofibres external surfaces. Also illustrated are the numerous longitudinally running blood capillaries (**7**) with their transverse inter-connections. Figure adapted from an illustration in **Skeletal Muscle Pathology** Eds. F.L. Mastaglia and J. Walton, Churchill-Livingstone, London (1982) p2.

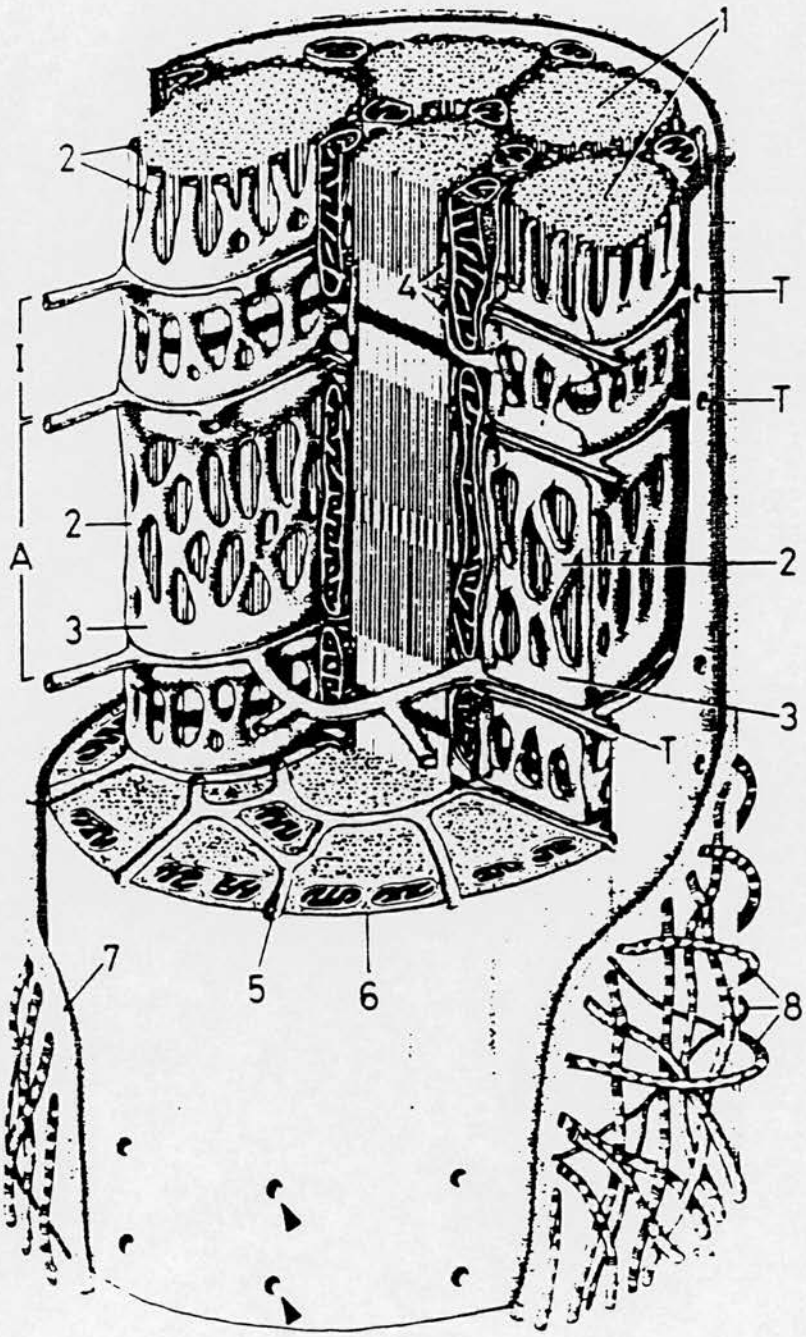

Figure 1.2 A diagrammatic dissection of a single mammalian myofibre. The myofibrils (**1**), with their arrays of thick and thin myofilaments are ensheathed by the complex internal membrane system of the sarcoplasmic reticulum (SR), which is structurally and functionally divided into central fenestrated zones (**2**) and the terminal cisternae (**3**). The transverse tubular (T) system contacts with the terminal cisternae of the SR to form triads (**4**), a three part structure which is attached to the continuous myofibril surface (**5**) via sub-surface caveolae (not shown) and the sarcolemma (**6**). The myofibre is surrounded externally by an amorphous extracellular component, the basal lamina (**7**) and endomysial collagen/reticulin fibrils (**8**). Figure adapted from an illustration in **Skeletal Muscle Pathology** Eds. F.L. Mastaglia and J. Walton, Churchill-Livingstone, London (1982) p10.

Morphology

Skeletal muscle cells (myofibres) are collectively arranged into groups of between 10-20 fibres to form muscle fascicles (**See Figure 1.1**). Within the fascicle, individual myofibres are separated by thin connective tissue septa (endomysium) and surrounded by a denser connective tissue envelope (perimysium) which together confer mechanical support and provide a structural framework for the process of muscle contraction. The outer surface of the individual myofibre (**See Figure 1.2**) is composed of a tightly-linked three component complex, endomysial tissue layer, basal lamina and plasma membrane (sarcolemma). A second internal membrane component, the sarcoplasmic reticulum (SR) and the T-tubule system is found branching between the muscle contractile units (myofibrils) forming a complex reticular network which plays functional role in the control of the activation of contraction and relaxation of the myofibrils.

Myofibrils

The major subcellular component of the skeletal muscle fibres are the myofibrils (**see Figure 1.2**) which occupy approximately 80-90% of total cell volume. The myofibrils are composed of serially repeating identical units (sarcomeres) of thin and thick contractile elements (myofilaments). Thin I-band (isotropic) actin filaments are attached to the Z-disk and interdigitate with the thick myosin filaments of the A-band (anisotropic). The interaction between the actin of the thin filaments and the myosin of the thick filaments shortens the sarcomeres. The thin filaments also contain the proteins tropomyosin and troponin that mediate the interaction between actin and myosin through the action of Ca^{2+} (Ebashi, 1980). During contraction the actin filaments slide into the centre of the A-band decreasing I-band length and the myosin region (H-zone) without actin filaments and thereby increasing filament overlap (**See Figure 1.3**).

T- system

The muscle T-tubules (T-system) represents a network of tubular invaginations of the plasma cell membrane (sarcolemma) that forms specific functional associations with the SR. The T-system has the function of transporting the electrical depolarising signal into the cell to activate muscle contraction through the release of calcium ions (Ca^{2+}) from the SR.

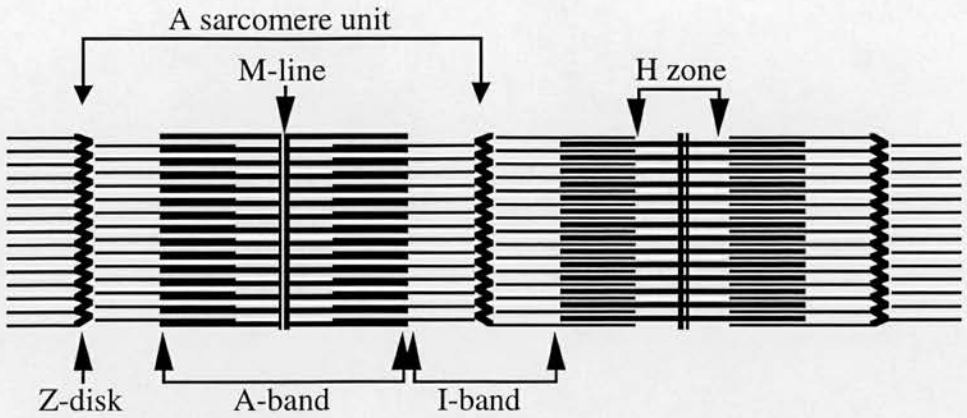

Figure 1.3 Diagrammatic representation of the muscle cell myofilaments.

Sarcoplasmic Reticulum (SR)

The SR is a completely internal membrane system that forms a 3-dimensional network of cisternae and tubules around the myofibrils, with localised areas of membrane differentiation adjacent to specific band of myofibrillar striations (**See Figure 1.2**). SR structural differentiation occurs in close proximity to the Z-line within the I-band of the myofibril, where the T-tubular system and SR terminal cisternae form a 3 part structure (triad), and where longitudinal tubules originating from the SR terminal cisternae converge to form a fenestrated area adjacent to the centre of the myofibrillar A-band (**See Figure 1.2**).

T-system and SR functional coupling

The SR association with the myofibrils plays a major role in controlling the state of activation of the contractile myofilaments by regulating the concentration of calcium in the sarcoplasmic space (Eisenberg 1983). The SR engages in two separate activities while carrying out this important function. First, the SR pumps calcium into its interior and holds it there, thus lowering the free calcium concentration in the sarcoplasmic space to below 10^{-7} M. Secondly, the SR releases sufficient calcium into the sarcoplasm to bind to troponin to release contraction inhibition. The pumping activity of the SR takes place continuously whereas Ca^{2+} release is triggered only when the muscle cell is stimulated. Autoradiographic studies have demonstrated that SR release of calcium is specific to the terminal cisternae (Winegrad, 1970), in contrast the process of Ca^{2+} re-pumping into SR during the relaxation phase of the contraction cycle is distributed evenly throughout the SR (Meissner, 1975). These observations are consistent with the fluorescent antibody studies of the relative distribution of two important SR proteins, Ca^{2+}-ATPase and calsequestrin, both of which are integral to the processes of SR calcium uptake and storage. SR

calsequestrin has been shown to be localised adjacent to the myofibril A-I band interface, whereas, Ca^{2+} ATPase activity is more uniformly distributed along the I band as well as to the centre of the A-band. Suggesting that Ca^{2+} is stored and released from the terminal cisternae, whereas its uptake occurs along the entire surface of the SR (Jorgensen *et al.*, 1979: Junker and Sommer, 1979; Junker and Sommer, 1980).

Excitation-contraction (EC) coupling

The evolution of a structural and functional coupling of the T-system and the SR with the muscle myofibrillar contractile elements, the so called process of excitation-contraction (EC) coupling, has been extensively researched. EC coupling describes a process of rapid signal transduction that occurs in skeletal muscle following depolarisation of the surface membranes propagates down the T-tubular network to lead to the release of Ca^{2+} from the SR. The precise mechanism of EC coupling is still unknown, however recent advances have lead to a better definition of some of the processes involved (Rios and Pizarro, 1991; Rios *et al.*, 1992; Franzini-Armstrong and Jorgensen, 1994). It is currently recognised that there are at least three discrete distinguishable stages involved in EC coupling. Firstly, a process of voltage sensing which occurs at the level of the T-tubule membranes, followed by a transmission process which links voltage sensing to changes in the SR that allow the release of Ca^{2+}, and finally the release of Ca^{2+} itself. Advances in understanding the primary event of voltage sensing and that of the ultimate release of Ca^{2+} from the SR from biochemical and molecular studies, and through studies of intra-membrane charge movements have identified two molecular species associated with these events, namely the dihydropyridine receptor (DHPR) and the ryanodine receptor (RyR) as the protagonists of voltage sensing and Ca^{2+} release respectively (Leung *et al.*, 1987; Rios and Brum, 1987; Block, 1988; Rios *et al.*, 1992; Pozzan *et al.*, 1994).

Fibre characteristics

It has long been recognised that vertebrate skeletal muscles do not consist of a single homogeneous population and that they are divisible on the basis of a number of morphological and physiological characteristics into at least two major sub groups. The most obvious visible difference being a variation in red coloration which correlates to the presence of the intrinsic muscle protein myoglobin (Mastaglia and Walton, 1982). On the basis of this muscles can be loosely classified as "red" or "white" muscle types. Early comparative studies established that both types of muscle differed in their physiological properties in that red type muscles were slower in

contraction and relaxation than the white muscle and required lower frequencies of electrical stimulation to generate a tetanic response (Lawrie, 1952).

The initial observation of differences in myoglobin content of red muscle were found to reflect a capacity to attract and store oxygen that was associated with a highly developed cytochrome oxidase system (oxidative) to support the aerobic synthesis of energy-rich compounds. "White" muscles on the other hand were found to lack these properties and to be more efficient in the anaerobic synthesis of energy rich substrates from intracellular stores in the form of glycogen (glycolytic) (Mastaglia and Walton, 1982).

Fibre types

The identification of functional and metabolic differences between individual muscles was followed by the recognition that the degree of 'redness' or 'whiteness' of a muscle reflected the sum of the properties of its constituent fibres (Brooke and Kaiser 1970). Through the development of histochemical techniques it has been possible to determine the metabolic characteristics of individual muscle fibres. Most of this type of work has primarily focused on mammalian skeletal muscle. Mammalian muscle fibre types are now commonly classified into convenient categories such as those described by Brooke and Kaiser (1970), i.e. types I (slow, oxidative), IIA (fast oxidative) and IIB (fast glycolytic) fibres. The classification of avian skeletal muscle is essentially the same as mammalian skeletal muscle (Barnard, 1980). However, it has been shown in studies by Asiedu and Shafiq (1972) that avian skeletal muscle has a third class of tonic fibre (type III), which is further divided into two sub-types, IIIA and IIIB .

Avian type I fibres

Prior to histochemical studies undertaken by Barnard *et al.*, (1982) it had originally been thought that slow-twitch type I fibres did not occur in avian skeletal muscle (Ashmore *et al.*, 1978). However, Barnard *et al.*, (1982) identified slow-twitch type I fibres in the chicken comparable in function to those found in mammals. Confirmation of this avian fibre type was based on histochemical similarities with human type I fibres. Furthermore, evidence of multiple nerve innervations with *en plaque* terminations in this fibre type had been demonstrated by Buchthal and Schmalbruch (1980) which was consistent with the physiological properties of motor units in human slow-twitch type I fibres.

In broiler chickens, type I slow-oxidative fibres have been shown to be localised in the muscles of the leg that are required to maintain posture and work

against gravity namely, the *pubo-ischio femoralis-(pars medialis)*, and "red" *sartorius* (Barnard *et al.*, 1982; Iwamoto *et al.*, 1993) These muscles have been found to contain approximately 30-45% type I fibres and tend to predominantly occur within fast-twitch but not in tonic muscles (**See Table 1.2**). No muscle has yet been found in chicken to contain a predominance of type I fibres like in mammalian skeletal muscle (e.g. *soleus*).

Avian type II fibres

It is well established in mammals that there are two predominant subclasses of fast-twitch myofibre; type IIA (fast oxidative) and IIB (fast glycolytic) fibres (Brooke and Kaiser, 1970). More recently a third class of fast-twitch muscle fibre (type IIC,~1%) has been identified (Dr. I. Nimmo *pers comm.* 1997). The two major type II fibres sub-classes (A and B) have been demonstrated biochemically and histochemically in avians skeletal muscle (Ashmore and Doerr, 1971; Talesara and Goldspink, 1978). As with avian type I fibres the morphological and histochemical characteristics of avian type II subclasses were very similar to those observed in human type II fibres. Cross-species type II fibre confirmation was based on action potential characteristics and responses to denervation in avian fast-twitch type II fibres which were demonstrated to be very different to the responses observed in avian 'type I' fibres (Cullen *et al.*, 1975; Buchthal and Schmalbruch, 1980).

In the broiler chicken, it has been shown that pectoralis muscle (breast muscle) is comprised almost entirely (99%) of type IIB fast-twitch fibres (**See Table 1.2**). The very high proportion of type IIB fibres in broiler chicken pectoral muscle is atypical, as in most avian species the relative proportion of IIB fibres in pectoral muscle is between 20-50% (Rosser and George, 1986). The high proportion of IIB fibres in chicken pectoral muscle has been brought about by the commercial intensive breeding of broiler chickens for high breast meat yield. In most avians the pectoralis muscle normally provides the power for the down stroke during flight (but not broiler chickens which are unable to fly), and is massive relative to other appendicular muscles in other flighted species (Rosser and George, 1986). The pectoral muscles of the modern broiler chicken typically comprise over 50% of total muscle mass and therefore has a high commercial relevance (Ross Breeders, 1996). The greater proportion of chicken muscles contain type II fibres of differing relative proportions of type A and B fibres, and account for over 80% of a chicken's muscle mass.

Avian type III fibres

Type III muscles are defined as "tonic muscles" due to their contraction behaviour and histochemical characteristics which applies to all tonic muscles. Type III fibres are multiply innervated, the density of which is on average greater than that observed in type I fibres in both mammals and birds (Barnard *et al.*, 1982). The difference between type IIIA and IIIB fibre types stems from the relative differences in phosphorylase, NADH-TR (nicotinamide adenine dinucleotide-tetrazolium reductase) and ATPase concentrations. Type IIIB fibres possess a higher concentration of these enzymes than type IIIA, but innervation in these fibre types does not vary (Ashmore *et al.*, 1978).

Tonic fibres are very rare in mammals except in the specialised structures of the extraoccular muscles and oesophageal muscles (Hess, 1970), but are more prevalent in avian skeletal muscle. In the broiler chicken, *anterior latissimus dorsi*, *plantaris* and *adductor profundus* muscles have all been shown to be composed entirely of tonic type III fibres (**See Table 1.2**), but containing differing proportions of type A and B fibres (Rouaud and Toutant, 1982). Like type I slow-oxidative fibres, tonic muscles only represent a small percentage (10-15%) of total muscle mass in the commercial broiler chicken.

Function

The primary function of skeletal muscle is the generation of force . The organisation of muscle into motor units with the appropriate neural control, and connections to the skeleton allow the developed force to potentiate movement (Block, 1994). Skeletal muscle also provides heat via shivering and non-shivering mechanisms of thermogenesis and supplies gluconeogenic precursors through sarcoplasmic protein degradation during fasting (Abbott and Howath, 1973; Homsher and Kean, 1978; Clausen, 1986; Clausen *et al.*, 1991).

Table 1.1 Fibre characteristics in chicken skeletal muscle (from Barnard *et al.*, 1982)

	Skeletal muscle fibre types				
	Twitch fibres			Tonic fibres	
Histochemical criteria	I	IIA	IIB	IIIA	IIIB
ATPase (pH 9.4)	○	●	●	◍	●
ATPase (pH 4.6)	●	○ or ◍	◍	◍	◍
ATPase (pH 4.3)	●	○	○	◍	◍
NADH-TR	◍	◍ or ◍	○	◍	◍ or ●
Phosphorylase	○ or ◍	●	●	◍	◍
Fibre innervation	Multiple	Focal	Focal	Multiple	Multiple
Histology Characteristics					
Fibre shape	Polygonal	Polygonal	Polygonal	Rounded	Rounded
Fascicle shape	Polygonal	Polygonal	Polygonal	Rounded	Rounded
Mitochondria density	Very high	High	Low	Very high	Very high
Fibre lipid droplets	No	Yes	No	No	No
Relative fibre size	Small/med	Medium	Medium	Large	Medium
Nuclei distribution	Peripheral	Peripheral	Central	Peripheral	Peripheral

Relative stain intensities: ○ = 0 ◍ = 1+ ◍ = 2+ ● = 3+ on an arbitrary scale, increasing from 0 to 3 . The pH of pre-incubation of the specimen prior to standard ATPase reaction (Dubowitz and Brooke, 1973) is given in parenthesis.

Table 1.2 Relative muscle fibre type composition (%) from 50-80 day old chickens (from Barnard *et al.*, 1982).

	Muscle fibre composition (%)				
	Twitch fibres			Tonic fibres	
Muscle	I	IIA	IIB	IIIA	IIIB
Pectoralis	0	<1	>99	0	0
Latissimus dorsi (P)	<3	5-20	80-95	0	0
Sartorius (red)	30-45	15-30	15-25	0	0
Sartorius (white)	0	10-20	80-90	0	0
Adductor superficialis	<5	80-95	5-15	0	0
Latissimus dorsi (A)	0	0	0	65-80	20-35
Adductor profundus	0	0	0	70-85	15-30
Plantaris	0	0	0	65-75	25-35

CREATINE KINASE (CK)

Function

Creatine kinase (CK; ATP creatine *N*- phosphotransferase, E.C. 2.7.3.2) is an key enzyme of cellular energetics, representing an enzyme system with a number of isoenzyme forms which are, in part, compartmentalised in tissues associated with a high energy utilisation and demand (Wallimann *et al*, 1992). The primary role of CK in these tissues is to catalyse the reversible transphosphorylation of adenosine di-phosphate (ADP) and creatine to maintain intracellular energy supplies through spatio-temporal buffering of adenosine tri-phosphate (ATP) concentrations (Kuby and Noltmann, 1962). See following reaction:

$$\text{Creatine phosphate} + \text{ADP} + \text{H}^+ \underset{\text{Mg}^{2+}}{\overset{\textbf{Creatine kinase (CK)}}{\rightleftharpoons}} \text{Creatine} + \text{ATP}$$

CK isoenzymes

In vertebrate tissues, it has long been recognised that CK is a dimeric molecule composed of two distinct monomer subunits M-CK and B-CK (Eppenberger *et al.*, 1967). These subunits combine to give three dimeric 'cytosolic' isoenzymes with an approximate M_r of 80,000-86,000 designated: MM-CK (skeletal muscle type), BB-CK(brain type) and MB CK (cardiac muscle type), owing to their apparent differential distribution as the predominant isoform in these tissues (Hamburg *et al.*, 1991). A fourth CK isoenzyme, a mitochondrial form Mi-CK has also been characterised. It consists of interconvertable dimeric and octameric structures composed of a third CK subunit (CK-Mi) and is clearly distinguishable from its cytosolic counterparts (Hossle *et al*, 1988).

MM-CK

In humans and other mammalian species it has been demonstrated that skeletal muscle contains the highest activity of CK of all tissues studied. Approximately 90% of the total skeletal muscle CK activity is present in the sarcoplasm (muscle cytosol), the remainder is associated with subcellular structures or organelles within the intermyofibrillar space (Farrell and Baba, 1980). More than 99% of this activity has been identified as MM-CK isoenzyme (Wallimann *et al.*, 1992). A functional coupling of this MM-CK with the processes of glycogenolysis and glycolysis in

skeletal muscle has been proposed by Wallimann and co-workers (1992) as MM-CK and other glycolytic enzymes have been found to be concentrated together at the I-band (**See Diagram 1.3**) where they form multi-enzyme complexes which are loosely associated with the thin actin filaments. A small amount of cytosolic MM-CK isoenzyme (3-5%) has also been found to be localised in the M-line of the muscle myofibril (**See Diagram 1.3**) where it is believed that the isoenzyme is functionally coupled to the myofibrillar actin-activated Mg^{2+}-ATPase. The interaction of these two enzyme systems facilitates the regeneration of ATP hydrolysed during muscle contraction (Wallimann *et al.*, 1977, 1983). In addition to its function as an ATP regenerator, it has also been suggested that M-line bound MM-CK may have a structural role by forming interlinking m4 and m4´ m-bridges which position the thick myosin filaments within the myofibril (Strehler *et al.*, 1983).

In addition, localisations of MM-CK have also been found specifically associated with the SR and T tubule system (Rossi *et al.*, 1990), where again it is thought to be locally coupled to the ATP-dependent Ca^{2+} pump supporting ATP-driven Ca^{2+} uptake in SR vesicles (Levitsky *et al.*, 1978). Relatively small, but significant, amounts of MM-CK have also been found at the sarcolemmal membrane, where MM-CK has been shown to be functionally associated with the ATP-dependent Na^+/K^+ -pump (Wallimann, 1989).

BB-CK

BB-CK has been found to be the predominant cytosolic CK isoenzyme present in the brain (Eppenberger *et al.*, 1967). However, the presence of small quantities of MM-CK in specific regions of the brain has been reported (Hamburg *et al.*, 1991). Although abundant in the entire brain, higher BB-CK activities have been demonstrated in the cerebellar cortex notably in the cerebellum, striatum and pyramidal tracts (Walliman *et al.*, 1992). Unlike the situation in skeletal and cardiac muscle, little is known about the subcellular distribution and specific function in the brain. Limited evidence tends to suggest that some brain CK isoenzyme activity may be functionally coupled to the co-processes of acetylcholine release, Na^+/K^+ -ATPase function and membrane potential maintenance (Walliman *et al.*, 1992).

Unlike MM-CK, which has been shown to be very specific for post-embronic skeletal muscle, BB-CK whilst found predominantly in the brain has also been found to be expressed numerous other tissues including the prostate gland, urinary bladder, gastro-intestinal tract (Oguni *et al.*, 1992), kidney (Ikeda, 1988), amnion, decidua, placenta, uterus (Weisman *et al.*, 1986), retina (Wallimann *et al.*, 1985a) electrocytes (*Torpedo sp.* Wallimann *et al.*, 1985b) and spermatozoa (Tombes *et al.*, 1987).

MB-CK

In cardiac muscle, CK has been found to be localised in an isoenzyme-specific way at different subcellular structures or in different sub-compartments similar to that observed in skeletal muscle (Walliman *et al.*, 1986, 1992). Mammalian cardiac muscle has been shown to contain significant levels of MB, MM and Mi-CK isoenzyme activity with MB-CK reflecting the predominant isoform (Jacobus, 1983). In contrast to skeletal muscle where MM-CK activity is coupled to primary energy production associated with glycolysis and anaerobic ATP production, it has been postulated by Wallimann *et al.*, 1992 that cardiac muscle ATP is mainly derived from fatty acid oxidation, and ATP regeneration provided by Mi-CK within cardiac mitochondria. This hypothesis is consistent with the observation that Mi-CK is expressed at relatively high levels in the mitochondrial membranes of cardiac muscle (Jacobus, 1983).

Myocardial cells are also devoid of the electron-dense material associated with the M-line region in which MM-CK is usually localised in skeletal muscle (Perriard *et al.*, 1982) The lack of a discernible M-line in cardiac muscle is consistent with the suggestion that the presence of MM-CK is important in maintaining the structure of the M-Line (Strehler *et al.*, 1983).

Mitochondrial CK (Mi-CK)

Mitochondrial CK (Mi-CK) is accumulated specifically in mitochondria in a wide variety of tissue types (Jacobus and Lehninger, 1973; Walliman and Eppenberger, 1985). Two distinct subunit isoforms have been identified and are expressed in a tissue-specific manner, sarcomeric Mi_b-CK specific to skeletal muscle and a non-tissue specific ubiquitous Mi_a-CK (Hössle *et al.*, 1988; Schlegel *et al.*, 1988).

In contrast to the cytosolic CK isoenzymes, which are always dimeric, Mi_b-CK and Mi_a-CK are found as octameric entities composed of four dimeric units with M_r of approximately 86,000 (Schlegel *et al.*, 1988). The specific location of Mi-CK between the mitochondrial membranes has prompted suggestions that Mi-CK is functionally coupled to the process of oxidative phosphorylation and to the conservation of mitochondrial phosphorylation potentials (Walliman *et al.*, 1992). Subfractionation studies have shown that Mi-CK is tightly associated with the outer face of the inner mitochondrial membrane, and remains bound even in hypo-osmotically swollen mitochondria (Schlegel *et al.*, 1988), and therefore does readily not leak out of mitochondria into muscle sarcoplasm.

CK and muscle damage

The majority of enzyme catalysed reactions tend to occur within cells. Whenever an imbalance occurs as a result of exposure to infective agents, toxins, physical damage or other noxious stimuli, some of these enzymes 'leak out' through the cell membrane (plasmalemma) in to the extracellular environment (Foster, 1982). This usually leads to an increase in the circulating levels of these enzymes above those normally present as a result of cellular metabolism and turnover. Measurement of the type, extent and duration of these raised circulating enzyme activities provides information on the identity of the damaged cell type and indicates the degree of injury and its response to corrective treatment (Kenyon and Reed, 1983).

It is recognised that damage to skeletal muscle can be caused by many factors including exercise, drugs, physical trauma, inflammatory diseases, microbial infection or metabolic dysfunction or genetic predisposition (Mastaglia and Walton, 1982). Several muscle derived enzymes are routinely measured in order to aid in the diagnosis of myopathies in clinical situations these include creatine kinase (CK) lactate dehydrogenase (LDH), aldolase (ALD) and aspartate aminotransferase (AST). Creatine kinase (CK) is the enzyme elevated in plasma with the highest frequency in virtually all myopathies and is therefore the one most frequently assayed in the diagnosis of muscle related disorders (Foster, 1982). In addition to its widespread diagnostic value as a measurement of muscle myopathies and dystrophies, the measurement of CK activity and isoenzyme profiles in plasma or serum is also well established as an important diagnostic technique in a number of other pathologies including, myocardial infarction, lesions of the central nervous system and certain neoplastic diseases (Deluca *et al.*, 1981; Capocchi *et al.*, 1987; Vretou-Jockers and Vassilopoulos, 1989; El Mallakh *et al.*, 1992; Karkela *et al.*, 1993).

Chapter 2

General Materials & Methods.

Subjects

A commercial line of broiler chicken (*Gallus domesticus*) was used in the studies described in this thesis (**See Figure 3.1**). These birds represent a line of fowl especially selected for rapid growth rate and food conversion efficiency. Typically, these birds grow from approximately 50 g at 1 day old to 2.2-2.4 kg at slaughter age (42 d).

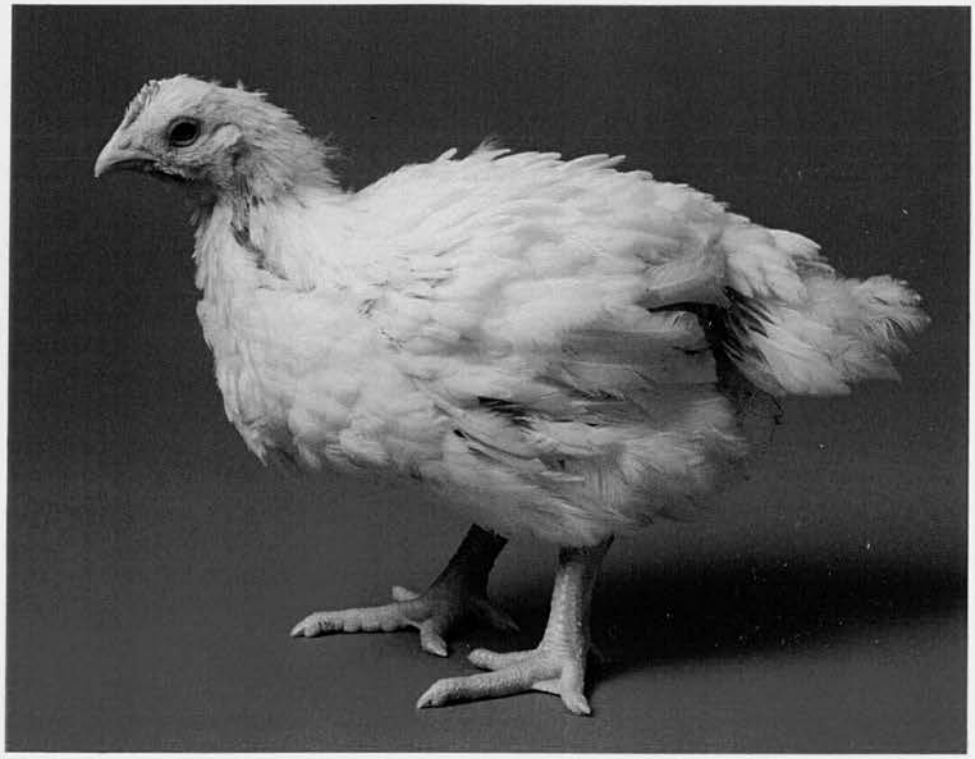

Plate 2.1. A 42 day old commercial broiler chicken (*Gallus domesticus*)

Housing and rearing

All birds were reared from 2 weeks of age under good husbandry procedures and kept in standard battery units at 22°C/50% relative humidity (RH), with a photoperiod of 14h light:10h dark. The birds had *ad libitum* access to a commercial broiler diet[1] and water throughout. Birds were only removed from this environment prior to use.

[1] See Appendix I

Blood sample collection

Blood samples (2 ml) were obtained by venepuncture (brachial vein) and transferred to 5 ml blood collection tubes (Teklab) containing 50 units Li-heparin anti-coagulant and placed on ice to arrest erythrocyte metabolism (Harris, 1983). Plasmas were prepared by centrifugation at 1500g (MSE-Mistral 2000R) for 5 minutes, immediately frozen and stored at -20°C pending analysis. All whole blood analyses were performed immediately after blood collection, prior to preparation of plasma.

Biochemical Assays

Enzyme activities and metabolite concentrations in incubation medium, homogenates and blood plasma samples were measured using modified commercially available diagnostic kits and automated spectrophotometry/microplate system (Titertek Twin Reader Plus, ICN).

Creatine kinase

Creatine kinase (CK) activity was measured using a kinetic coupled enzyme assay method developed by Rosalki (1967) using a modified commercially available kit (Biotrol CK monoreactif, Alpha Laboratories). In this assay, creatine phosphate (CP) present in the assay buffer solution is utilised as substrate together with adenosine -5'-di-phosphate (ADP) to yield creatine and adenosine-5'-triphosphate (ATP) in the presence of (CK). The ATP thus formed is determined spectrophotometrically as the production of reduced nicotinamide adenine dinucleotide phosphate (NADPH) resulting from the coupled enzyme reaction involving hexokinase (HK) and glucose-6-phosphate dehydrogenase (G-6-PDH). The NADPH produced in the final reaction is proportional to the creatine produced in the initial reaction. The rate of NADPH increase measured at 340 nm is directly proportional to the CK activity in the sample. The assay reactions are illustrated below.

$$\text{Creatine phosphate} + \text{ADP} + H^+ \xrightarrow{\textbf{CK}} \text{creatine} + \text{ATP}$$

$$\text{ATP} + \text{D-glucose} \xrightarrow{\textbf{HK}} \text{ADP} + \text{glucose-6-phosphate}$$

$$\text{Glucose-6-phosphate} + NADP^+ \xrightarrow{\textbf{G-6-PDH}} \text{6-phospho-gluconate} + NADPH + H^+$$

Assay samples were pipetted into individual microplate wells. CK reagent was added and changes in the rate of absorbance were measured over a 5 minute period at 340 nm by automated spectrophotometry at 37°C. The average rate of change in absorbance over 1 minute was determined (ΔA/min). CK activity in the sample was determined using the calculation shown in **Appendix III** and expressed

in international enzyme units of activity per litre (IU litre^{-1}). One IU of enzyme activity is defined as the amount of enzyme required to catalyse the reaction of one µmole of substrate per minute under normal conditions. The assay method is linear to 1000 IU litre^{-1} at 37°C with an average sensitivity of 0.0002 absorbance units per minute (A/min) per unit of enzyme activity (IU litre^{-1}). Within assay variation was 1.8%, between assay variation 4.5%.

Lactate

Lactate concentration was measured using a modified end-point enzymatic assay in kit form (Sigma Chemical Co. Ltd, UK) which utilises the catalytic action of the enzyme lactate dehydrogenase (LDH) and derives the concentration of lactate from the generation of reduced nicotinamide adenine dinucleotide (NADH). The principle of the assay is shown below:

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xleftarrow{\textbf{LDH}} \text{Lactate} + \text{NAD}^+$$

The reaction is carried out from right to left with excess nicotinamide adenine dinucleotide (NAD^+) utilised as substrate. Hydrazine (pH 9.2) present in the assay buffer forces the reaction in this direction by preventing the re-conversion of the pyruvate to lactate. The increase in NADH produced in the reaction is a measure of the lactate concentration in the sample. Lactate concentrations were determined by measuring the absorbance at 340 nm after 15 minutes reaction development time at 37°C. Lactate concentrations were calculated from calibration curves using lactate (L[+] lactic acid) working standards (1.33-6.66 mmol litre^{-1}). The lowest accurate measurement of lactate concentration using this method was considered to be 0.3 mmol litre^{-1}. Within assay variation was 1.3%, between assay variation 3.0%.

Total (bound and free) calcium

Total calcium concentrations were measured using a modified end-point assay (Calcium C, Alpha Laboratories) which is based on an ortho-cresolphthalein complexon (OCPC) colour development method (Wako, 1989). Calcium specific colour development was achieved when colour reagent containing 8-hydroxyquinoline was added to samples in which the calcium had initially been complexed in OCPC buffer. The calcium content of the sample was determined by measuring the absorbance at 560 nm by automated spectrophotometry after 20 minutes of colour development at 25°C. The absorbance of the colour reaction produced by OCPC is proportional to the calcium content of the sample. Calcium concentrations were calculated from calibration curves using calcium working standards (0.625 to 5.0 mmol litre^{-1}). Within assay variation was 2.7%, between assay variation 6.5%.

Calcium can exist in blood in two separate states, either as "free" (ionic) calcium (Ca^{2+}) or as "bound" calcium where it is attached to a variety of blood proteins and metabolites. Calcium concentrations measured using the Total Calcium assay represent all the calcium that is present in a given sample, irrespective of its state either as "free" or "bound" form. Calcium concentrations determined using the 634 pH/Ca^{2+} analyser (**see Unbound calcium**) represent only the ionised or unbound "free" calcium concentration in the sample. As total and free calcium were measured using widely differing methodologies it was necessary to cross-calibrate one method against the other to be able reliably to compare values derived from the two techniques Validation of this comparison has been examined in these laboratories by Rennie (1996) which established a highly significant correlation (r^2=0.99) of calcium values obtained by the two methods across a range of concentrations (0.5 to 5.0 mmol $litre^{-1}$)

Protein

Total protein concentrations were measured using an end-point colorimetric assay (Bio-rad Laboratories Ltd, UK.) which utilises a dye-binding method developed by Bradford (1976). The assay is based on the colour change of Coomassie Brilliant blue G-250 dye from 465 nm to 595 nm in response to changes in the concentration of protein. The dye is bound to basic or aromatic residues, which exist as three visual light absorbing species: cationic, neutral and anionic. At the assay pH the dye molecules are in the cationic (red) form. Binding of the dye to protein stabilises the anionic (blue) form, which is detected at 595 nm by spectrophotometry. This assay method was favoured due to its high sensitivity, speed of product development and relative ease of use compared to other methods such as Lowry or Biuret procedures. Total protein concentrations were calculated from calibration curves using bovine γ-globulin (Sigma Chemical Co. Ltd, UK) as standard (range 0.125 to 2.0 mg ml^{-1}).

Blood metabolite and electrolyte analysis

Unbound calcium (Ca^{2+})

Concentrations of unbound calcium (Ca^{2+}) in whole blood were measured using 634 pH/Ca^{2+} analyser (Ciba-Corning Diagnostics Ltd). Blood samples were collected into tubes containing 50 units Li-heparin and held on ice. Ca^{2+} concentrations were determined from a 35 µl sample volume using a Ca^{2+} selective electrode. The measurement range of the Ca^{2+} analyser was between 0.20 and 5.00 mmol $litre^{-1}$. Where possible, all blood measurements were taken within minutes of collection.

pH and carbon dioxide (CO_2)

Blood pH and carbon dioxide (CO_2) concentrations were measured using a 238 pH/Blood Gas auto-analyser (Ciba-Corning Diagnostics Ltd). Blood samples for pH/gas analysis were put into pre-coated Li-heparin (50 units) blood collection tubes and held on ice. Blood pH was measured by glass capillary pH electrode selective to hydrogen ions (H^+).which has a pH measurement range 6.00-8.00 (10.0 nmol - 1.0 μmol litre^{-1} H^+). CO_2 concentration measured as the partial pressure of carbon dioxide (pCO_2) in blood was determined by capillary electrode containing a selective membrane permeable to gaseous CO_2. The range of sensitivity for the determination of blood pCO_2 by this method was 5 - 250 mmHg (0.7-33.3 kPa).

Sodium and potassium

Blood concentrations of sodium (Na^+) and potassium (K^+) were measured using a 614 Na^+/K^+ auto-analyser (Ciba-Corning Diagnostics Ltd). Blood samples for Na^+ and K^+ analysis were put into blood collection tubes containing Li-heparin (50 units) anti-coagulant and held on ice. Blood Na^+ was measured by a glass capillary electrode selective to sodium ions. Na^+ measurement range was 80-200 mmol litre^{-1}. Blood potassium concentration was measured using a glass capillary K^+ selective electrode consisting of a valinomycin based membrane which is in contact with the sample on one side and with an electrode fill solution (saturated KCl) on the other side. K^+ measurement range was 0.50-9.99 mmol litre^{-1}

Muscle radio-labelled ^{45}calcium incorporation

Medium Labelling

Where stated in the *in vitro* isolated muscle incubation experiments (**See Chapters 6-10**), the radio-isotope ^{45}calcium (as $CaCl_2$ in aqueous solution) was added to stock incubation medium 199 prior to experimentation (16.4 kBq ml^{-1}). ^{45}Calcium was obtained from Amersham International plc, Bucks, UK. Where possible the isotope was used within 1 month of receipt (half-life = 163 days) to minimise the effects of radio-active decay.

Preparation of muscle homogenates

At the end of each *in vitro* experimental run, muscles were carefully removed from the radio-active pre-labelled incubation medium, blotted dry on absorbent paper, and rapidly frozen in small individual polythene bags in liquid nitrogen at -180°C. Muscles were removed from liquid nitrogen and stored at -20°C pending analysis.

Prior to homogenisation, frozen muscles were transferred into 10 ml homogenisation tubes containing 4 ml of 0.9% saline and allowed to thaw. The muscles were then homogenised for 2-3 minutes at 1200 rpm using a Braun tissue homogeniser (FT scientific, UK). A aliquot of muscle homogenate suspension (0.5 ml) was pipetted into a 5 ml scintillation vial containing 4 ml liquid scintillation cocktail (Optiphase X, BDH Chemicals). Homogenate isotope activity was measured as radio-active disintegrations of ^{45}calcium over a 1 minute period (Dpm) using an automated scintillation counter (Wallac 500 Betamaster Beta Counter, UK).

The calculations used to determine *in vitro* muscle ^{45}calcium uptakes and CK effluxes can be found in **Appendix IV**, along with two GWBASIC programs which were developed to handle the large volume of data generated by these experiments (**see Appendices V** and **VI**).

Statistical Analysis

All statistical analyses were performed using Minitab Data Analysis Software (Ver. 6.1) for Apple Macintosh Systems. Data from experiments performed as balanced designs were analysed using one-way or two-way analyses of variance. Comparisons of differences between two sample means was examined using a two-tailed un-paired Students t-test (pooled two-sample). Examination of the correlation of relationships or associations between variables was performed using regression analysis.

Experimental Procedures

All experimental procedures performed on the birds were properly licensed according to Home Office Standards.

Chapter 3

Creatine kinase isoenzyme profiles in the broiler chicken: effects of acute heat stress.

Introduction

Creatine kinase (CK) is a key enzyme of cellular energetics, representing an enzyme system with a number of isoenzyme forms (**see Chapter 2**) which are compartmentalised in tissues associated with a high energy utilisation and demand such as cardiac and skeletal muscle, neuronal tissue and brain. The intracellular compartmentalisation of these isoenzymes in specific tissues promotes the functional coupling of the production and utilisation of energy and the integration and control of cellular metabolism (Wallimann *et al.*, 1992).

Consequently, measurements of the activity and isoenzyme profiles of CK in plasma or serum are now well established as an important diagnostic indicator of a wide number of pathologies including myopathies and dystrophies, myocardial infarction, lesions of the central nervous system and neoplastic disease (DeLuca *et al.*, 1981; Capocchi *et al.*, 1987; Vretou-Jockers and Vassilopoulos 1989, El Mallakh *et al.*, 1992; Karkela *et al.*, 1993). Diagnostic interpretation is based upon the assumption that the relative tissue-specific distinguishable isoenzymes are released in response to cellular damage, resulting in characteristic blood CK profiles (Kenyon and Reed, 1983; Hamburg *et al.*, 1991). As a result a number of electrophoretic and chromatographic techniques have been developed to facilitate the separation of the isoenzymes of CK (Marshall *et al.*, 1991; Luque de Castro and Fernandez-Romero, 1992).

Changes in total plasma CK activity have been reported in a number of avian species which occur in response to various pathologies (Hollands *et al.*, 1980; Lumeij *et al.*, 1988a, b; Cardona *et al.*, 1992; Itoh *et al.*, 1993; Baird *et al.*, 1997), acute heat stress (Ostrowski-Meissner, 1981) and transportation (Mitchell *et al.*, 1992) have been reported but without reference to the associated isoenzyme profile. Whilst it might be assumed that the CK isoenzymes in the tissues of avians are analogous to those identified in mammals, it has been reported that the CK-M monomer is not expressed in post embryonic avian cardiac muscle (Schafer and Perriard, 1988). As a result of this no MB-CK dimer (**see Chapter 2**) is found in this tissue which contains only BB-CK (Quest *et al.*, 1990). The relative distribution of the activities of the isoenzymes of CK may therefore differ markedly from those described in mammals, in either normal diseased or stressed individuals.

Experimental aims

This aim of the work presented in this chapter of the thesis was to develop and validate a simple solid phase chromatographic (SPC) technique for the separation of the CK isoenzymes from avian tissue extracts and plasma. Using this technique, the objectives of this study were to identify the origin of the isoenzyme/s responsible for basal CK activity in the plasma of unstressed broiler chickens, then establish the origin of the isoenzyme/s responsible for the increase in total plasma CK activity in broiler chickens following exposure to acute heat stress. The identification of the origin of the CK isoenzymes is imperative if we are to be able to understand why, under varying stress situations, the stress response is specific to that tissue.

Materials and Methods

Subjects

The birds used in this study were 6 week old immature, females broiler chickens approximately 1.8-2.2 kg. in body weight (**see Chapter 2**). From 3 weeks of age to the time of the experiment the birds were individually caged in a controlled climate chamber (22°C/50% RH) with a photoperiod of 14h-light:10h-dark. A commercial broiler diet was fed *ad libitum* throughout with free access to water. The birds were only removed from this environment immediately prior to use.

Preparation of tissue homogenates

Birds were killed by an intravenous injection of sodium pentobarbitone (80 mg/kg body weight) and samples (approximately 2g) of skeletal muscle (*m. pectoralis*) and whole heart and brain were removed rapidly and placed in ice-cold 0.9% saline. Tissues were chilled, dissected in to smaller pieces and weighed, then transferred to 10 ml of cold homogenisation medium (buffer A): 100 mM Tris, 5 mM magnesium sulphate, 0.4 mM EDTA, 20 mM sodium chloride and 2.75 mM dithiothreitol (DTT) adjusted to pH 7.9 (at 20°C). Tissues were homogenised (2 minutes at 1200 rev/min) in a cooled Braun tissue homogeniser (FT Scientific Ltd. UK.) and the resulting extract was centrifuged (5000g for 20 min, 0°C). The supernatant was removed and stored at -20°C. Prior to chromatographic analysis equal volumes of thawed supernatants from each tissue from the 5 different birds were combined.

Separation of CK isoenzymes

The CK isoenzymes were separated by anion exchange chromatography on Sep-Pak cartridges containing Accell Plus QMA (Millipore UK Ltd.) using stepwise gradients of pH and ionic strength. The method was based upon a procedure described by Kaye *et al.* (1981) and Somjen *et al.* (1984 a, b) but using a quaternary methylamine (QMA) sorbent containing acrylamide co-polymer on diol silica in preference to diethylaminoethyl (DEAE)-cellulose. The QMA column was chosen because of its reported improved specificity for molecular separation and extraction of acidic and weakly acidic proteins and enzymes (Millipore UK Ltd.). Each QMA cartridge was preconditioned with 10 "hold-up" volumes (approximately 8.0 ml) of buffer A. Eluting buffers B and C were prepared to the same basic formulation as buffer A but contained 40 mM and 150 mM sodium chloride respectively and were both eventually adjusted to pH 6.4 (at 20°C). An aliquot of buffer A (1 ml) was added to the cartridge and the eluant was collected and designated fraction Ao. Sample (supernatant or plasma; 1.0 ml) was applied to the cartridge resulting in the elution of fraction S. Three further aliquots, each of 1 ml of buffer A were introduced to the cartridge at 30 second intervals and fractions A1, A2 and A3 were collected. Fractions B1, B2, B3, C1, C2 and C3 were collected by corresponding appropriate elutions with buffers B and C in that order.

Enzyme and protein analysis

Creatine kinase activities and protein concentrations in tissue extract supernatants, fractions and plasmas were measured using the methods described in **Chapter 2**.

Statistical Analysis

Where appropriate data are presented as the means ± S.D. Levels of significance were determined by analysis of variance or unpaired Student's t-test.

Results

Separation of CK isoenzymes in individual and combined tissues extracts (Experiment I)

The total creatine kinase activities in each of the three tissue extracts is shown in **Table 3.1**. Whilst cardiac muscle and brain tissue contain approximately the same amounts of CK on both bases, skeletal muscle CK activity is approximately 10 and 5 fold higher per unit of tissue weight and protein content respectively.

Table 3.1. Tissue activities of creatine kinase (CK) expressed as IU g^{-1} wet weight and IU mg^{-1} supernatant protein. Values represent means ± S.D., (n=5).

CK activity (IU)	Skeletal muscle	Cardiac Muscle	Brain
g^{-1} wet weight	1196 ±81	115 ±16	117 ±3
mg^{-1} supernatant protein	4.49 ±0.42	0.99 +0.19	1.36 ±0.05

Separation of CK isoenzymes in individual tissue extracts

The elution profiles of CK in the individual tissue supernatants are presented in **Figure 3.1**. Almost all of the enzyme activity in skeletal muscle (99.4%) appeared in the early fractions eluted at low ionic strength with buffer A and was designated MM-CK. The remaining activity (0.6%) was present in fractions B1-B3. In cardiac muscle some CK was present in the MM-CK fractions (4.1% S-A3) but the majority of the activity eluted with increasing ionic strength, the primary peak occurring in fractions B1-B3 (67.4%), with a second peak (28.5%) eluting with buffer C (C1-C3). Based on the reported absence of the CK-M monomer from avian cardiac tissue the CK activities eluted by buffers B and C were designated BB-CK1 and BB-CK2 respectively. Brain extract also contained CK activity in the eluants of all three buffers. 13.4% was present as the MM-CK form, 39.9% in fractions B1-B3 (BB-CK1), but with the principal peak of eluted activity (46.7%) occurring following the application of the high ionic strength buffer C (BB-CK2).

Separation of CK isoenzymes in combined tissue extracts

The elution profile of a mixture of equal volumes of the three tissue supernatants is presented in **Figure 3.2**. The three peaks of CK activity observed in the individual supernatants are apparent, indeed the elution patterns and retention volumes are unaltered. Recoveries of each isoenzyme were calculated from their activities in the original supernatants, corrected for dilution and summation of the fraction activities for each eluting buffer. Approximately 98% of the MM-CK activity was recovered, the corresponding values for BB-CK1 and BB-CK2 being 107% and 111% respectively. Essentially identical profiles were obtained from chromatographic analysis of two separate combined supernatant preparations.

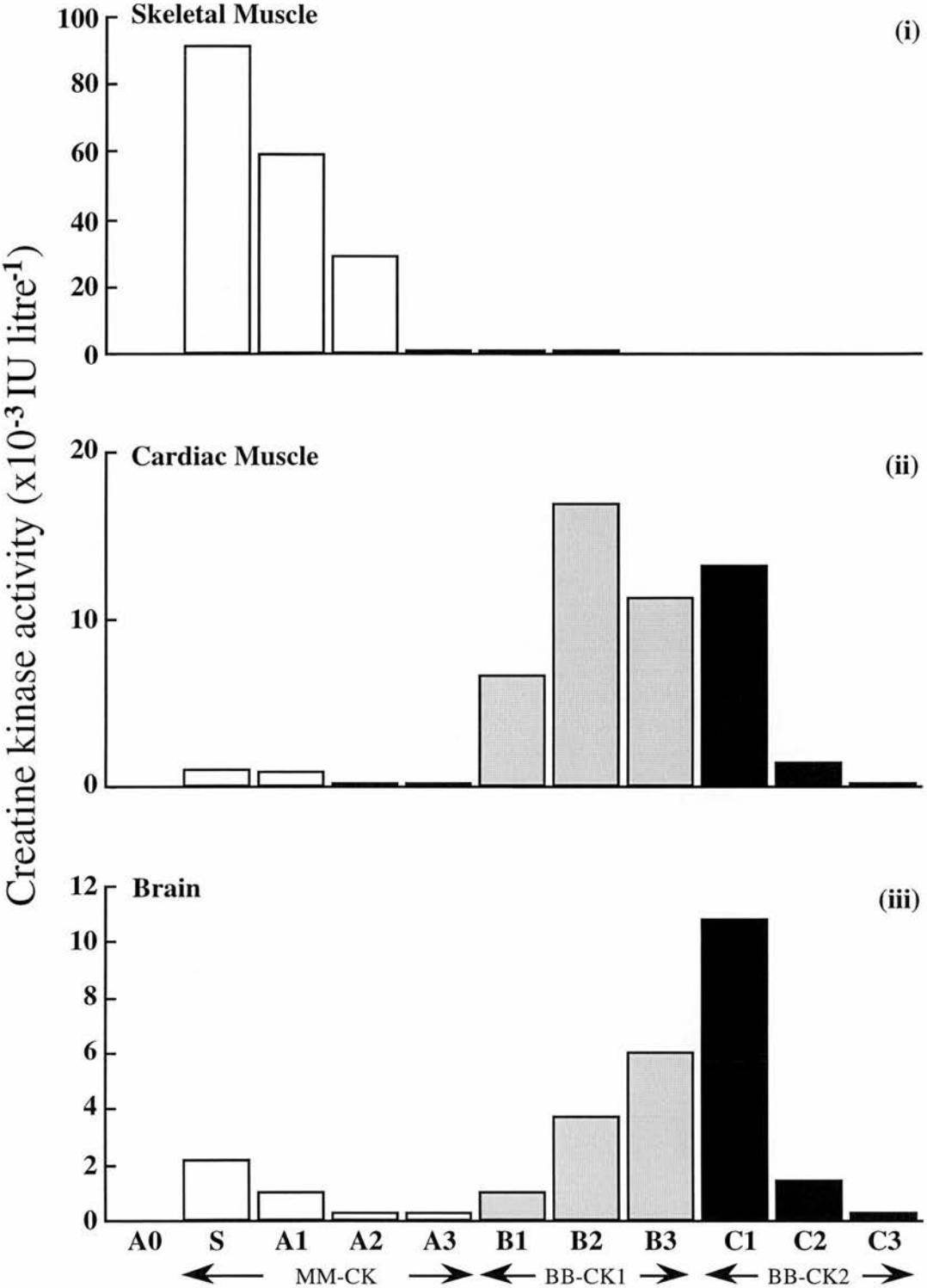

Figure 3.1 Creatine kinase (CK) activity in extract supernatants of skeletal muscle (i), cardiac muscle (ii) and brain tissue (iii) from the broiler chicken. CK eluting in the buffers A, B, and C, in ascending ionic strength are distinguished by differences in shading. The profile is derived from the combination of five separate extracts of each tissue.

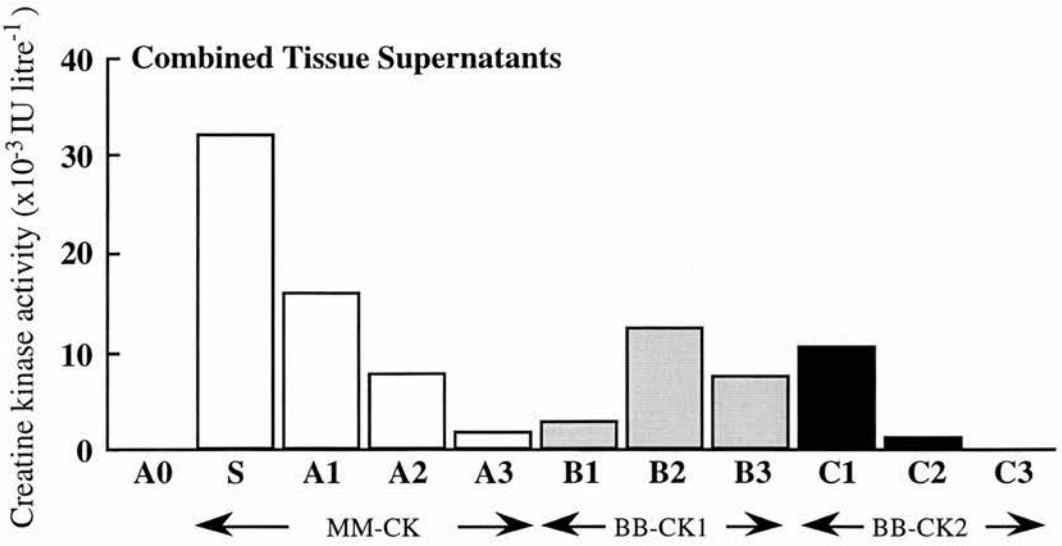

Figure 3.2 CK activity elution profile of an isovolumetric mixture of supernatants from extracts of skeletal muscle, cardiac muscle and brain tissue from the broiler chicken. Supernatants were derived from five preparations for each tissue. CK activities eluting in the buffers A, B, and C, in ascending ionic strength, are distinguished by differences in shading.

Determination of the origin of CK isoenzymes in the plasma of broiler chickens: effects of acute heat stress (Experiment II)

Acute heat stress protocol

A group of 5 birds was transferred to a commercial poultry transport container (1.3 x 0.7 x 0.25m) and placed in a controlled climate chamber at a temperature of 30°C and a relative humidity of 80% for 2 hours. Exposure for this length of time to this combination of temperature and relative humidity is typical of the conditions encountered by these birds during transportation (Mitchell *et al.*, 1992). Container microenvironment was monitored at 1 minute intervals using a temperature-humidity probe (Vaisala HMP31 UTA) connected to a Squirrel 1201 data logger (Grant Instruments, Cambridge, UK.). Immediately prior to, and at the end of heat stress exposure, blood samples and body temperature measurement were taken. Blood samples (2 ml) were obtained by venepuncture (brachial vein) and transferred to pre-heparinised tubes (Teklab 5 ml) and placed on ice. Plasmas were prepared from these samples by centrifugation at 1500g (MSE-Mistral 2000R) for 10 minutes, immediately frozen and stored at -20°C pending analysis (**see Chapter 2**). Deep body temperature measurements were obtained using a thermistor probe inserted 5 cm into the birds rectum.

Effects of heat stress

Exposure to acute heat stress induced a profound hyperthermia, i.e. a highly significant elevation of deep body temperature of 2.8°C ($p<0.001$) concomitant with a 65.4% increase ($p<0.01$) in total plasma CK activity (**see Table 3.2**). Average container microenvironment measurements of temperature and relative humidity (RH) were 31.6°C and 84% respectively.

Table 3.2. Effects of acute heat stress on rectal temperature (°C) and total creatine kinase (CK) activity (IU litre^{-1}). Values represent means ± S.D., n=5.

Measurement	Before heat stress	After heat stress
Body temperature (°C)	41.7 ±0.2	44.5 ±0.3
Plasma CK activity (IU litre^{-1})	518±96	859 ±146

Plasma CK isoenzyme distribution before and after heat stress

The distribution of CK isoenzyme activity in broiler chicken plasma before and after exposure to high thermal load is presented in **Figure 3.3**. The majority of CK "normally" circulating in the plasma (96.8%) appears to be of the MM-CK isoenzyme form eluting in fractions S and A1, with only minor components of CK-BB1 (1.5%) and CK-BB2 (1.7%). The summated isoenzyme activity recovered in all fractions was 528 ±23 IU litre^{-1} compared to an original total plasma CK of 518 ±13 IU litre^{-1}, a recovery of 102%. In order to confirm the quantitative detection and recovery of the BB-CK isoenzymes from plasma, a 1.0 ml. aliquot was seeded with a 20 µl volume of the original cardiac muscle extract supernatant containing approximately 50 IU litre^{-1} CK activity as BB-CK1 and BB-CK2. This procedure increased the total CK activity eluted by buffers B and C from 20 IU litre^{-1} to 74. IU litre^{-1}, a recovery of added BB-CK of approximately 105%.

Exposure to acute heat stress produced a significant increase in the plasma activity of the MM-CK form (63.3%; $p<0.05$) with no detectable change in the plasma activities of the BB-CK1 or BB-CK2 isoenzymes (**see Figure 3.3**). Summation of the fractional enzyme activities in post-heat stress plasma indicated a total CK activity of 863 ±156 IU litre^{-1} compared to a measured value of 859 ±133 IU litre^{-1} indicating a recovery, primarily of MM-CK, of 100%.

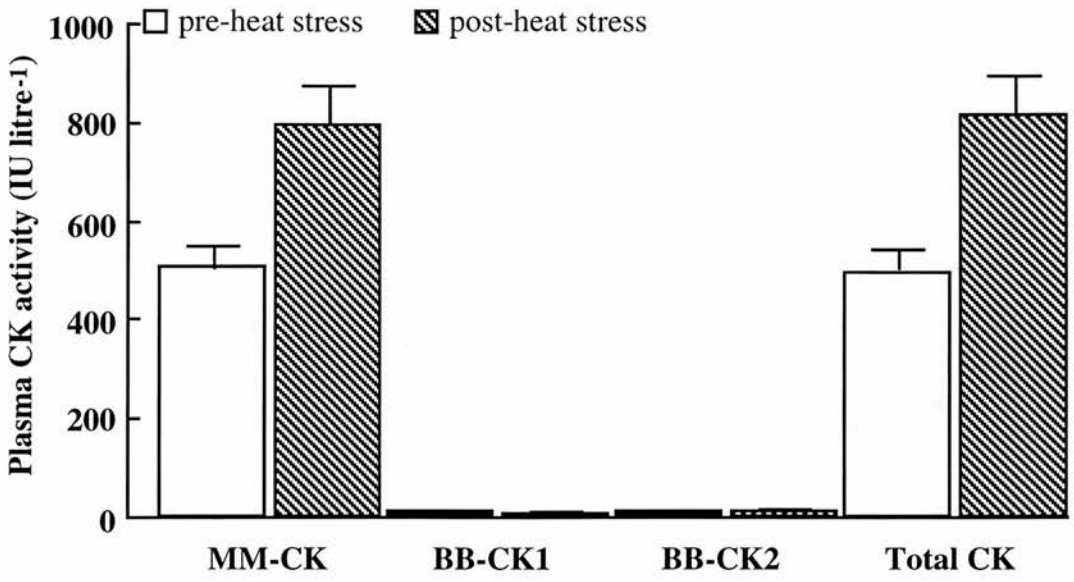

Figure 3.3 Plasma CK isoenzyme and total enzyme activities in plasma of broiler chickens before (pre) and after (post) exposure to acute heat stress. The values are presented as means ± S.D. for 5 birds.

Discussion and conclusions

The CK activities observed in the three individual tissue extract supernatants (**see Figure 3.1**) exhibited a fundamentally similar distribution to those previously described in other species (Farrell and Baba 1980; Lumeij *et al.*, 1988a). Skeletal muscle containing significantly higher enzyme activity than heart or brain tissue whether expressed on the basis of tissue wet weight or supernatant protein content (**see Table 3.1**). In the broiler chicken, the CK distribution is present in the ratio 10:1:1 in muscle, heart and brain respectively calculated per g wet weight and as 3.3:0.7:1 per mg supernatant protein. Corresponding ratios of 4.4:1.3:1 and 5.1:2.0:1 have been reported in the racing pigeon (Lumeij *et al.*, 1988a).

The extraction procedures and chromatographic techniques employed in the present study were designed to facilitate the separation of the cytosolic CK isoenzymes predominating and exhibiting characteristic distributions in skeletal muscle, heart and brain. Contamination of these cytosolic CK dimers, derived from the CK-M and CK-B monomers in the tissue extracts by the mitochondrial sub-types (CK-Mi) will be negligible under the conditions selected and thus will not adversely influence the elution profiles. The majority of intact mitochondria will be removed by the centrifugation step (Schneider, 1961). In addition, firmly bound mitochondrial CK represents only 3-5% of total activity in chicken skeletal muscle (Wallimann *et al.*, 1977) and requires hypotonicity induced mitochondrial swelling, phosphate ion solvation and detergent treatment, or sonication for its efficient extraction (Farrell and

Baba, 1980; Wallimann *et al.*, 1986; Wegemann *et al.*, 1991). Anion exchange chromatography of the skeletal muscle supernatant (**see Figure 3.1**) resulted in the elution, at low ionic strength, of a predominant single form (99%) of CK isoenzyme activity. This finding is in agreement with the proposed exclusive presence of the MM-CK isoenzyme as previously reported for this tissue and ascribed to the expression of only the M-CK monomer (Wallimann *et al.*, 1977, 1983; Perriard *et al.*, 1978; Quest *et al.*, 1989).

In both cardiac muscle and brain supernatants (**see Figure 3.1**) small, but significant CK activities (4.1 and 13.4%) eluting coincident with the MM-CK form were detected. This would appear a direct contradiction of the reported absence of the CK-M monomer from these tissues (Schafer and Perriard, 1988). The observations may however reflect a small amount of expression of the muscle type sub-unit or the previously documented conversion of the predominant BB-CK isoenzyme, during short term storage, to a form with different physical properties but which migrates with the MM-CK isoenzyme on electrophoresis (Hallberg *et al.*, 1979). Attempts were made in the present protocol to minimise proteolytic and oxidative degradation of the enzyme by optimal sample handling during supernatant preparation and storage, rapid freezing and inclusion of DTT in the medium, however a small degree of conversion cannot be entirely discounted.

The vast majority of the CK activity in both muscle and brain tissue was eluted by the higher ionic strength buffers B and C. The resulting profile differed between the two tissues in that whilst cardiac activity eluted in the ratio 2.4:1 in buffers B and C the corresponding ratio was 0.85:1 for brain CK. These tissue specific elution patterns, under the conditions employed, may reflect the presence of two isoforms of the BB-CK isoenzyme (here designated BB-CK1 and BB-CK2), separable by anion exchange chromatography and differentially expressed in cardiac and neural tissue. This hypothesis is consistent with previous reports of a high degree of heterogeneity of the B-type creatine kinase isoproteins in chicken tissue (Wirz *et al.*, 1990) and the existence of two distinct CK-B sub-units, identified as Ba and Bb, differing in N-terminus structure and a number of physical properties, including isoelectric point and distinguishable by FPLC-anion exchange (Quest *et al.*, 1989, 1990). It is therefore tempting to propose that BB-CK1 and BB-CK2 correspond to the isoforms resulting from the regulated association of the Ba and Bb monomers, and that the current technique represents a simple and efficient method for their separation in avian tissue extracts and plasma. The resolution of BB-CK1 and BB-CK2 might be improved by the use of increased fraction volumes for each buffer and further adjustments of the elution conditions. Such refinements were considered unnecessary, however in

relation to the objectives of part of the study. Absolute confirmation of the correspondence of the BB-CK1 and BB-CK2 activities to the isoforms derived from different combinations of Ba and Bb sub-units would require immunological characterisation of the monomer composition of the isoenzymes. It is clear, however, that the separation technique described efficiently separates the isoenzyme activities designated MM-CK, BB-CK1 and BB-CK2 in both mixtures of tissue extract supernatants (**see Figure 3.2**) and plasma (**see Figure 3.3**) with good recoveries. Seeding of the plasma with exogenous BB-CK indicated accurate detection of the increased isoenzymes and total recovery of the added activity.

Characterisation of the effects of acute heat stress on plasma CK isoenzyme activities demonstrates the importance of the application of the separation technique. The predominance of MM-CK in plasma of control birds might be predicted from the high CK weight specific activity present in skeletal muscle and the large mass of the tissue relative to heart and brain. This observation serves to emphasise the necessity of a technique to separate the MM-CK and BB-CK isoenzymes in avian plasma as the nature of their distribution might result in the obscuring of relatively large changes in activity of cardiac or brain origin by proportionately smaller but absolutely greater alterations in the muscle form. This is particularly vital in the absence of a readily distinguishable avian analogue of the diagnostically important mammalian MB-CK cardiac specific isoenzyme (Schafer and Perriard 1988; Quest *et al.*, 1990).

In the heat stress experiment (**Experiment II**) exposure to a temperature of 32°C and humidity approaching saturation, under simulated transport conditions, induced a profound hyperthermia, rectal temperature increasing by 2.8°C to 44.5°C and accompanied by a 65.4% increase in total plasma CK activity (**see Table 3.2**). A rapid elevation of plasma CK in broiler chickens subjected to severe heat stress (ambient temperature = 42°C) has previously been described (Ostrowski-Meissner 1981). Isoenzyme separation indicates that the increase in plasma CK following heat stress is entirely attributable to changes in the MM-CK form, probably as a result of efflux from muscle tissue due to changes in muscle membrane (sarcolemma) integrity (Lumeij *et al.*, 1988a, b). However, the finding that only the muscle isoenzyme was elevated immediately after an acute heat stress does not preclude the possibility of stress-induced dysfunction in the other tissues. It is possible that skeletal muscle may be more sensitive to the stressor or, its relative contribution may reflect its proportional mass at the earliest stages of cellular damage. Circulating CK activities continue to rise for up to 12 hours after exposure to high thermal loads in broiler chickens (Mitchell *et al.*, unpublished results; **see Chapter 12**) and during this period enzyme efflux from cardiac, neural and other tissues may be increased.

In summary, it would therefore appear that using this simple chromatographic separation method it is possible to determine CK isoenzyme activities in tissue extracts and plasma of the broiler chicken. The results of this study clearly demonstrate that the elevation of plasma CK immediately resulting from acute heat stress is almost entirely attributable to an increase in the skeletal muscle isoenzyme, MM-CK.

On the basis of these findings, the main focus of the research subsequently undertaken and presented in this thesis has been to investigate the mechanisms that mediate the release of CK specifically from skeletal muscle. The findings of this experiment provided a sound justification for developing an *in vitro* chicken muscle preparation with which to further investigate the potential mediators of cellular damage in this tissue.

Chapter 4

Development of an isolated chicken skeletal muscle preparation for the study of the mechanisms of creatine kinase (CK) release.

Introduction

The technique of incubating isolated tissues *in vitro* has long proved useful in the investigation of many aspects of cell physiology and biochemistry. Isolated skeletal muscle preparations have long been used to examine such physiological processes as muscle contractility (Palmer *et al.*, 1981), protein and amino acid metabolism (Baracos *et al.*, 1989), and oxygen consumption (Maltin and Harris, 1985; Van Breda *et al.*, 1990). Processes involved in the development of muscle pathology and dysfunction have also been studied using this experimental approach (Duncan and Jackson, 1987; Amelink *et al.*, 1990; McArdle *et al.*, 1991).

The majority of research using isolated skeletal muscle preparations has generally been confined to studies in small rodents (mouse and rat), using small thin muscles such as *m. soleus* and *m. extensor digitorum longus* . A small number of studies using isolated muscle preparations from the domestic chicken have been reported, examining rates of muscle protein turnover (Klasing and Jarrell, 1984; 1985). There are however, few reports of the use of isolated skeletal muscle preparations for the study of the processes of muscle pathology or damage in the domestic chicken (Dawson 1966).

Conditions for incubating skeletal muscle

It is recognised that there are several important factors which should be considered when incubating isolated skeletal muscle *in vitro*, if any loss of tissue viability is to be avoided. In order to obtain results from *in vitro* studies that are meaningful as a model for the situation *in vivo*, it is essential that the physiological stability and integrity of the muscle is retained. It has long been recognised that a major difficulty of incubating tissues *in vitro* is to ensure sufficient O_2 and metabolites reach the cells by diffusion in the absence of a patent circulatory system (Goldberg *et al.*, 1975). In incubated rat and mouse muscle preparations it has been demonstrated that inadequate O_2 and metabolite availability increases net protein catabolism (Baracos *et al.*, 1989), diminishes contractile properties (Segal and Faulkner, 1985), and promotes the loss of intracellular constituents through alterations in cell membrane permeability (Jackson *et al.*, 1984; Jones *et al.*, 1983). Inadequete tissue O_2 diffusion can lead to depletion of glycogen reserves in the inner-most fibres of the muscle

(coring), thereby reducing substrate availability for energy metabolism (Maltin and Harris, 1985). The degree of core formation is largely dependent on the relative cross-sectional thickness of the tissue, but is also influenced by the composition and temperature of the incubation buffer, and the metabolic rate of the donor animal (Van Breda *et al.*, 1990). In addition, the provision of adequate tissue O_2 and metabolite availability is much greater in actively contracting muscle where the metabolic requirements are significantly increased (Segal and Faulkner, 1985). Studies on incubated isolated skeletal muscle preparations have generally employed minimal physiologic buffers as the choice of incubation medium. In the majority of cases, muscles have been incubated in Krebs-Ringer bicarbonate buffer[1] equilibrated with 95% O_2 and 5% CO_2 at pH 7.4. Whilst there is good evidence to suggest that muscles incubated in this buffer remain viable for 3-6 hours post dissection (Goldberg *et al.*, 1975; Jones *et al.*, 1983), it is recognised that muscles undergo net protein breakdown under these conditions (Fulks *et al.*, 1974). The addition of metabolites such as glucose, insulin and amino acids to the buffer helps inhibit protein degradation and maintain intracellular amino acid pools (Baracos *et al.*, 1984). The addition of glucose and other metabolites to the incubation buffer has proved especially important in studies in which muscles have been required to contract (Jones *et al.*, 1983, 1984, Baracos *et al.*, 1989).

On this basis, it was decided that muscles used in the present study would be incubated in a complete physiologic buffer in order to minimise the extent of tissue degradation. Medium 199 + Earles salts[2] was the choice of incubation buffer. It is a complete growth medium widely used in cell culture and tissue incubation which contains the necessary metabolites and nutrients to ensure optimum cellular function and viability. To further minimise any loss of tissue viability, muscles were not stimulated to contract like in other studies, as the loss of intracellular constituents has been shown to be elevated in contracting isolated skeletal muscles (Jones *et al.*, 1983).

Experimental aims

The work presented in this chapter describes the development of a novel isolated chicken skeletal muscle preparation (*m. tensor patigialis*) and incubation system and evaluates the viability of the preparation by examining the characteristics of basal CK release or 'efflux' from the muscle under the described experimental conditions.

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[1] Buffer contains 119 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM KH_2PO_4 and 25 mM $NaHCO_3$ (pH 7.4)

[2] See Appendix II

Materials and methods

Subjects

All muscles used in this study were taken from 4 weeks old immature female broiler chickens (**See Chapter 2**). Birds were reared from 2 weeks of age under good husbandry procedures and kept in standard battery units (4 birds per unit) at 22° C/50% RH with a photoperiod of 14h light: 10h dark. The birds had *ad libitum* access to a commercial broiler diet and water throughout. At the time of experimentation birds weighed between 0.8 -1.0 kg.

Study muscle

A great deal of consideration was given to the type of muscle used in this study. Examination of the superficial muscles of the wing offered the possibility of utilising one of a number of small flexor or extensor muscles, which given their appropriate size and morphology made them suitable for use in *in vitro* incubation studies (Goldberg *et al.*, 1975). *M. tensor patigialis* (Van den Berge, 1975) was eventually chosen because of the relative ease of its intact removal during dissection (**see Plate 4.1**). *In situ*, the muscle emerges complete with an aponeurotic sheath over the main belly of the *m biceps brachii* distal to its emergence from beneath *m. pectoralis,* and extends dorsally and distally into the *propatigium* or wing web ending in a diffuse tendon which extends towards the wrist joint. Histochemical examination of *m tensor patigialis* myofibre composition was kindly performed by Dr. Bob Green of the Muscle Pathology Unit at the Edinburgh Western and General Hospital and revealed that the muscle contained >99% type IIB fast-glycolytic fibres, a composition identical to that of the commercially important *pectoralis* muscle.

Dissection procedure

On completion of euthanasia (**see Chapter 3, Materials and Methods**) birds were placed in a recumbent position with their wings slightly extended outwards and clamped in position to keep them steady. The skin adjacent to the dorsal side of the elbow was pinched and raised and an initial 3-4 cm incision was made along the skin parallel with the *ulna*. Another 6-8 cm incision, starting from the same origin, was then made parallel to the *m. biceps brachii*, up to its tendonous junction beneath the *m. pectoralis* to form a v-shaped skin flap. Using blunt dissection, the flap was carefully drawn back to reveal the wing web muscle held *in situ* within the *propatigium* (**see Plate 4.2**). The muscle was then carefully dissected out from with in the wing web area complete with distal tendon attached (**see Plate 4.3**). At this point the proximal head of the muscle was still attached to the myotendonous junction

at the head of the *m. biceps*, just beneath the *m. pectoralis*. A 4 cm incision was made into the pectoral muscle to expose the underlying tendonous area (**see Plate 4.4**). The wing web muscle was then excised completely with a small amount of the proximal tendon attached (**see Plate 4.5**).

Incubation protocol

Upon removal, muscles were washed in ice-cold oxygenated (95% O_2 + 5% CO_2) 0.9% NaCl, blotted, weighed and transferred to sealable polypropylene beakers (Fisons, UK) containing 2.5 ml of medium 199 + Earles salts (Sigma Chemical Co.) as shown in **Plate 4.6.**, and then placed in a shaking waterbath pre-set to normal broiler body temperature (41.5°C). The muscles were continually gassed with 95% O_2 + 5% CO_2 in order to keep both medium pH (7.4) and pO_2 (>98% O_2 saturation) constant during incubation (**see Plate 4.7**). During experimentation 4 muscles were incubated at any one time, thus allowing for one muscle from each bird to act as a contralateral control to a corresponding treatment muscle.

All muscles used *in vitro* were incubated using a standard incubation procedure. Initially, the freshly excised muscles were given a 30 min pre-'wash-off' incubation period prior to any stimulus or treatment to remove any exogenous enzyme present following dissection. After this wash-off period, muscles were removed from the medium, blotted and subsequently placed back into new beakers containing 2.5 ml of fresh medium and re-incubated for another 30 minutes. This procedure was repeated for a further 3 x 30 minute incubations up to a total of 150 minutes incubation. Any pharmacological challenge or incubation modification was initiated after 60 minutes of incubation and either continued through to 150 min of incubation and or administered for only 30 minutes after 60 minutes of incubation.

At the end of each muscle incubation, a 1 ml aliquot of medium was removed and pipetted into an appropriately labelled stoppered sample collection tubes (Teklab 5 ml) and immediately stored and frozen at -20°C prior to enzyme and metabolite determination. Muscles were blotted and snap frozen in individually labelled plastic bags in liquid nitrogen at -180°C and stored at -20°C pending homogenisation and analysis.

Determination of CK activity

CK activities in the incubation medium and in homogenised muscle supernatants were determined by the methods described in **Chapter 2** and quantified using the calculation outlined in **Appendix III** (**see Equation 3**). The preparation of muscle homogenates for CK analysis was by the procedure outlined in the materials and methods section of **Chapter 3**.

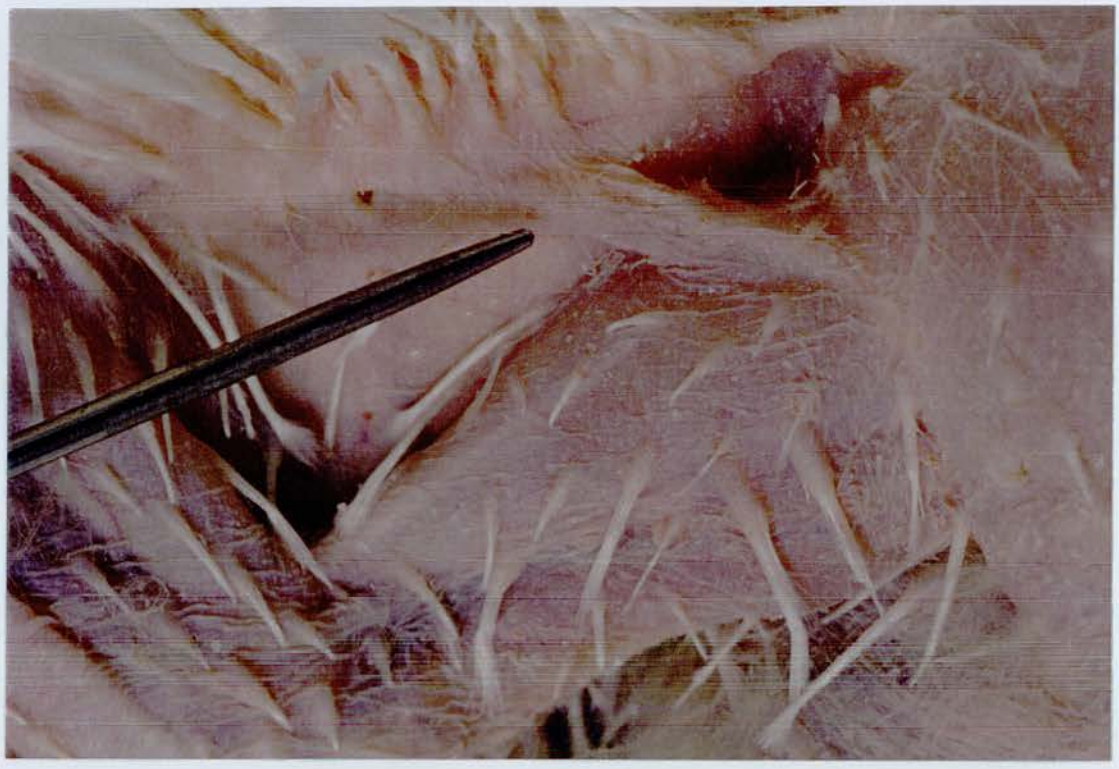

Plate 4.1 Pre-dissection view of the right wing of a 4 week old female broiler chicken showing *m. tensor patigialis* held *in situ* just beneath the skin surface. Ventral view. x3.5 magnification.

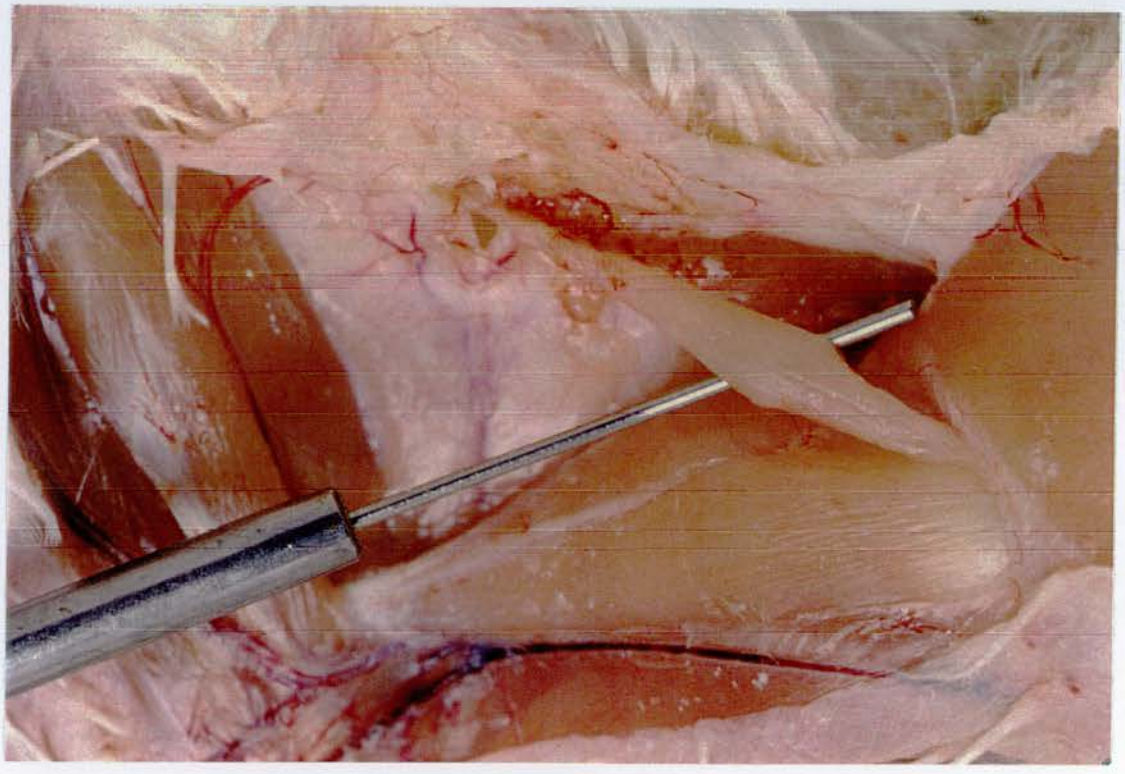

Plate 4.2 Exposed *tensor patigialis* muscle after initial skin incisions. Note the distal and proximal tendon insertions into the propatigium, and beneath the pectoral muscle. Ventral view. x3.5 magnification.

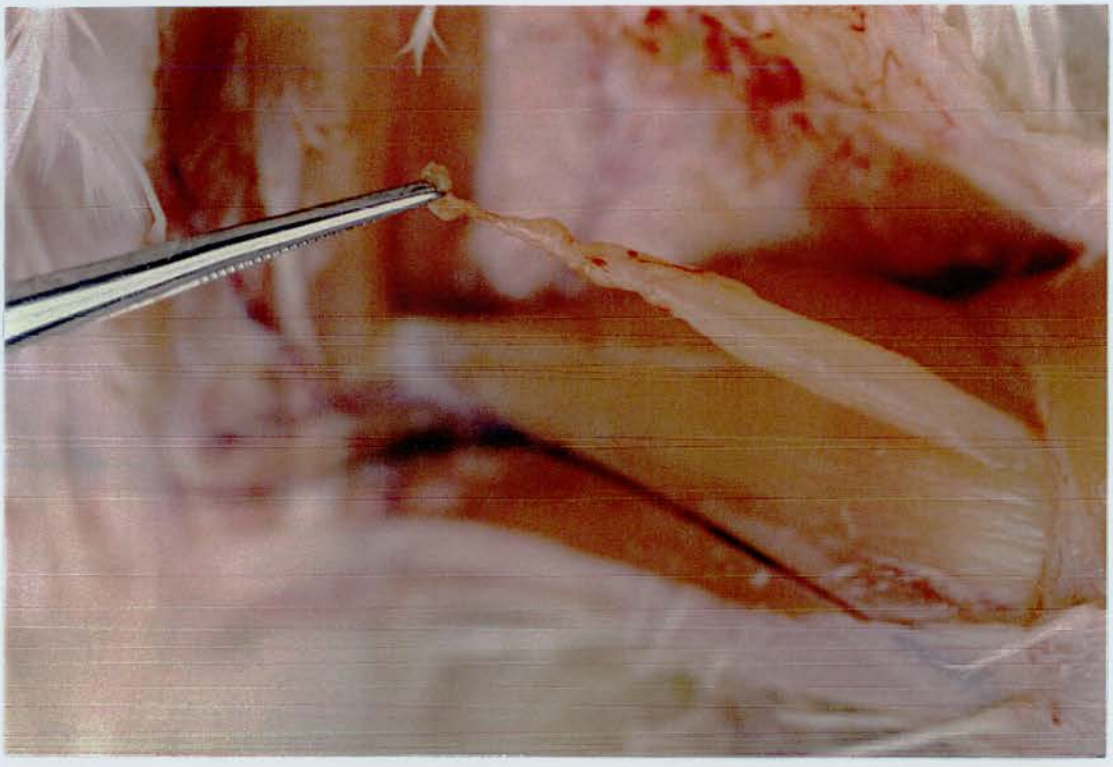

Plate 4.3 Partially excised *tensor patigialis* muscle complete with distal tendon attached. Ventral view. x3.5 magnification.

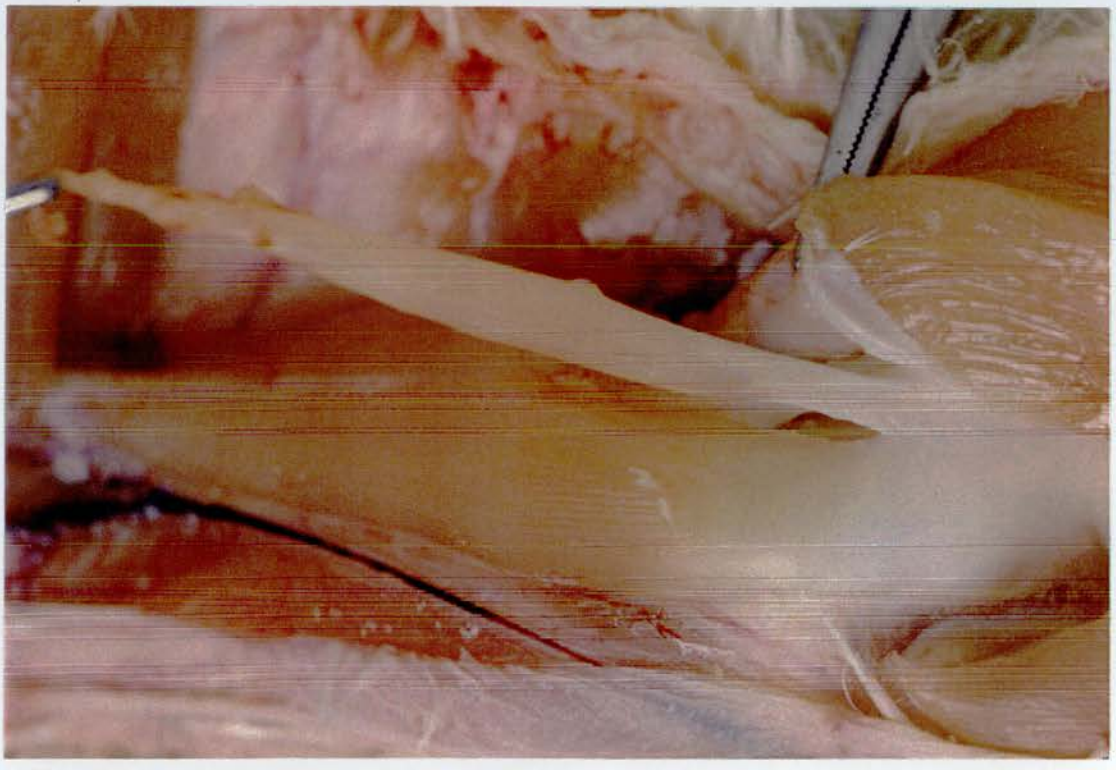

Plate 4.4 Incision made into the *m. pectoralis* to reveal the underlying proximal tendon insertion of *tensor patigialis* at the myotendonous junction at the head of the *m. biceps brachii.* Ventral view. x3.5 magnification.

Plate 4.5 Isolated intact *tensor patigialis* with tendon shown against a metric scale. Actual average muscle diameter and length (excluding tendon) is shown in the bottom right hand corner . Ventral view. x7 magnification.

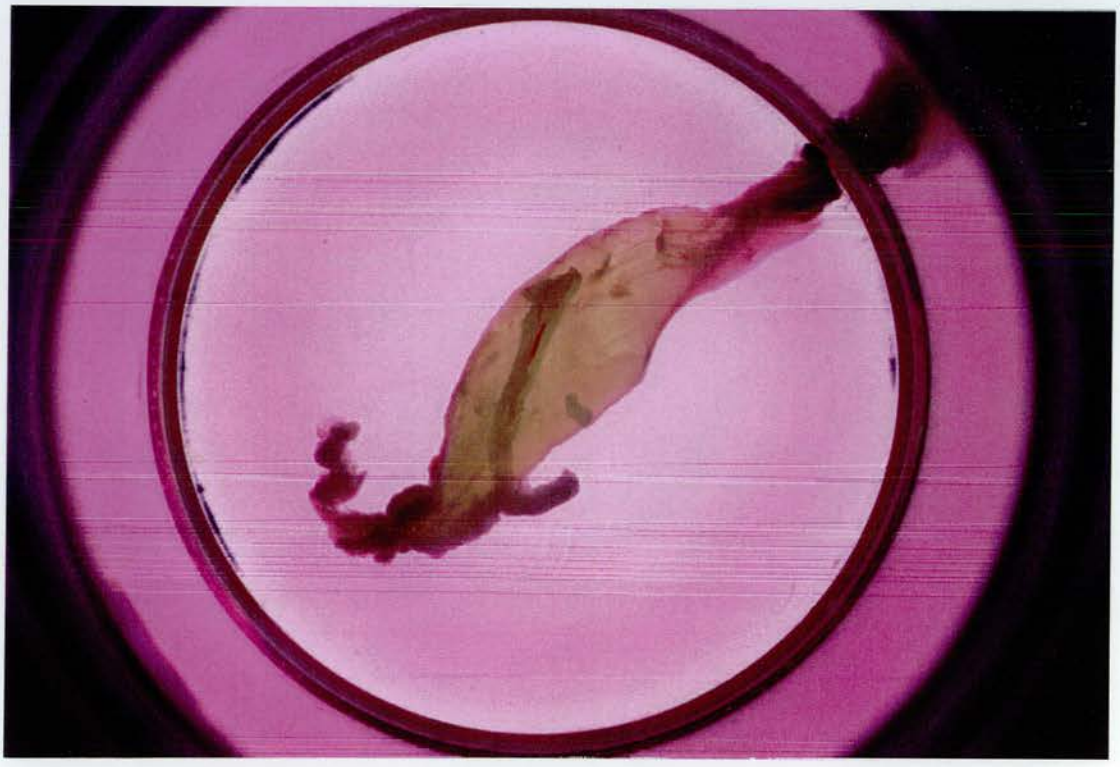

Plate 4.6 Overhead view of *tensor patigialis* muscle in beaker containing incubation medium 199 with Earles salts. x4 magnification.

Results

A profile of CK efflux from 32 muscles incubated over a 5 x 30 minutes incubation period (150 minutes) calculated in mU g^{-1} min^{-1} is shown in **Figure 4.1**. The highest rate of CK loss was observed during the first 30 minutes of incubation. The rate of CK efflux was subsequently reduced by approximately 50% after 60 minutes of incubation. Thereafter, the rate of CK efflux remained relatively stable and constant after over the remainder of the incubation time, representing an approximate rate of CK release of less than 20 mU g^{-1} min^{-1}.

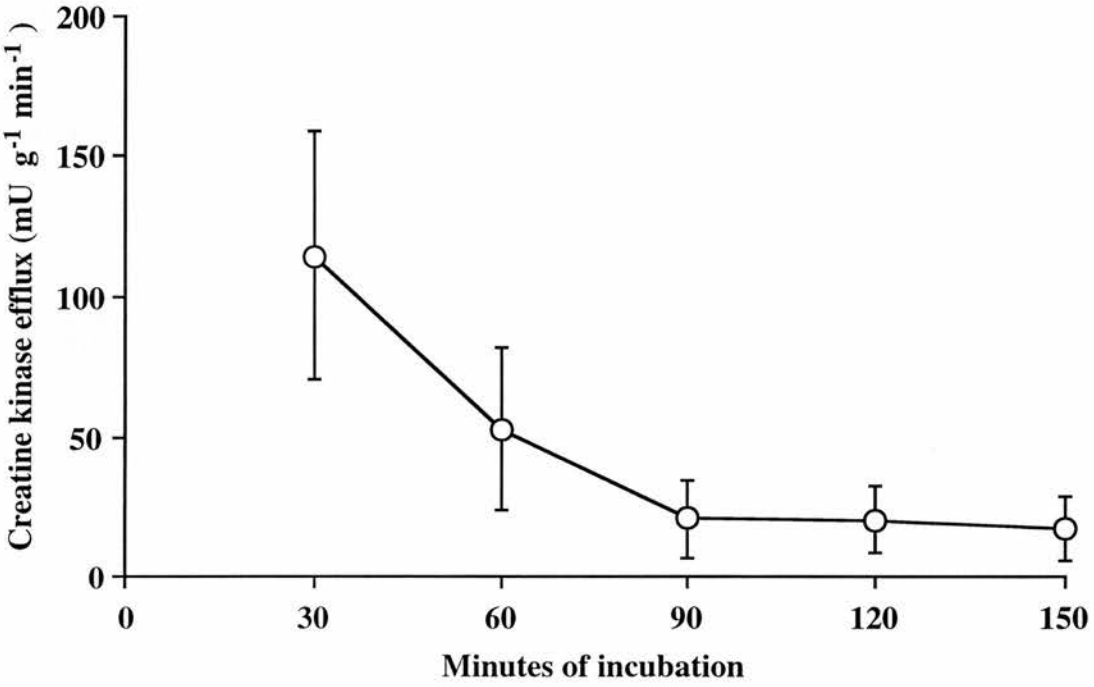

Figure 4.1 "Baseline" rate of creatine kinase (CK) efflux from isolated chicken *tensor patigialis* muscles incubated under control experimental conditions. Muscles were incubated in medium 199 with Earles salts at 41.5°C for 150 minutes and continually gassed with 95% O_2 + 5% CO_2. Values represent means ± S.D for 32 muscles.

A profile of total cumulative muscle CK loss expressed as IU g^{-1} wet weight of tissue is shown in **Figure 4.2**. Of the total enzyme released over the 150 minutes of incubation (**see Table 4.1**) greater than 75% was accounted for during the first 60 minutes of incubation, with the remaining proportion of the enzyme being evenly released over the remaining 3 x 30 minute incubation periods. Mean values ± S.D. for total muscle CK loss and muscle activity, and total CK loss expressed as a percentage of total muscle CK are given in **Table 4.1**. Total muscle CK loss over the duration of incubation (150 minutes) was minimal, representing 0.3 % of total muscle CK content.

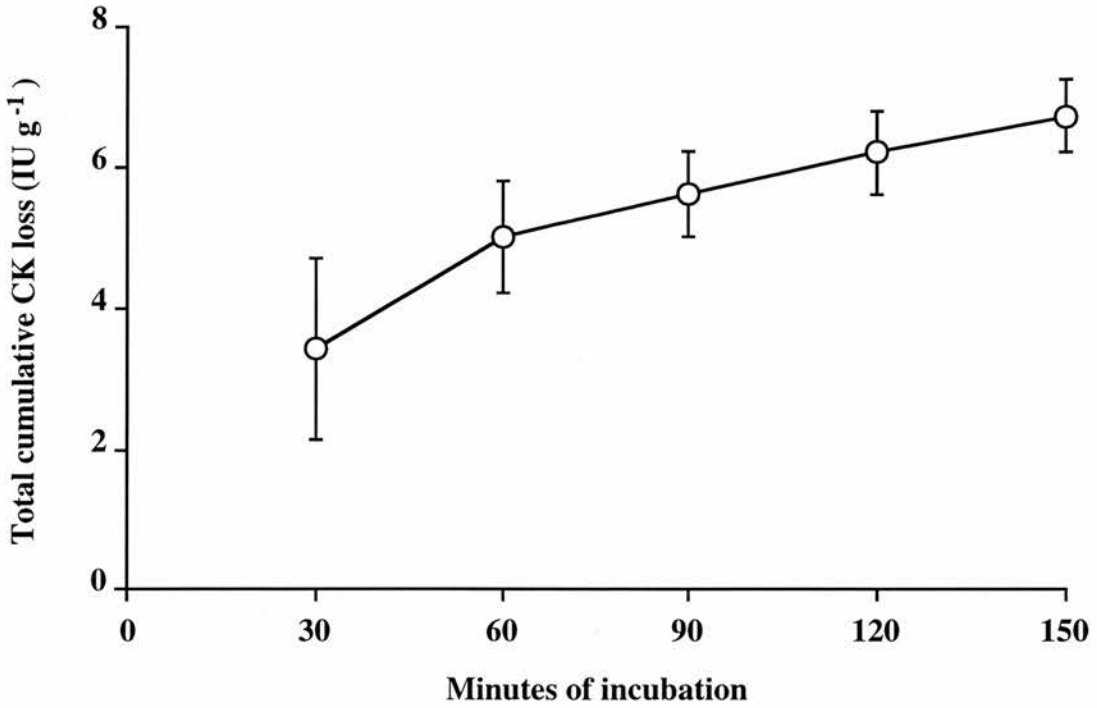

Figure 4.2 Total cumulative CK efflux from isolated chicken tensor *patigialis* muscle incubated under control experimental conditions, enzyme loss is expressed in IU g^{-1} wet weight of tissue. Values represent means ± S.D for 32 muscles.

Table 4.1. Mean (± S.D.) muscle weight, total CK loss, total CK activity and CK loss calculated as a percentage (%) of total activity. Values are derived from 32 isolated muscles incubated *in vitro*.

Muscle weight (g)	Total CK loss (IU g^{-1} muscle)	Total Muscle CK (IU g^{-1} muscle)	Muscle CK loss (% of total muscle CK)
0.111 ± 0.018	6.4 ±1.8	2485.2 ±380.9	0.3 %

Discussion and conclusions

The release of CK from chicken skeletal muscle incubated under the described experimental conditions (**see Figure 4.1**) showed a similar pattern of release to those described in other species (Suarez-Kurtz and Eastwood, 1981; Jones *et al.*, 1983, 1984). The pattern of efflux clearly shows that the greatest proportion of muscle enzyme release (>75%) occurred during the first 30 to 60 minutes of incubation and remained constant and stable for up to 90 minutes of incubation thereafter. The initial high level of enzyme loss most probably reflects measurement of exogenous enzyme activity ("wash-off") from cut muscle fibres damaged during dissection, a view supported by Suarez-Kurtz and Eastwood (1981) from studies on amphibian skeletal muscle. It is also possible that the enhanced release of enzyme

during the early stages of incubation may constitute a loss of intracellular CK occurring as a result of transient reduced post-dissection muscle viability.

It is also clear that from **Figure 4.1** that following this initial high level of enzyme measurement there is a steady low level rate of enzyme release (20 ±12 mU g^{-1} min^{-1}) which presumably reflects actual basal CK release from the muscle under these experimental incubation conditions. Corresponding basal rates of CK release of 1.3 U g^{-1} min^{-1} and 16.7-20.0 mU g^{-1} min^{-1} have been reported in studies on frog (Suarez-Kurtz and Eastwood, 1981) and mouse (Jones *et al.*, 1983) skeletal muscle. It has long been recognised that incubated muscle preparations lose cytoplasmic enzymes in to the incubation medium (Zierler, 1958; Goldberg *et al.*, 1975). What mechanisms mediate this low level basal release of enzyme from incubated skeletal muscle is still unclear. It is conceivable that the release could occur by a process of diffusion (either simple or carrier mediated) or by some active transport mechanism. Since CK synthesised within skeletal muscle is only utilised in that tissue (Walliman *et al.*, 1992) there is no apparent requirement for its active transport out of the cell. This would suggest that the low level release of CK from incubated muscles is by some process of diffusion. Any potential inward movement of CK from the medium (i.e. CK re-entry) may be discounted due to the large concentration gradient between medium and muscle which would always favour the outward movement of enzyme.

Whilst it is recognised *in vivo* that the loss of CK from skeletal muscle is normally associated with damaged or dysfunctional cells (Hamburg *et al.*, 1991), it is conceivable that the basal efflux of CK observed in this and other *in vitro* studies does not reflect true muscle damage, but represents loss via diffusion which is "normal" in tissues incubated in this manner. Whilst no attempt was made to quantify tissue viability, it is reasonable to assume that overall tissue viability was good, given that the total amount of CK released as a percentage of the total muscle activity was extremely low (0.3%). It is apparent that membrane integrity remained high and constant throughout the duration of incubation. It may therefore be suggested that the viability of the preparation was acceptable.

In summary, the aim of the present study was to develop and assess a suitable novel isolated skeletal muscle preparation and incubation system to examine the mechanisms of CK release in chicken skeletal muscle. The study has shown that the basal rate of CK release from skeletal muscle under the described experimental incubation conditions was low, and typical of basal enzyme loss in incubated tissues. It is therefore proposed that the *tensor patigialis* muscle preparation and incubation system represents an appropriate model for the investigation of the mechanisms of CK release in the domestic chicken.

Chapter 5

Effect of elevated incubation temperature on the release of creatine kinase (CK) from isolated chicken skeletal muscle

Introduction

The physiological effects of elevated body temperature have been extensively examined in many species including poultry (Yousef, 1985), since a rise in core body temperature can result from several causes including metabolic derangements (e.g. hyperthyroidism, fever), exercise, certain drugs or anaesthetics or exposure high environmental temperatures and humidities (Hardy, 1980). Studies on isolated cell and tissue preparations (including skeletal muscle) have examined the effect of temperature on many aspects of cellular physiology and biochemistry such contractility (Palmer *et al.*, 1981), protein catabolism (Baracos *et al.*, 1989), and oxygen consumption (Maltin and Harris, 1985; Van Breda *et al.*, 1990), anti-cancer therapy (Burkhardt and Ghosh, 1977) and gene expression of heat shock proteins (Stevenson *et al.*, 1986).

In **Chapter 3** of this thesis it has been demonstrated that skeletal muscle memebrane damage in broiler chickens caused by exposure to acute heat stress (2h; 32°C/80%RH) leads to a significant increase in the plasma activity of MM-CK. In addition, it was also shown that acute heat stress exposure induced a highly significant elevation of deep body temperature from 41.7± 0.2°C to 44.5 ± 0.3 °C. which was associated with a significant increase in total plasma CK activity from 518±96 to 859±146 IU litre^{-1}. Similar excursions in body temperature and changes in plasma enzyme activity have also observed in broiler chickens by Ostrowski-Meissner (1981) and Mitchell *et al* (1992) in previous studies on acute heat stress exposure and transportation.

Experimental aims

The aim of this experiment was to examine the effect of incubation at 45°C on the profile of CK release from isolated chicken *tensor patigialis* muscles. This temperature representing the greatly elevated deep body temperature attained by some broiler chickens exposed to acute heat stress (**See Chapter 3**).

Materials and methods

The muscles used in this experiment were incubated under a slightly different incubation protocol to that described in **Chapter 4** due to the technical difficulty of incubating muscles at different temperatures in the same waterbath. Four muscles of the same treatment were incubated each day per experimental run over a period of two days (16 muscles in total). Muscles incubated at control body temperature (41.5°C) were incubated under the conditions described in **Chapter 4.** Muscles incubated at the elevated incubation temperature of 45°C were initially incubated at control temperature for 60 (2 x 30) minutes. After this time the temperature setting of the waterbath was adjusted to 45°C, some of the water in the bath was removed and replaced with pre-heated water at 45-50°C to adjust the water to the correct temperature. The gas line supplying the muscles with 95% O_2 + 5% CO_2 was coiled and submerged in the water bath to pre-heat the gas mixture and thus minimise a reduction in incubation medium temperature. As a further measure to ensure the muscles were being incubated at 45°C, the incubation medium temperature was monitored using a mercury thermometer (Macfarlane Robson Ltd. UK) inserted through a small sealed hole in the lid of a separate adjacent beaker containing 2.5 ml (gassed) incubation medium. In addition to this, the incubation medium in the beaker was seeded with 20µl broiler plasma (CK activity; 551±9 for 5 replicates) to examine the possibility of enzyme degradation at the higher temperature.

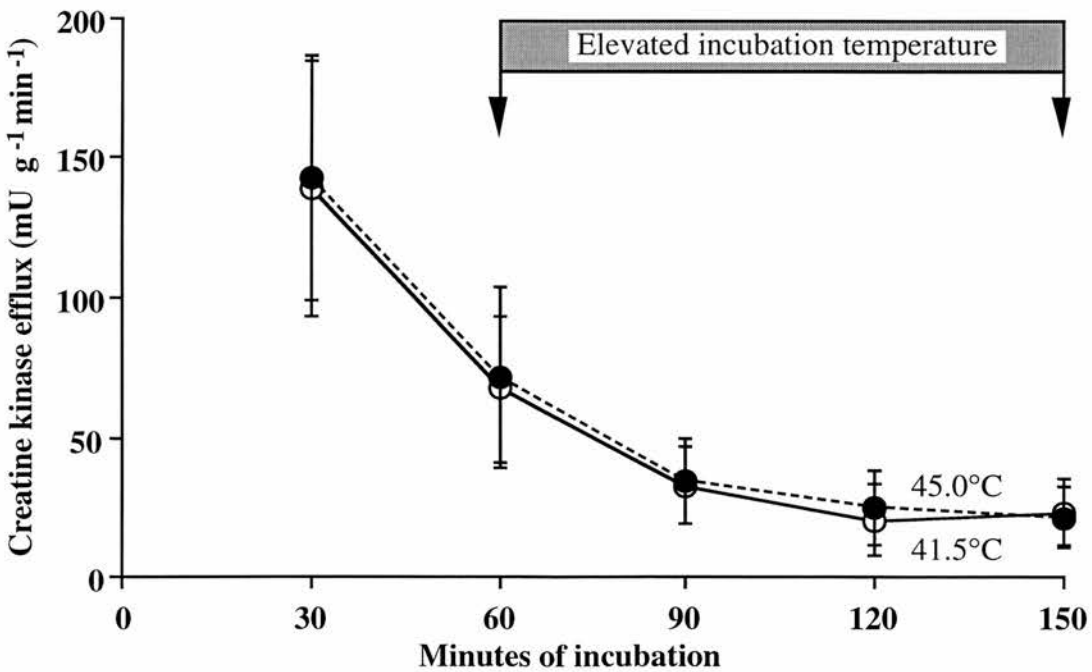

Figure 5.1 The effect of incubation at control (41.5°C) and "heat-stress equivalent" temperature (45°C) on the release of CK from isolated skeletal muscle. Values represent means ± S.D for 8 muscles per treatment.

Results

The effect of incubation at 45°C incubation temperature on muscle CK release is shown in **Figure 5.1**. The results clearly show that incubation at the elevated incubation temperature had no effect on the rate of muscle efflux above that released from control muscles incubated at 41.5°C. The total loss of CK from the muscles incubated at control and elevated temperature representing 6.8 ±0.9 and 7.0 ±1.1 IU g^{-1} respectively. CK activity in the seeded medium was unaffected by an increase in incubation temperature showing a 98.9% recovery (539±12 IU $litre^{-1}$) of initial enzyme activity.

Discussion and conclusions

It is clear from the results of the present experiment that there was no effect of elevated incubation temperature on the release of CK from isolated incubated chicken skeletal muscle. The apparent lack of effect of incubation at 45°C was not attributable to inaccuracies in the control of incubation temperature, as monitoring of the incubation medium temperature using a mercury thermometer showed that the muscle were incubated at the required temperature. Similarly, any potential effect of the raised incubation temperature on enzyme inactivation can also be discounted as 98.9% of the enzyme activity was recovered in a seeded incubation medium at the elevated temperature.

It is also possible that skeletal muscle damage caused by exposure to elevated thermal loads *in vivo*, may be mediated by a humoral component present in the whole animal that is induced by heat stress and which is absent *in vitro*. One must also consider the possibility that the muscle preparation may be not be representative of those muscles which are affected by heat stress and contribute to an increase plasma CK activity (**see Chapter 1**). In addition, is also possible that the incubation buffer composition may influence the effects of elevated incubation temperature, in that the incubation of the muscles in a complete physiological medium may off-set any effect of incubation at a higher temperature (**see Appendix II**).

In conclusion therefore these results would suggest that elevated incubation temperature (45°C) *per se* had no effect on the release of CK from isolated chicken skeletal muscle.

Chapter 6

Characteristics of muscle creatine kinase (CK) release under conditions of impaired oxidative energy metabolism.

Introduction

Disruptions of the mechanisms that maintain intracellular energy metabolism (i.e. adenosine triphosphate (ATP) production) have long been regarded as an early event in the development of damage in many cells and tissues (Bowman and Rand, 1980). It is universally recognised that ATP is essential for maintaining normal cellular function and is utilised in many energy dependent processes such as trans-membrane ion exchange, protein synthesis, and contraction. Factors known to adversely influence changes in oxidatively-derived (aerobic) ATP production such as tissue hypoxia and mitochondrial poisoning have been reported to promote the development of cellular deregulation and even cell death in a variety of tissue types including hepatocytes (Sakaida *et al.*, 1992; Synder *et al.*, 1993), cardiomyocytes (Eisner *et al.*, 1989; Jennings and Reimer, 1991) and skeletal muscle (Duncan and Jackson, 1987). Morphological changes associated with impaired oxidative metabolic function include mitochondrial swelling, formation of amorphous matrix densities, and structural disruptions to myofilaments and cell membranes.

Inadequete cellular O_2 availability (hypoxia) has been shown to increase the release of cytosolic enzymes from isolated mouse skeletal muscle (Zierler, 1965; Jones *et al.*, 1983). Similarly, it has also been reported by Jackson *et al.* (1984) that inhibitors of oxidative energy metabolism such as sodium cyanide and 2,4 dinitrophenol produce detrimental changes in oxidative metabolic function in skeletal muscle analogous to those associated with tissue hypoxia, including altered sarcolemmal permeability and augmented cytosolic enzyme release.

Whilst it is acknowledged that the effects of impaired oxidative energy metabolism have been extensively examined and characterised in isolated mouse skeletal muscle preparations (Jones *et al.*, 1983; Jackson *et al.*, 1984). To date, there is no evidence that these effects have been examined in whole isolated chicken skeletal muscle. A full characterisation of the effects of impaired oxidative metabolism in chicken skeletal muscle is required if we are to be able to contrast and compare findings that may be analogous to those described in isolated mouse skeletal muscle.

Experimental aims

The objective of this study was to re-examine the effects of the impaired of oxidative energy metabolism on the release of CK from incubated isolated chicken *tensor patigialis* using methods previously described by Jones *et al.* (1983) and Jackson *et al.* (1984). In the first instance, muscles were incubated in the absence of O_2 under an atmosphere of 95% N_2+5% CO_2 (anoxia), conditions known to produce a profound tissue hypoxia that exceeds any clinical level of hypoxia encountered *in vivo* (Jones *et al.*, 1983). In addition, a second group of muscles were incubated with the metabolic inhibitor 2,4 dinitrophenol (Jackson *et al.*, 1984) an agent known to uncouple the oxidative phosphorylation of adenosine diphosphate (ADP) to ATP in mitochondria (Loomis and Lipman, 1948). In tissues where O_2 dependent ATP availability is compromised, there is a significant shift towards glycolytic (anaerobic) energy metabolism. This results in an increase in formation of lactic acid which readily diffuses from the cell into the external milieu (Bowman and Rand, 1980). Measurement of muscle lactic acid release (as lactate) in to the incubation medium was used to indicate a shift from oxidative to glycolytic metabolism.

Essential differences in the characteristics of the isolated skeletal muscle preparation and experimental methods in the present study, and in previously described isolated skeletal muscle studies by and Jones *et al.* (1983) and Jackson *et al.* (1984) are shown in **Table 6.1**.

Table 6.1 Differences in the characteristics of the isolated skeletal muscle preparation, and experimental methods in the present study, and in previously described isolated skeletal muscle studies by and Jones *et al.* (1983) and Jackson *et al.* (1984)

Measurement	Present study	Previous studies
Species	Chicken	Mouse
Ave. muscle size (mm)	18 x 5	10 x 2
Ave. muscle weight (mg)	100	10
Predominant fibre type (%)	IIB fast glycolytic (99%)	IA slow oxidative (85%)
Buffer type	Complete	Minimal

Materials and methods

Anoxia (Experiment V)

Muscles subjected to anoxic incubation were initially incubated under normal oxygenation conditions of 95% O_2 + 5% CO_2 (normoxia) for 60 (2 x 30) minutes,

then gassed with 95% N_2 + 5% CO_2 for the remainder of the experiment. Controls were incubated under normoxic conditions throughout.

2,4 dinitrophenol (Experiment VI)

Muscles receiving treatment with the mitochondrial inhibitor 2,4 dinitrophenol (2,4 DNP) were incubated under the same conditions as control muscles for the first 60 (2 x 30) minutes. After this time, treatment muscles received 80 µl of 2,4 DNP (5 mmol litre^{-1} in 90% ethanol) added to the incubation medium. Controls received an equal volume of 90% ethanol. Both groups of muscles were incubated for 30 minutes with their respective treatments. After this period both treatment groups were returned to control incubation medium for the remainder of the experiment.

All muscles were incubated according to the incubation protocol previously described in **Chapter 4**. CK activities and lactate concentrations in the incubation medium were determined by the methods described in **Chapter 2**. Data are presented as means ± S.D.. Levels of significance were determined by analysis of variance or unpaired Students t-test

Results

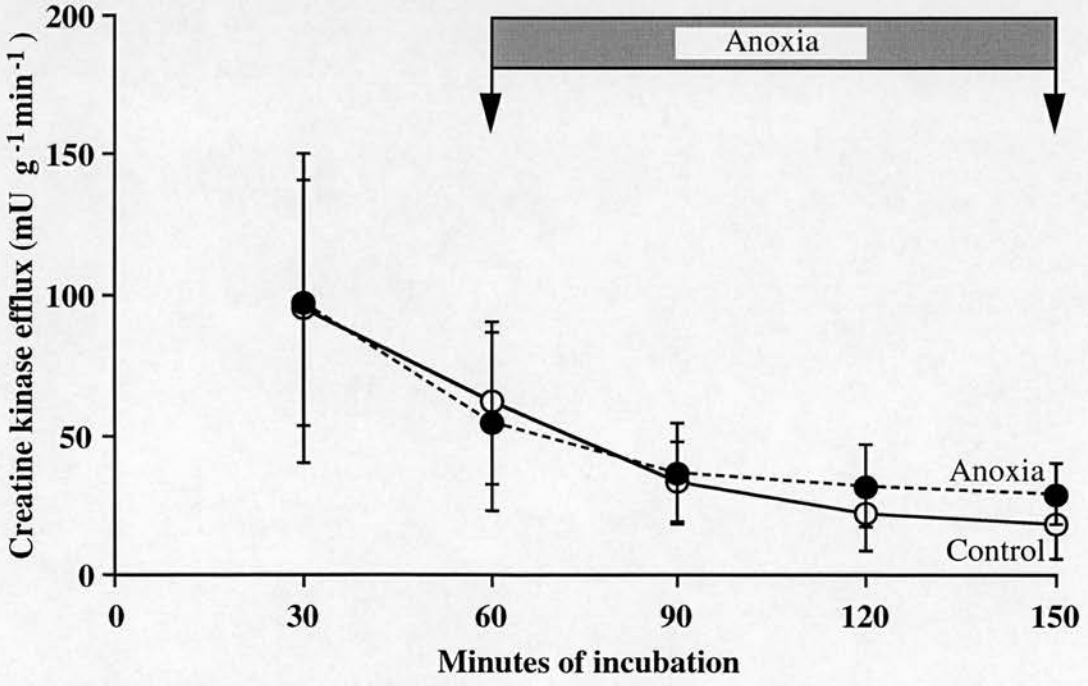

Figure 6.1 Effect of anoxia on the rate of CK efflux from chicken *tensor patigialis* muscle. Anoxia treatment muscles were initially incubated under normoxic conditions for 60 minutes, then gassed with (95% N_2 + 5% CO_2) for the remainder of the experiment. Control muscles were incubated under normoxic conditions (95% O_2 + 5% CO_2) throughout. Values represent means ± S.D for 8 muscles.

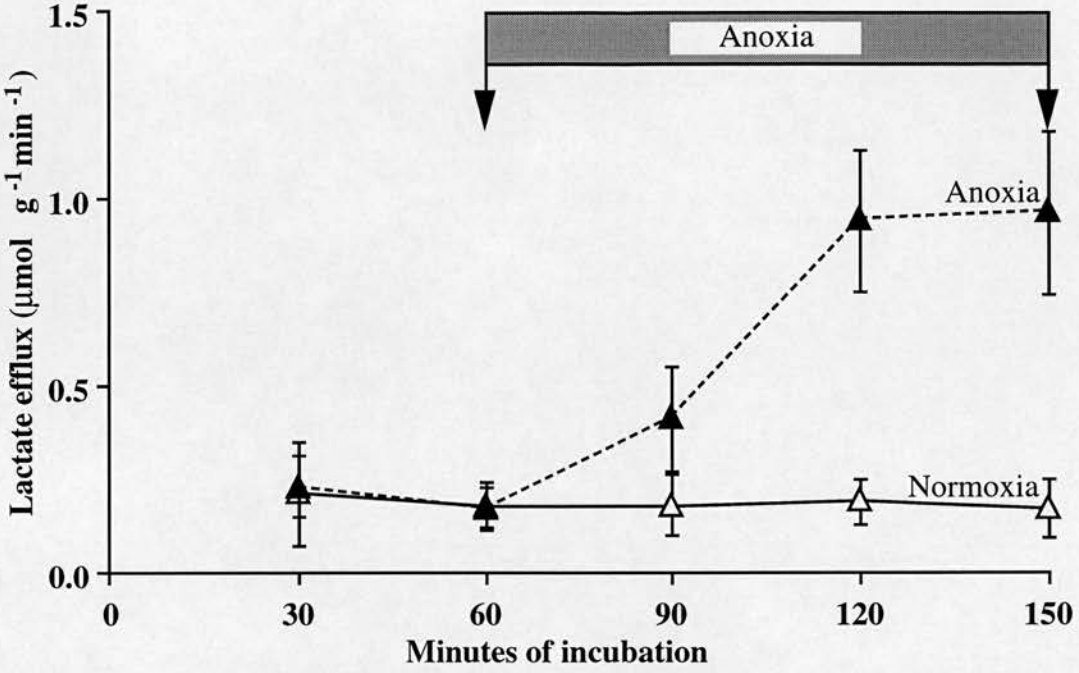

Figure 6.2 Effects of anoxia on the rate of lactate release from chicken *tensor patigialis* muscle. Anoxia treated muscles were initially incubated under normoxic conditions for 60 minutes, then gassed with (95% N_2 + 5% CO_2) for the remainder of the experiment. Control muscles were incubated under normoxic conditions (95% O_2 + 5% CO_2) throughout. Values represent means ± S.D for 8 muscles.

Effects of anoxic incubation

The effects of anoxic incubation on muscle CK and lactate release are shown in **Figures 6.1** and **6.2**. The results in **Figure 6.1** clearly show that incubation in the absence of O_2 had no effect on the rate of muscle efflux above that released from control muscles. Total CK loss from control and anoxia muscles was 6.9 ±0.9 and 7.5 ±1.2 IU g^{-1} respectively.

Corresponding effects on muscle lactate release during incubation under anoxic conditions are illustrated in **Figure 6.2**. Lactate release from control and anoxia treated muscles was the same during the initial 60 minutes of incubation under normoxic conditions. Incubation in the absence of O_2 caused a 3.8-fold increase in the rate of lactate release ($p<0.001$) which plateaued after 60 minutes of anoxic incubation at a maximal lactate release rate of approximately 1.0 µmol g^{-1} min^{-1}. Lactate release from control muscles remained stable and constant throughout the total incubation period reflecting a lactate release rate of approximately 0.27 µmol g^{-1} min^{-1}. Total muscle lactate release following anoxic incubation was 3.3-fold higher ($p<0.001$) than lactate loss from controls, constituting total losses of 76.5 and 23.4 mmol g^{-1} of lactate in anoxia and control treated muscles, respectively.

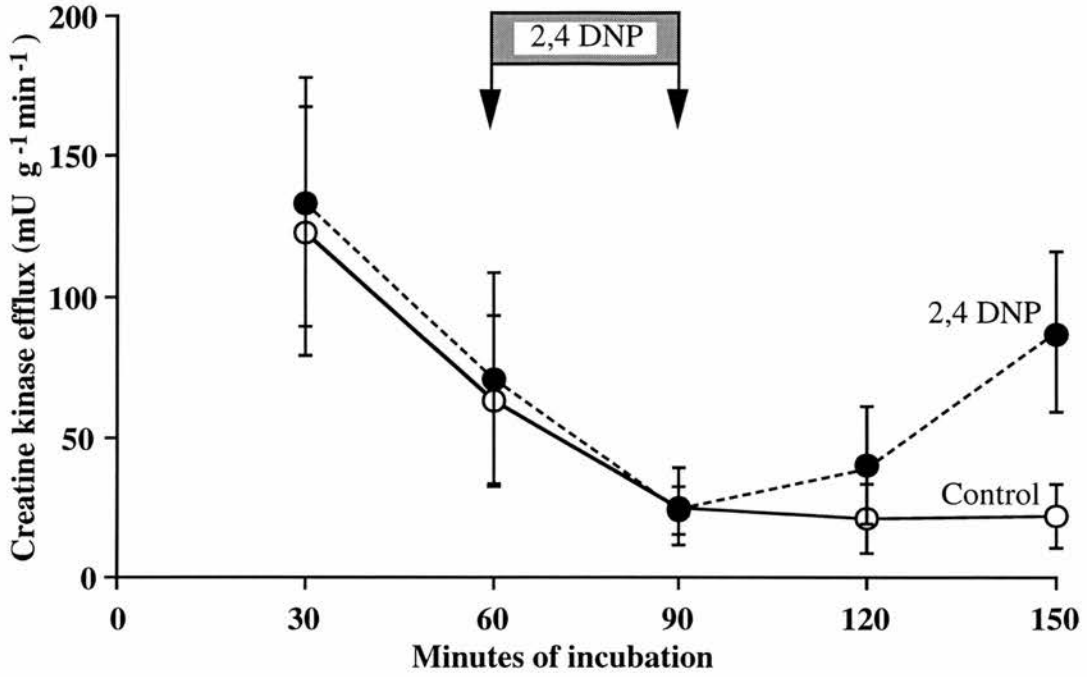

Figure 6.3 Effects of 2,4 dinitrophenol (2,4 DNP) on the rate of CK efflux from incubated isolated chicken *tensor patigialis* muscle. Muscles were incubated with 2,4 DNP (5 mmol litre^{-1}) for 30 minutes, following 60 minutes of incubation in control medium, then returned to control medium for the remainder of the experiment. Values represent means ± S.D for 8 muscles.

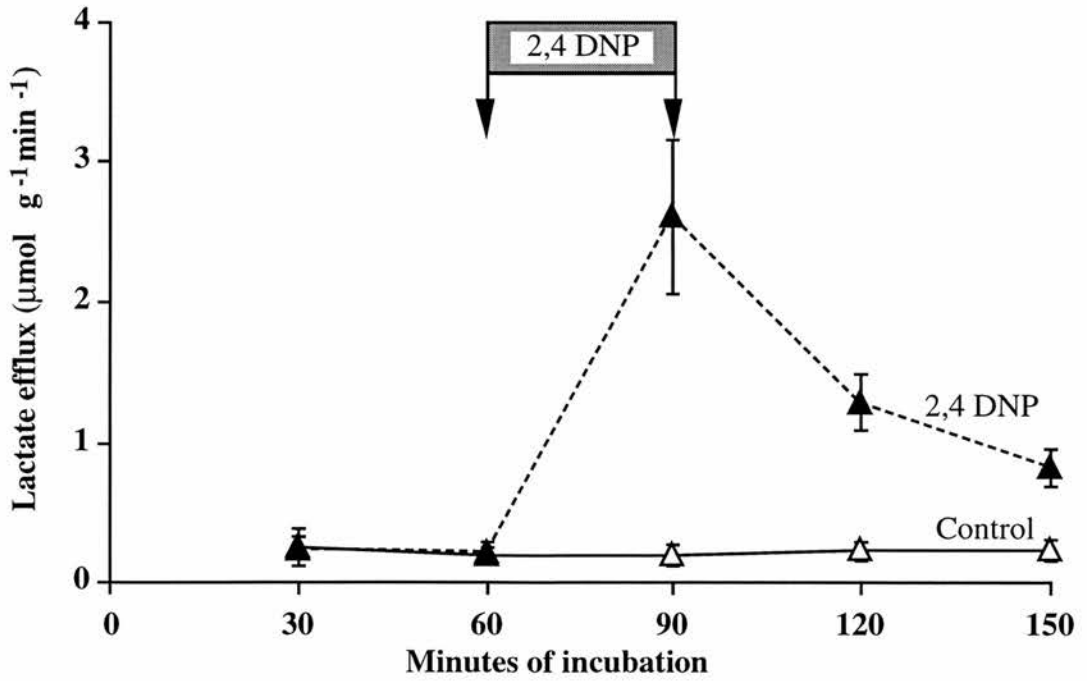

Figure 6.4 Effects of 2,4 dinitrophenol (2,4 DNP) on the rate of lactate efflux from chicken *tensor patigialis* muscle. Treatment muscles were incubated in the presence of 2,4 DNP (5 mmol litre^{-1}) after 60 minutes incubation in control medium for 30 minutes then returned to control medium for the remainder of the experiment. Values represent means ± S.D for 8 muscles.

Effects of 2,4 dinitrophenol (2,4 DNP)

The effects of treatment with 2, 4 DNP (5 mmol litre^{-1}) on muscle CK efflux and lactate release and are shown in **Figures 6.3** and **6.4**. The results in **Figure 6.3** show there was no observed difference in the rate of CK release during the 30 minutes of incubation with 2,4 DNP. However, following the return of these muscles to control incubation medium, there was a gradual increase in the rate of enzyme loss culminating in a significant 4-fold increase ($p<0.05$) in the rate of CK release after 90 minutes of post-treatment incubation. In the ethanol-treated control muscles the profile of CK release was identical to that previously observed in control incubated muscles in **Experiment III (Chapter 4)**. Total CK loss in the 90 minutes of incubation following treatment with ethanol or inhibitor was 2.1 ±0.3 and 4.5 ±0.6 IU g^{-1}, representing a 114.3 % greater loss of CK from the 2,4 DNP treated muscles during this period.

The effects of 2,4 DNP treatment on muscle lactate release are illustrated in **Figure 6.4**. During the 30 minutes of incubation with 2,4 DNP there was a rapid maximal 14-fold increase ($p<0.001$) in the rate of lactate released which diminished over subsequent incubations, yet remained significantly higher than control rates of lactate efflux after muscles had been returned to control incubation conditions. The rate of lactate efflux from ethanol-treated muscles was stable and constant throughout the duration of incubation, representing a release rate of 0.22 ±0.03 μmol g^{-1} min^{-1}.

Discussion and conclusions

Previous work on isolated mammalian skeletal muscle preparations by Jones *et al.* (1983) and Jackson *et al.* (1984, 1987) has suggested that disruption of the mechanisms that maintain cellular ATP production through oxidative metabolism play an important part in the development of muscle damage and enzyme release. The results of the present study, which has investigated the effects of impaired oxidative metabolism in isolated chicken skeletal muscle through anoxic incubation of muscles (**see Figure 6.1**) and treatment with 2,4 DNP (**see Figure 6.3**) essentially agree with the findings of the previously cited authors.

The results in **Figure 6.3** showed there was no difference in the rate of CK release from isolated chicken *tensor patigialis* muscle during incubation in the absence of O_2 (anoxia; 95% N_2 + 5% CO_2) compared with muscles incubated under control oxygenation conditions (95% O_2 + 5% CO_2). These results sharply contrast with the findings of Jones *et al.*(1983) which demonstrated a large and rapid increase in the release of CK (and LDH) from incubated isolated mouse soleus muscle under the

same anoxic experimental conditions. However, the disparity between the findings of the present study, and previous work by Jones *et al.* (1983), may possibly be explained by morphological differences (i.e. differences in relative muscle fibre composition) in the two types of muscle preparation used (**see Table 6.1**). It is well recognised that muscles composed of varying fibre types differ can appreciably in their metabolic and pharmacological responses (**see Chapter 1**). It is therefore possible that the role of oxidative energy metabolism may be less important in a muscle such as *tensor patigialis* which is better equipped metabolically to maintain intracellular energy production thorough glycolytic metabolism, than *soleus* muscle which is predominantly oxidative in function. This is supported by the lactate measurements of **Experiments V** and **VI**. In addition, it is likely that differences in incubation buffer composition may also influence the effects of anoxia in the two muscle types. Incubation in a complete medium may off-set the effect of any potential glycogen depletion by providing the muscle with renewed reserves of glycolytic substrates at physiological concentrations (**see Appendix II**). This contrasts with the set-up of Jones *et al.* (1983) in which muscles were incubated in modified Krebs-Ringer bicarbonate buffer.

The results illustrated in **Figure 6.2** clearly show there was a sustained increase in muscle lactate release during the period of anoxic incubation which plateaued at a maximum release rate of approximately 1.0 $\mu mol\ g^{-1}\ min^{-1}$. This rate of lactate release was approximately one third the maximum rate of lactate release observed in muscles treated with the mitochondrial inhibitor 2,4 DNP (**see Figure 6.4**). Given that the muscle used in the present study apperently had the capacity to maintain intracellular energy production at much higher rates of glycolytic metabolism without an immediate effect on CK release. It is suggested that the effect of anoxic incubation in this muscle was therefore not sufficient to reduce cellular energy production below a rate which could be adequately maintained through glycolytic metabolism. The low level rate of lactate efflux from muscles incubated under control (normoxic) conditions (**see Figure 6.2**) most likely reflects a low level rate of lactic acid production, which would suggest that during incubation under control conditions, *tensor patigialis* muscles were largely functioning aerobically.

Unlike the muscles incubated in the absence of O_2, muscles incubated with the mitochondrial inhibitor, 2,4 DNP demonstrated an significant 114.3 % increase in total CK release (**see Figure 6.3**). The effect of 2,4 DNP on muscle enzyme loss was consistent with the findings of Jackson *et al.* (1984) and support the view that conditions leading to impaired mitochondrial function may mediate the development of muscle damage and subsequent CK release. Whilst the outcome of the effect of

mitochondrial inhibition on the release of CK was the same in this, and earlier studies, the pattern of enzyme release was different to that previously reported in isolated mouse skeletal muscle studies. In the present study there was a gradual increase in the rate of CK efflux from the muscles following incubation with 2,4 DNP which sharply contrasts with the rapid release of CK observed in the studies by Jackson *et al.* (1984, 1987). Furthermore, the total amount of CK released from muscles in the current study was much less than that reported by Jackson *et al.* (1984, 1987) despite a 5-fold difference in inhibitor concentration. Disparity between studies in the pattern and amount of CK efflux in muscles treated with 2,4 DNP may be due to size differences in the muscle preparations (10-fold), but may also reflect differences due to muscle morphology and buffer composition as discussed previously. The gradual increase of CK release observed in the present study may be a function of muscle size. In a larger muscle, the effective diffusion distance is much greater, thereby extending the diffusion time for the released enzyme into the incubation medium, and the diffusion of DNP inwards. This diffusion hypothesis is consistent with the findings from a previous study by Zierler (1957) which showed that larger incubated muscles tended to have a lower rate of enzyme release compared to smaller muscles. This explanation may account for any delay in the time course of CK efflux, however it is clear from the results in **Figure 6.4** that it may not be true in the case of lactic acid release where the apparent effects of 2,4 DNP on lactic acid production and subsequent efflux were immediate. This disparity may however be a function of the large difference in CK (81000) and lactate (90.08) molecular size or differences in transport/efflux mechanisms.

Large differences in the total amount of CK released from the muscles treated with 2,4 DNP in the present study compared with the amount of enzyme released in previous studies may also be attributed to the combined effects of differences in muscle morphology and buffer composition as discussed previously. As with the situation in anoxia treated muscles, it is likely that the effects of mitochondrial inhibition and a reduction in oxidative metabolic function may be less pronounced in a muscle that is functionally equipped to maintain cellular ATP production through glycolytic metabolism.

Any possible interference effect of 2,4 DNP treatment on the determination of medium CK activity can be discounted. Preliminary tests on medium samples containing high initial CK concentrations (results not shown) demonstrated that increasing concentrations of 2,4 DNP had no effect on the determination of CK activity in vitro. This observation is consistent with the findings of Brazeau and Fung (1989).

In summary, it is clear from the results of this and other *in vitro* studies that conditions leading to impaired mitochondrial function and diminished intracellular energy production can lead to an increase in the efflux of CK from isolated skeletal muscle. How a reduction cellular ATP availability mediates an increase in muscle enzyme release is unclear, however it is widely believed that a reduction in cellular energy status may initiate a sequence of biochemical events which culminate in the development of altered muscle cell membrane (sarcolemma) permeability and increased muscle CK release (Wrogman and Pena, 1978).

For a long time it has been recognised that a large proportion of the ATP used in many cells is utilised in maintaining trans-membrane ion gradients a process which is mediated by a family of membrane localised ATP-dependent exchange proteins collectively known as ion motive ATPases (Pedersen and Carafoli, 1987a, b). Under conditions leading of reduced intracellular ATP availability, the normal functioning of these exchange proteins starts to breakdown, thereby reducing the ability of the cell to maintain ionic gradients. If sustained, this can eventually lead to a significant net influx of several mono and di-valent cations including sodium (Na^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}) (Trump *et al.*, 1989). Increasingly, the role of the influx of Ca^{2+} and its reported effects on the inhibition of mitochondrial function and the activation of several calcium-activated degenerative processes has received a great deal of attention and has dominated research into the mechanisms of damage in cardiac muscle for several years (Tani, 1990). The involvement of extracellular Ca^{2+} in development of skeletal muscle damage has also been proposed by Soybell *et al.* (1978), Jones *et al.* (1984) and Duncan and Jackson (1987).

Chapter 7

The role of external calcium entry on the release of creatine kinase (CK) from isolated chicken skeletal muscle

Introduction

The role of calcium (Ca^{2+}) as an intracellular mediator of many physiological process in a whole variety of cell types is well established (Bowman and Rand, 1980). It is becoming increasingly clear however, that disruptions of the mechanisms that regulate intracellular Ca^{2+} homeostasis may play a pivotal role in the development of cellular injury or pathology (Schanne *et al.*, 1979; Farber, 1982; Nicotera *et al.*, 1990). There is already great deal of evidence from studies in a wide range of cell and tissues to suggest that conditions leading to sustained increases in intracellular Ca^{2+} concentration may initiate many degradative cellular processes which include alterations in cytoskeletal organisation, activation of Ca^{2+}-dependent degradative enzymes, impairment of mitochondrial function, and activation of cell self-deletion or "programmed cell death" (Jewell *et al.*, 1982; Nicotera *et al.*, 1986; Bellomo *et al.*, 1991; Van Rooijen, 1991).

Early studies investigating the mechanisms of muscle damage associated with various muscular dystrophies have linked increases in intracellular calcium ion concentration with development of muscle damage (Bodenstein and Engel, 1978; Duncan, 1978). *In vitro* studies using isolated rat and mouse skeletal muscle preparations have also reported muscle dysfunction and damage associated with raised intracellular calcium concentrations these include: increased muscle proteolysis (Baracos *et al.*, 1984; Foruno and Goldberg, 1986), perturbations in myofilament structure (Duncan and Jackson, 1987), changes in cell membrane integrity or permeability and loss of intracellular constituents such as enzymes and metabolites (Jones *et al.*, 1984; Claremont *et al.*, 1984; Jackson *et al.*, 1984, Phoenix *et al.*, 1989).

In order to investigate the effects of elevated calcium on various aspects of cellular function, researchers have often used divalent cation ionophores such as A23187 and ionomycin to increase intracellular calcium content. A23187 has been extensively used in many studies on various cell and tissue types including skeletal muscle (Publicover *et al.*, 1978; Furuno and Goldberg, 1986; Albano *et al.*, 1991). The ionophoretic properties of A23187 facilitates the transport of calcium ions (Ca^{2+})

across biological membranes through a process of proton (H^+)/cation exchange (Wheeler *et al.*, 1994).

In order to assess the extent of trans-membrane calcium re-distribution in response to ionophore treatment, it has often proved necessary to quantify the increase in intracellular calcium. The range of techniques available for the measurement of calcium in isolated cells and tissue preparations is considerable (McCormack and Cobbold, 1991). The choice of method used is largely dependant on the type of preparation used and the nature of the biological process studied. Many studies have measured intracellular calcium concentrations in cultured cell preparations using Ca^{2+} sensitive fluorescent dyes and spectrofluorometry (Tsien, 1980; Grynkiewicz *et al.*, 1985) or Ca^{2+} sensitive micro-electrodes (Thomas, 1978; 1982). Studies using isolated whole tissue preparations have tended to favour the measurement of intracellular Ca^{2+} content either by methods such as atomic adsorption spectrometry (Jackson *et al.*, 1985; Phoenix *et al.*, 1987) or radio-isotope uptake using ^{45}calcium (McArdle *et al.*, 1991).

Measurement of calcium by this latter method has been employed in the experiment described in this chapter. This method offers several advantages of measuring calcium in isolated whole muscle preparations over other techniques in that it is non-destructive, simple in its application and does not require sophisticated analytical technology.

Experimental aims

The aim of this experiment was to examine the role of external calcium entry on the release of muscle CK from isolated chicken skeletal muscle utilising the properties of a calcium specific ionophore (**Experiment VII**). A halogenated derivative of the calcium ionophore A23187 (4Br-A23187, Sigma Chemical Co., UK) was used as it is reported to exhibit a 10-fold greater transport selectivity for Ca^{2+} over Mg^{2+} than A23187 (Debono *et al.*, 1981). Consistent with previous experiments (see Chapter 6), the release of muscle lactate in to the incubation medium was measured as indicator a shift from oxidative to glycolytic metabolism due to the reported effects of Ca^{2+} on mitochondrial function (Nicotera *et al.*, 1992).

Materials and methods

1 mg of 4Br-A23187 calcium ionophore was solubilised in 90% ethanol (1 ml) to give a final ionophore concentration of 25 μmol litre^{-1}. Ionophore treated muscles were initially incubated under the same conditions as the control muscles for 2 x 30

minutes. After this period, ionophore treatment muscles received 40 µl of 4Br-A23187 solution added to the incubation medium. Control muscles received the same volume of 90% ethanol. Muscles were then incubated with their respective treatment for 30 minutes. After this time, muscles were incubated back in normal medium for the remainder of the experiment. All muscles were incubated and processed according to the incubation protocol previously described in **Chapter 4.**

Muscle calcium content was determined from the uptake of radio-labelled ^{45}calcium ($CaCl_2$; 16.4 kBq ml^{-1}) added to the incubation medium. Isotope-labelling of the medium with ^{45}calcium and calculation of calcium uptake is described in the general methods section (**See Chapter 2**). Enzyme activities and lactate production were also assayed as previously described in the general methods section.

Results

The effect of 4Br-A23187 calcium ionophore on muscle CK efflux is illustrated in **Figure 7.1**. Incubation with ionophore (25 µmol $litre^{-1}$) caused a profound 60-fold increase ($p<0.001$) in the release rate of CK. This high level rate of efflux was sustained throughout the duration of experiment even when the muscles had been returned to control medium. Ethanol treated control muscles showed no evidence of any increase in CK efflux.

Figure 7.2 shows of the effect of calcium ionophore on muscle ^{45}calcium uptake and total CK efflux . Incubation with 4Br-A23187 induced a 79.7% increase in muscle ^{45}calcium uptake compared to controls. This increase was associated with an 8.6-fold greater release of total muscle CK. Total CK loss was calculated at 54.3±6.6 IU g^{-1} in ionophore treated muscles compared to 6.5±3.1 IU g^{-1} in the controls. Despite the dramatic elevation in muscle CK efflux following treatment with ionophore, the total enzyme loss constitutes only 2.38% of total muscle CK activity.

The effect of 4Br-A23187 on muscle lactate release is shown in **Figure 7.3.** Treatment with the ionophore produced a 47.2% increase ($p<0.05$) in the rate of lactate release, which was sustained throughout the remainder of incubation time. This increase constituted a 26.4% greater total lactate release ($p<0.05$) in the ionophore treated muscles compared to ethanol treated controls.

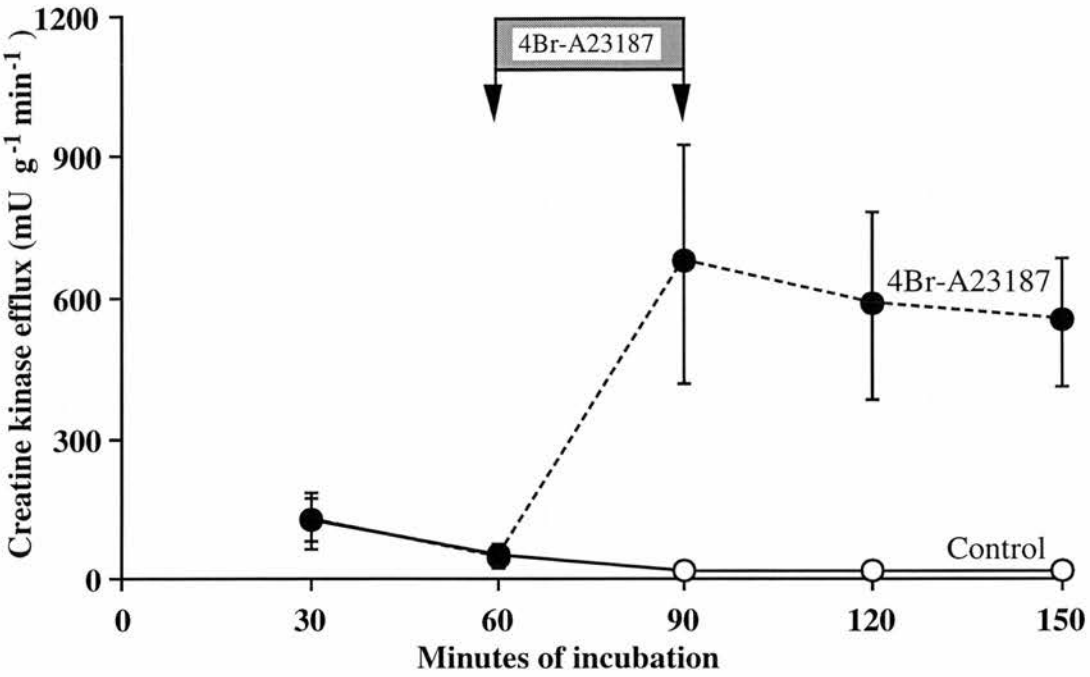

Figure 7.1 Effect of 4Br-A23187 calcium ionophore on the rate of CK efflux from incubated isolated chicken *tensor patigialis* muscle. Treatment muscles were incubated with ionophore (25 µmol litre^{-1}) for 30 minutes, following 60 minutes of incubation in control medium, then returned to control medium for the remainder of the experiment. Values represent means ± S.D for 8 muscles.

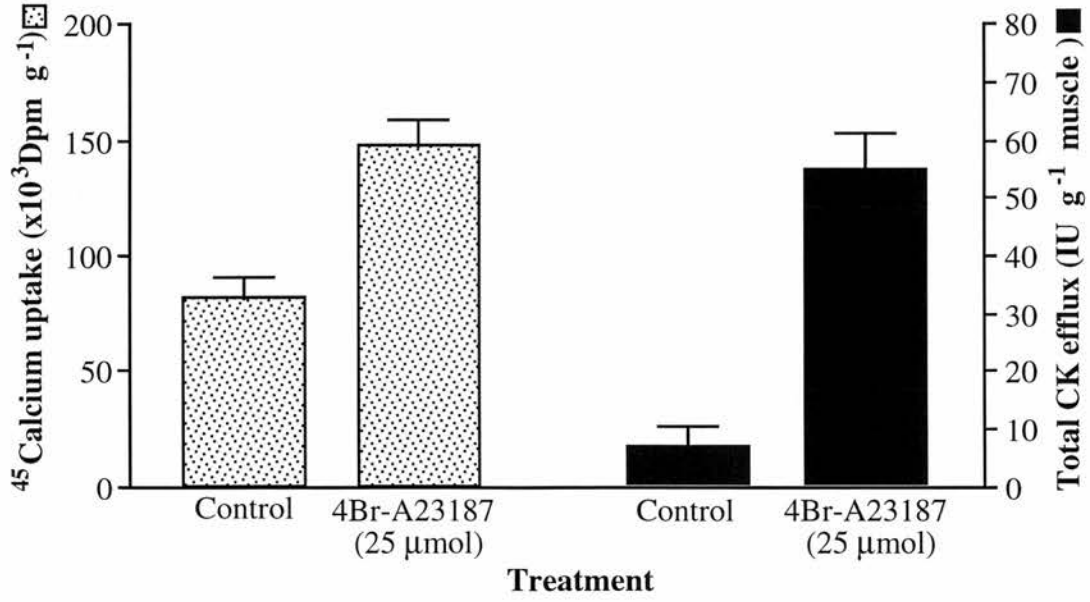

Figure 7.2 Effect of 4Br-A23187 calcium ionophore (25 µmol litre^{-1}) on muscle ^{45}calcium uptake and total creatine kinase (CK) efflux. Values represent means ± S.D for 8 muscles per treatment.

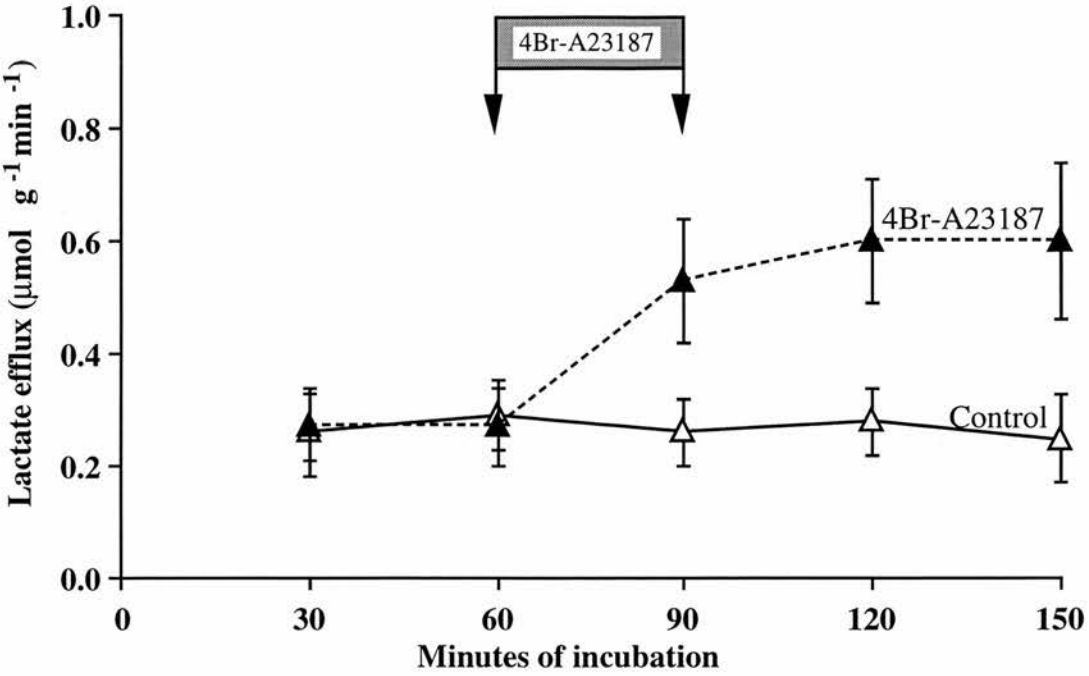

Figure 7.3 Effects of calcium ionophore (4Br-A23187) on the rate of lactate efflux from incubated isolated chicken *tensor patigialis* muscle. Treatment muscles were incubated with ionophore (25 µmol litre^{-1}) for 30 minutes, following 60 minutes of incubation in control medium, then returned to control medium for the remainder of the experiment. Values represent means ± S.D for 8 muscles.

Discussion and conclusions

It is clear from the results in **Figure 7.1** that increasing muscle calcium content by incubation with 4 Br-A23187 calcium ionophore had a profound effect on the release of muscle CK. The effects of 4Br-A23187 on the release of CK from isolated chicken skeletal muscle are similar to those reported in previous studies by Jones *et al.*(1984), Duncan and Jackson (1987) and Phoenix *et al.*(1989) on isolated mouse skeletal muscle using the calcium ionophore A23187. The results of this study support previous suggestions that sustained increases in external Ca^{2+} entry may play an important role in the development of skeletal muscle damage and subsequent CK loss. Further evidence to support the role of calcium in the mechanism of muscle CK release is illustrated in **Figure 7.2**, which clearly demonstrates a relationship between increased muscle Ca^{2+} accumulation (as measured by the uptake of radio-labelled ^{45}calcium) and elevated muscle CK release.

A striking observation of this experiment is the difference in the magnitude of the effect of 4Br-A23187 calcium ionophore on muscle CK and lactate release in comparison to effects of 2, 4 DNP (**Experiment VI**) reported in the previous chapter (**see Chapter 6, Figures 6.3 and 6.4**). Although it is apparent that the effect of

4Br-A23187 was clearly toxic to muscle cells causing significant damage as reflected by the large release of muscle CK. The metabolic consequences of the ionophore on oxidative energy metabolism were substantially lower than those caused by respiratory chain uncoupling with 2, 4 DNP. The effect of treatment with 4Br-A23187 on muscle CK efflux was approximately 8.2-fold greater than that induced by treatment with 2,4 DNP. This might suggest that despite a marked inhibition of oxidative metabolism by 2,4 DNP, the effects upon cellular ion balance (in particular intracellular Ca^{2+} concentration) may have been relatively low in comparison to the large effect on Ca^{2+} influx caused by treatment with 4 Br-A23187. With the benefit of hindsight, it is perhaps unfortunate that Ca^{2+} uptake was not measured in the earlier experiments.

In contrast to the effect of 4 Br-A23187 on CK release, the effect of ionophore on muscle lactate release (**see Figure 6.3**) was approximately 4.5-fold less that observed in 2,4 DNP treated muscles (**see Chapter 6, Figure 6.4**). Given that 2,4 DNP produced an inhibition of oxidative metabolism as evidenced by a very large increase in muscle lactate release, which suggests a stimulation of glycolysis. It might be concluded that the cellular energy available through glycolytic metabolism was sufficient to support those ATP-dependent transport processes required to maintain intracellular calcium homeostasis, such that the disturbances in intracellular Ca^{2+} concentration were much less during under conditions of metabolic inhibition than during treatment with calcium ionophore. Despite a contrast in the magnitude of the effect of these two treatments on oxidative metabolism , it is clear that treatment with 4Br-A23187 still caused a significant increase in lactate release. This would suggest that the increase in muscle Ca^{2+} accumulation consequent upon treatment with ionophore, may have had an effect on oxidative muscle metabolism possibly through the inhibition of the muscle mitochondria. This hypothesis is consistent with reports from other studies in which cells exposed to treatment with calcium ionophore (ionomycin) demonstrated an increase in the amount of Ca^{2+} taken up by the mitochondria (Richelmi *et al.*, 1989; Gunther and Pfeiffer, 1990; Bellomo *et al.*, 1991). It has been demonstrated by Gunther and Pfeiffer (1990) in studies on isolated mitochondria that mitochondrial membrane potentials decreased proportionally to the amount of Ca^{2+} taken up by the mitochondria, which in turn proportionally decreased mitochondrial ATP synthesis.

It is therefore clear that raising skeletal muscle intracellular Ca^{2+} concentration to a high level by treatment by a calcium ionophore released a large amount of enzyme from the cell. This probably occurs due to calcium-induced changes in muscle cell membrane (sarcolemmal) permeability (Soybell *et al.*, 1978), with little effect upon oxidative metabolism as reflected by the small increase in lactate production through

glycolysis. This is in contrast to the effect of 2,4 DNP where inhibition of oxidative phosphorylation substantially stimulated glycolytic metabolism and dramatically increased muscle lactate release but had little effect on total CK efflux, presumably because intracellular calcium was not raised to the same extent as that induced by treatment with 4Br-A23187. On this evidence it seems likely that the important step in the induction of muscle CK release would appear to be an elevation of intracellular calcium, and not an inhibition of oxidative metabolism *per se*.

Comparison of muscle calcium uptakes with previous isolated skeletal muscle studies

Whilst it might be proposed that the determination of muscle calcium from the uptake of radio-labelled ^{45}calcium (expressed in radio-active disintegrations per minute per gram of muscle) is perfectly valid as a unit of calcium measurement. It was recognised that it would be useful to convert values of Ca^{2+} uptake measured in Dpm g^{-1} into standard units of Ca^{2+} measurement (e.g. μmol g^{-1}). This would facilitate comparison of values with other studies where tissue calcium was measured by more conventional methods of ion analysis such as atomic absorption spectrometry (Claremont *et al.* 1984; Jackson *et al.*, 1987; Phoenix *et al.*, 1989). The degree of calcium uptake required to induce CK release can be easily estimated from the uptake of ^{45}calcium coupled with a knowledge of the medium total calcium concentration which in this study was 1.8 mmol litre^{-1} (**for calculation see Appendix IV**).

Before any valid comparisons of calcium uptake could be made, it was necessary to determine what proportion of the Ca^{2+} taken up by the muscles was intracellular, and what proportion was present in the extracellular space. This was achieved by incubating a group of eight muscles in the presence of a highly specific Ca^{2+} chelating agent 1, 2 bis(2-aminophenoxy)ethane- N, N, N', N' tetracetic acid (BAPTA, 5 mmol litre^{-1}). The BAPTA treated muscles were incubated in addition to the control and calcium ionophore treatment muscles (results not shown). At the concentration of chelator used, virtually all of the Ca^{2+} present in the incubation medium was complexed and therefore unavailable for translocation across the muscle plasma membrane into the intracellular compartment. Estimates of muscle Ca^{2+} uptake calculated as Dpm g^{-1} and μmol g^{-1} are shown in **Table 7.1**. It is clear from these values that over half (54%) of the calcium taken up by the control incubated muscles was present in the extracellular compartment as evidenced by the values obtained for the BAPTA treated muscles. Correcting for this puts estimates of rates of Ca^{2+} entry specific to intracellular Ca^{2+} uptake over the 150 min experimental period at 37.9 x10^3

Dpm g^{-1} or 0.54 μmol g^{-1} for the control muscles and 103.4 x10^3 Dpm g^{-1} or 1.48 μmol g^{-1} for muscles treated with 4Br-A23187 ionophore.

In a study by Claremont *et al.*(1984), the extracellular space in mouse skeletal muscle was calculated as approximately 28% of muscle mass, as determined by [^{3}H]inulin. A corresponding estimate of 32% has been reported by Mitchell and Smith (1991) for chicken muscle utilising the same technique.

Table 7.1 Calcium uptake in control, BAPTA (5 mmol litre^{-1}) and 4Br-A23187 (25 μmol litre^{-1}) treated muscles, expressed in x10^3 Dpm g^{-1} and μmol g^{-1}. Values represent means ± S.D. for 8 muscles per treatment.

	Estimate of calcium uptake	
Treatment	x10^3 Dpm g^{-1}	μmol g^{-1}
Control	82.0 ±8.3	1.18 ±0.12
BAPTA (5 mmol litre^{-1})	44.1 ±4.4	0.64 ±0.07
4-Br A23187 (25 μmol litre^{-1})	147.5 ±11.0	2.12 ±0.16

In studies by Jackson *et al.* (1987) and Phoenix *et al.* (1989) average rates of Ca^{2+} uptake were calculated at between 41.6 and 53.3 nmol g^{-1} dry weight min^{-1} in isolated mouse skeletal muscle following 30 minutes incubation with calcium ionophore A23187. This compares with a value of 19.6 nmol g^{-1} dry weight min^{-1} Ca^{2+} influx calculated in the present study (based on muscle dry weight = 25% of muscle wet weight). It is clear from this comparison that the estimates of rates of Ca^{2+} uptake were reasonably similar between studies. This serves to corroborate earlier claims made at the beginning of this section of the validity of determining calcium from radio-labelled ^{45}calcium uptake. Whilst the conversion from Dpm g^{-1} to μmol g^{-1} is useful for comparing muscle calcium uptakes with other studies, for most other purposes it is clear that measuring radio-labelled ^{45}calcium uptake (expressed as Dpm g^{-1}) is perfectly adequate as an quantitative measurement of total muscle calcium accumulation.

Chapter 8

The effect of inhibition of ionophore-induced Ca^{2+}-activated phospholipase A_2 (PLA_2) activity on CK release from avian skeletal muscle

Introduction

The possible manner in which perturbations in intracellular calcium regulation may mediate cell damage in skeletal muscle and other cell types has been the subject of much investigation in recent years. It is now well recognised that at high concentrations, Ca^{2+} is a potent activator of several degradative enzyme pathways which are involved in the catabolism of intracellular constituents such as proteins, phospholipids and nucleic acids (Murachi, 1983; Campbell, 1987; Arends *et al.*, 1990; Nicotera *et al.*, 1992). Such sustained increases in intracellular Ca^{2+} concentration above "normal physiological levels" may therefore result in an uncontrolled breakdown of intracellular macromolecules vital to maintaining normal cell structure and function. Previous studies examining the effects of Ca^{2+} on cardiac and skeletal muscle damage have suggested that increases in intracellular Ca^{2+} may trigger a series of events that ultimately leads to a condition of enhanced enzyme leakage. However, the exact site or sites of calcium action have yet to be fully established (Jackson *et al*, 1984; Jones *et al.*, 1986). Given that over 90% of CK present in skeletal muscle is found in the muscle sarcoplasm (Farrell and Baba, 1980), it has been hypothesised that increases in its release may reflect changes in muscle membrane (sarcolemma) permeability or integrity (Soybell *et al.*, 1978; Jackson *et al.*, 1987).

It recognised that there is family of Ca^{2+}-dependent enzymes that plays an important physiological role in many cell and tissue types known as the phospholipases (Dennis, 1983). These enzymes share the common property of catalysing the hydrolysis of phospholipids, a primary constituents of all biological cell membranes. A sub-group of phospholipases, collectively designated phospholipase A_2 (PLA_2) have recently received much attention due to their reported effects on cell plasma membranes (Nicotera *et al.*, 1992). It has long been known at a that the primary physiological function of PLA_2 was to facilitate the turnover of fatty acids in membrane phospholipids (Van Deenen, 1965), in particular to regulate the release of arachidonic acid (AA) from phospholipids for eicosanoid biosynthesis (Exton, 1990). PLA_2 has also been implicated in the detoxification of phospholipid hydroperoxides because of its ability to preferentially hydrolyse peroxidised fatty acid esters in phospholipid membranes (Van Kuijk *et al.*, 1987).

Due to the fact that PLA_2 represents a Ca^{2+} and calmodulin-dependent family of enzymes it is susceptible to activation after elevations in cytosolic calcium concentration. If this increase in Ca^{2+} is sustained, the activation of PLA_2 can result in extensive membrane breakdown and the generation of toxic metabolites (Nicotera *et al.*, 1992). Evidence of Ca^{2+}-dependent activation of PLA_2 has been reported by several workers during ischaemic and anoxic injury in isolated rat ventricular myocytes (Chien *et al.*, 1979; Sulieman *et al.*, 1992) and hepatocytes (Farber, 1981). Cellular membrane damage associated with Ca^{2+}-mediated activation of PLA_2 activity has also been reported by Rice *et al.* (1990) using the calcium ionophore A23187 in isolated rat type I and II alveolar epithelial cells. In addition to these in studies, there has also been extensive demonstration of the myotoxic effects of exogenous PLA_2 activity in snake venom on skeletal muscle (Gutierrez *et al.*, 1990, 1991; Fletcher *et al.*, 1991; Ownby *et al.*, 1993; Bultron *et al.*, 1993).

In order to investigate the effects of PLA_2 on cell membranes, many workers have employed the use of a variety of PLA_2 inhibitors such as quinacrine (mepacrine), p-bromophenacyl bromide (p-BPB) and various other long chain alkylamines (Fletcher *et al.*, 1991). These compounds have been shown to reduce wide range of PLA_2 mediated effects such as acute lung injury (Bachofen and Weibel, 1977) erythrocyte hemolysis (Bell and Majeros, 1979; 1980) and prostaglandin E_2 mediated skeletal muscle proteolysis and contracture (Rodemann *et al.*, 1982). Recently, a novel highly specific synthetic PLA_2 inhibitor has been developed (Ro-31-4494/001) which has been shown to substantially reduce intracellular protein and enzyme loss isolated guinea pig ventricular myocytes during Ca^{2+} repletion (Sulieman *et al.*, 1992).

Experimental aims

The aim of work presented in this chapter was to examine the effects of PLA_2 inhibition on CK release from isolated chicken skeletal muscle (**Experiment VIII**) using the novel PLA_2 inhibitor Ro 31-4493/001 (Sulieman *et al.*, 1992) Experimental muscle damage was induced using the calcium ionophore 4-Br-A23187 as has been previously demonstrated in **Chapter 7**.

Materials and methods

All muscles used in this experiment were incubated and processed as according to the experimental protocol outlined in **Chapter 4** with the inclusion of radiolabelled $^{45}CaCl_2$ in the incubation medium (**see Chapter 7**). The phospholipase A_2 inhibitor Ro-31-4493/001 (Ro-31) was kindly donated by Dr G Lawton (Roche

Research Centre, Welwyn Garden City, Hertfordshire, UK.). A stock solution of the inhibitor (50 μmol litre^{-1}) was prepared in 90 % ethanol and added to half of the labelled incubation medium. An equal volume of 90 % ethanol was also added v/v to the control medium stock. 4-Br-calcium ionophore was prepared in 90 % ethanol as previously described in **Chapter 7**.

All muscles were initially incubated under control conditions (no PLA_2 inhibitor or calcium ionophore) for the first 2 x 30 minutes. After this time, muscles were incubated in medium containing Ro-31-4493/001 (50 μmol litre^{-1}) or an equivalent volume of ethanol for the remainder of the experiment (3 x 30 minutes). Immediately following the initial 2 x 30 minute incubation, the muscles received either 0 (ionophore control), 5 or 25 μmol litre^{-1} calcium ionophore solution for 30 minutes in order to induce varying degrees of experimental muscle damage (**see Chapter 7**).

All muscles were incubated according to the incubation protocol previously described in **Chapter 4**. CK activities in the incubation medium and muscle radio-labelled ^{45}calcium uptakes were determined by the methods described in **Chapter 2**. Data are presented as means ± S.D. Levels of significance were determined by analysis of variance or unpaired Students t-test.

Results

No difference in the rate of CK release was observed between either of the two groups of muscles incubated with or without Ro-31 in the absence of any calcium ionophore administration (**see Figure 8.1**). These rates of CK release being identical to those reported in the preliminary baseline CK experiment (**see Chapter 4**). Incubation with 4Br-A23187 at 5 μmol litre^{-1} produced significant increases in the rates of CK loss from muscles incubated with or without PLA_2 inhibitor of 16.7-fold and 10.4-fold , respectively (**See Figure 8.2**). The difference in magnitude of the increase in the muscles incubated with Ro-31 was significantly lower (34.5%; $p<0.05$) than that observed in muscles incubated without the PLA_2 inhibitor. Even greater increases in the rates of CK loss were observed in muscles incubated with (18.3-fold) or without (22.2-fold) PLA_2 inhibitor following incubation with 4Br-A23187 at 25 μmol litre^{-1} (**See Figure 8.3**). However, the 13.3% reduction in the rate of CK release observed in the Ro-31 treated muscles at this concentration of ionophore failed to reach significance at $p<0.05$.

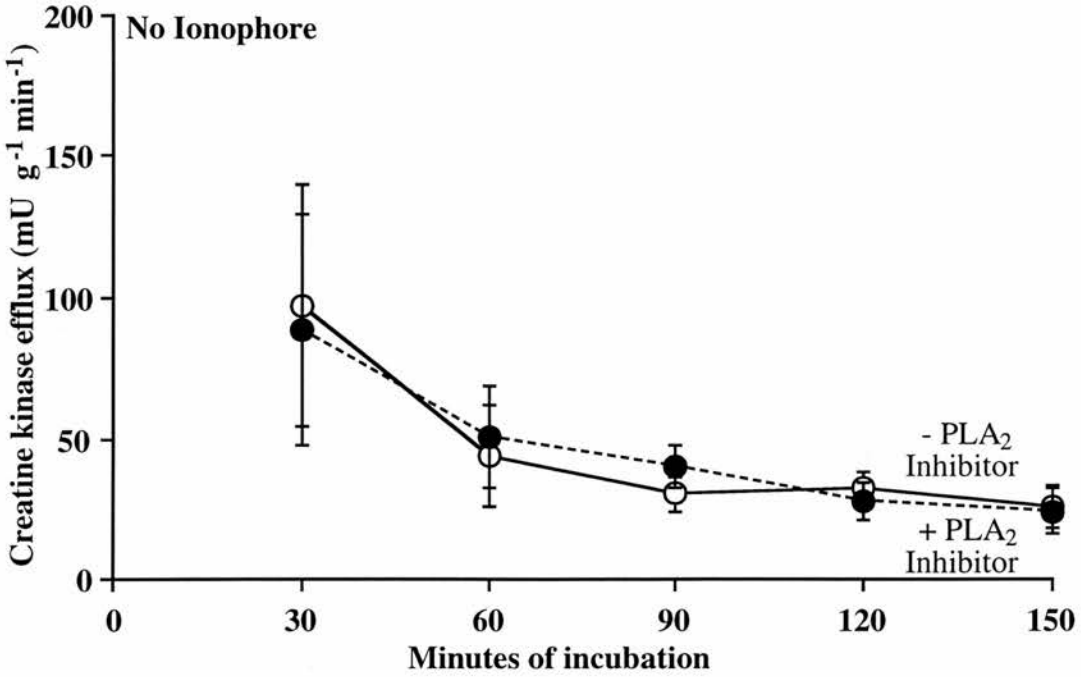

Figure 8.1 Effect of incubation with (+) or without (-) Ro-31 PLA_2 inhibitor (50 μmol litre^{-1}) on the rate of CK release from isolated chicken *tensor patigialis* muscle. Muscles were incubated with Ro-31 for 3 x 30 minutes after 60 minutes of incubation in control medium. Values represent means ± S.D for 8 muscles per treatment.

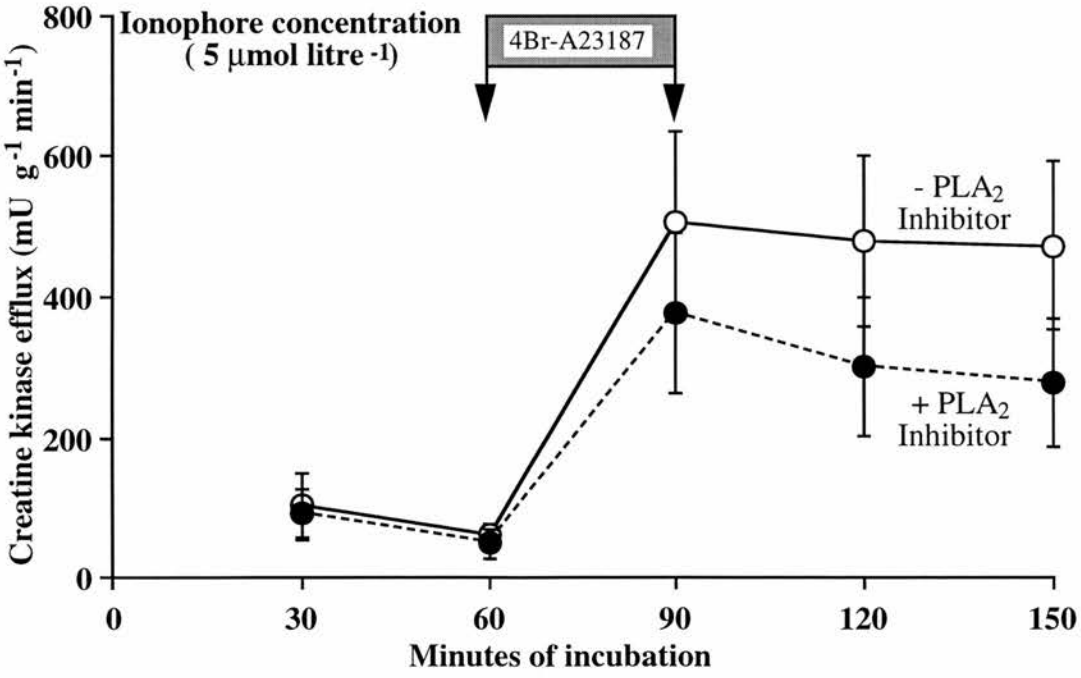

Figure 8.2 Effect of incubation with (+) or without (-) Ro31 PLA_2 inhibitor (50 μmol litre^{-1}) on the rate of CK release from isolated chicken *tensor patigialis* muscles, following incubation with 4Br-A23187 (5 μmol litre^{-1}). Muscles were incubated with PLA_2 inhibitor for 3 x 30 minutes following 60 minutes of incubation in control medium. Values represent means ± S.D for 8 muscles per treatment.

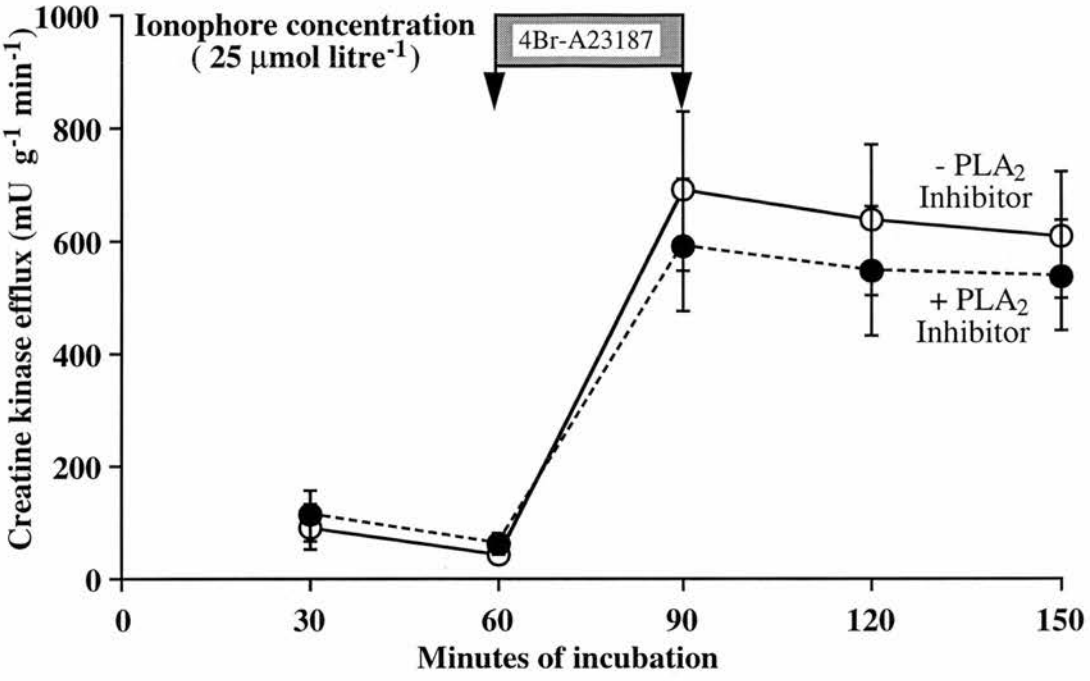

Figure 8.3 Effect of incubation with (+) or without (-) Ro31 PLA_2 inhibitor (50 µmol litre^{-1}) on the rate of CK release from isolated chicken *tensor patigialis* muscles, following incubation with 4Br-A23187 (25 µmol litre^{-1}). Muscles were incubated with PLA_2 inhibitor for 3 x 30 minutes following 60 minutes of incubation in control medium. Values represent means ± S.D for 8 muscles per treatment.

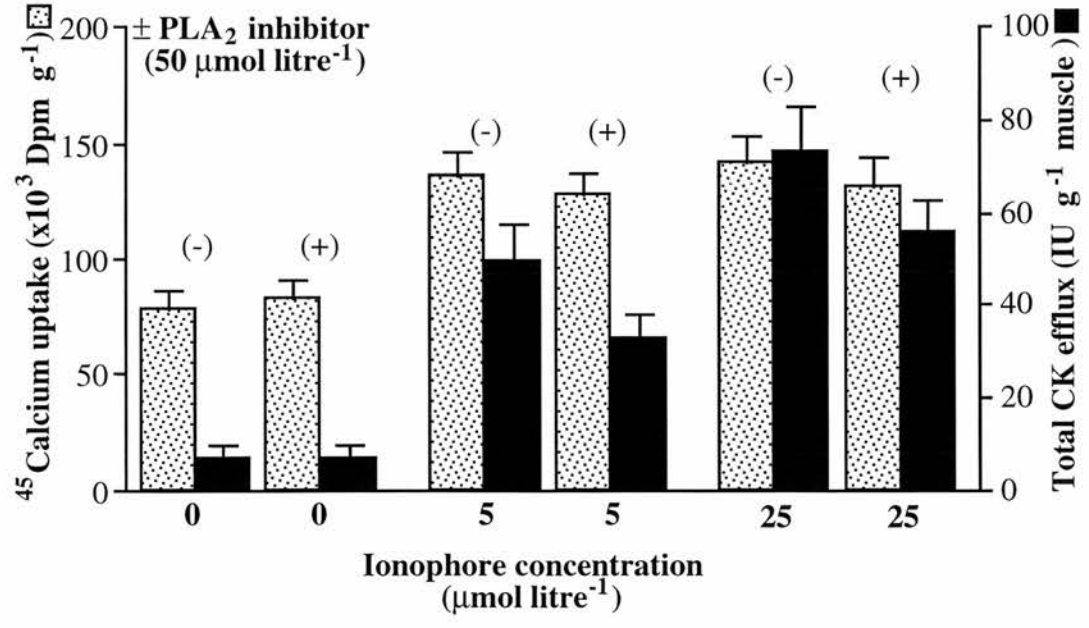

Figure 8.4 Effect of incubation with (+) or without (-) Ro31 PLA_2 inhibitor (50 µmol litre^{-1}) on muscle ^{45}calcium uptake and total CK efflux, following incubation with two concentrations of 4Br-A23187 calcium ionophore (5 and 25 µmol litre^{-1}). Values represent means ± S.D for 8 muscles per treatment.

The effects of incubation with or without Ro-31 on muscle ^{45}calcium uptake and total CK efflux are illustrated in **Figure 8.4**. The effect of the PLA_2 inhibitor appeared to have no effect on ^{45}calcium uptake in muscles treated without 4Br-A23187 or at either of the two ionophore concentrations used compared to respective non inhibitor incubated controls. The magnitude of the increases in ^{45}calcium uptake observed in the two groups of ionophore treated muscles were essentially the same despite signs of a slight reduction in the Ro-31 treated muscles. These increases reflecting elevations in ^{45}calcium uptake of 72.2% and 72.5% in the 5 and 25 μmol litre^{-1} calcium ionophore treated muscles respectively. In contrast, treatment with the PLA_2 inhibitor Ro-31 produced marked effects on total muscle CK loss in the ionophore treated muscles. Whilst no effect on CK release was observed in non-ionophore treated muscles, Ro-31 significantly reduced 4Br-A23187 mediated total CK loss by 44.6% ($p<0.001$) at an ionophore concentration of 5 μmol litre^{-1}. A significant reduction in total CK loss (23.8%; $p<0.05$) was also observed in the muscles treated at the higher ionophore concentration of 25 μmol litre^{-1}.

Discussion and conclusions

The aim of this study was to assess the effect of phospholipase A_2 (PLA_2) inhibition on muscle CK efflux, using the novel synthetic PLA_2 inhibitor Ro-31-4439/100. Its effects were examined in the face of a calcium ionophore challenge (4Br-A23187) which causes profound skeletal muscle damage and subsequent CK release see **Chapter 7** of this thesis and work by other researchers (Publicover *et al.*, 1978; Claremont et al., 1984; Jones *et al.*, 1984; Jackson et al., 1987; Phoenix *et al.*, 1989). In addition to this, the present study also evaluated the effects of phospholipase A_2 inhibition on extracellular calcium accumulation as measured by the uptake of ^{45}calcium.

The results of this experiment clearly show that the stimulation of PLA_2 activity in chicken skeletal muscle is implicated in the mechanism of CK release as demonstrated by the effective reduction in calcium ionophore induced CK loss caused by Ro-31 (**See Figures 8.2 and 8.3**) The ameliorating effects of the PLA_2 inhibitor on muscle CK release were consistent with the findings of previous studies examining Ca^{2+}-mediated mammalian cardiac and skeletal muscle injury using Ro-31 and other PLA_2 inhibitors such as mepacrine, dibucaine and chlorpromazine (Jackson *et al.*, 1984, 1985; Phoenix *et al.*, 1990, Sulieman *et al.*, 1992).

On the basis of the CK results presented in this study it might be suggested that Ro-31 inhibits the release of CK in chicken skeletal muscle via the inhibition of PLA_2 activity "down stream" of external calcium entry. However, despite the

constancy of calcium uptake and CK release observed in the muscles incubated at the two ionophore concentrations in the absence of Ro-31 (**see Figure 8.4**), there were marked difference in total CK loss in both groups of ionophore treated in the presence of Ro-31. This observation might suggest that the PLA_2 inhibitor may have an effect on the action of the calcium ionophore in relation to CK release other than through the simple entry of external calcium, upon which Ro-31 seems to be acting. How the PLA_2 inhibitor might affect the action of the ionophore is not clear. One explanation may involve the possible effect of the PLA_2 inhibitor on ionophore mediated calcium release from intracellular Ca^{2+} stores.

In conclusion it is suggested that PLA_2 activation plays a role in the mechanism leading to CK release. It likely that increases intracellular calcium cause the activation of PLA_2, which mediates alteration in plasma membrane integrity leading to enhanced CK efflux and possible further external calcium entry.

Chapter 9

The role of external sodium ions (Na^+) on trans-membrane Ca^{2+} flux and its effect on the release of CK from avian skeletal muscle

Introduction

The inability to maintain cellular ion balance has long been favoured as a primary determinant in the development of cellular damage (Trump and Ginn, 1969; Trump and Arstila, 1975; Trump and Berezesky, 1984). It has been suggested that initial disturbances in cellular Na^+ homeostasis may play a key role in the development of cell damage or injury through its interaction with other ions such as Ca^{2+} (Blaustein, 1974; Piper, 1988, Trump *et al.*, 1989). Several studies on isolated rat ventricular myocytes have demonstrated an increase in cell damage was caused by raised intracellular Na^+ mediated shifts in Ca^{2+} entry following the on-set of ischaemia (Osornio-Vargas *et al.*, 1981) and during metabolic inhibition (Eisner *et al.*, 1989). Similarly in other cell damage studies on human epidermoid A431 cells increases in calcium uptake have been shown to be mediated by an initial increases in Na^+ infux flowing heat shock exposure and metabolic inhibitors (Kiang *et al.*, 1992; Kiang and Smallridge, 1994). Whether the same mechanisms might operate in chicken skeletal muscle under conditions of heat stress exposure is not yet known.

It is recognised however, that a muscle myopathy exists that has been described in poultry and other species caused by the administration of monensin, which causes alterations in intracellular Na^+ balance that lead to disturbances in intracellular Ca^{2+} homeostasis (Van Vleet, 1983a, b; Dowling, 1992). Monensin is a polyether-carboxylic ionophore that is capable of collapsing Na^+ gradients that exist across cell membranes through its action of Na^+ selective transport across cell membranes (Ledger and Tanzer, 1984). Monensin has long been used in the field of veterinary medicine as a dietary supplement for the prevention of coccidiosis (Shumard and Callender, 1968). Muscle damage (myopathy) and necrosis associated Na^+ mediated changes in extracellular Ca^{2+} uptake following with monensin treatment have been reported by Sheir and Dubourdieu (1992) in isolated rat cardiomyocytes. Extensive examination of the effects of monensin on Na^+ and Ca^{2+} movements have been described by Hoya and Venosa (1992; 1994) in isolated frog skeletal muscle but not in relation any possible pathological effect on skeletal muscle. Other studies using monensin have also demonstrated Na^+-mediated changes in Ca^{2+} flux at a physiological level in isolated rabbit aorta (Ozaki *et al.*, 1982), facial vein (Herion *et*

al., 1994) and rat diaphragm (Zavecz and Anderson, 1992) and sertoli cells (Gorczynska and Handelsman, 1993).

Experimental aims

The aim of the present study was to examine the role of sodium ions (Na^+) on trans-membrane Ca^{2+} fluxes and CK release from avian skeletal muscle, using the Na^+ ionophore monensin (**Experiment IX**). As an aside to the *in vitro* investigation undertaken and presented in this chapter, a separate in vivo experiment was also set-up to examine the possible damaging effects of dietary monensin supplementation on broiler chicken skeletal muscle. The results of the *in vivo* study (**Experiment XVII**) are presented later in this thesis in **Chapter 11**.

Materials and methods

Monensin Na^+ ionophore was dissolved in methanol and added to four stock solutions of medium 199 to give final monensin concentrations of 25, 50, 100 and 200 μmol $litre^{-1}$. All stock solutions (including control) were then adjusted to contain 2% methanol by volume. Monensin treated muscles were initially incubated under the same conditions as the control muscles for 2 x 30 minutes. After this period, monensin treated muscles were incubated for 3 x 30 minutes in medium containing the ionophore at one of the four reported concentrations. Control muscles were incubated in control medium throughout.

All muscles were incubated and processed according to the experimental protocol described in Chapter 4. Isotope-labelling of the medium with ^{45}calcium and the methods for the determination of ^{45}calcium uptakes and CK efflux activities were the same as those outlined in Chapter 7. Data are presented as means ± S.D. Levels of significance were determined by analysis of variance or unpaired Students t-test.

Results

The results illustrated in **Figure 9.1** clearly demonstrate that with increasing concentrations of monensin Na^+ ionophore there was a corresponding increase in both muscle ^{45}calcium uptake and total CK efflux. The largest and maximal responses (108.2±9.2 $x10^3$ Dpm g^{-1} and 17.1±1.2 IU g^{-1}) occurring at monensin concentrations of 100 μmol or greater. At a monensin concentration of 100 μmol $litre^{-1}$ there was an 49.4% increase ($p<0.05$) in muscle ^{45}calcium uptake and an 2.4-fold increase ($p<0.001$) in total CK efflux, compared with control values.

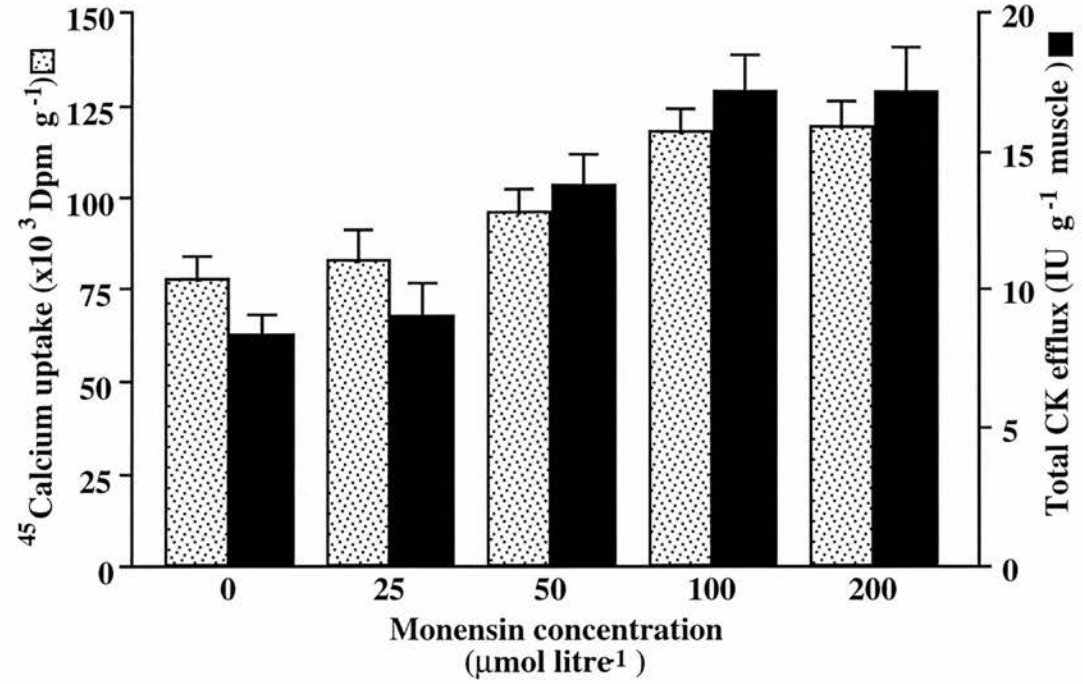

Figure 9.1 Effect of increasing monensin sodium ionophore (25-200 μmol litre^{-1}) on muscle ^{45}calcium uptake and total creatine kinase (CK) efflux. Values represent means ± S.D for 6 muscles at each concentration of ionophore.

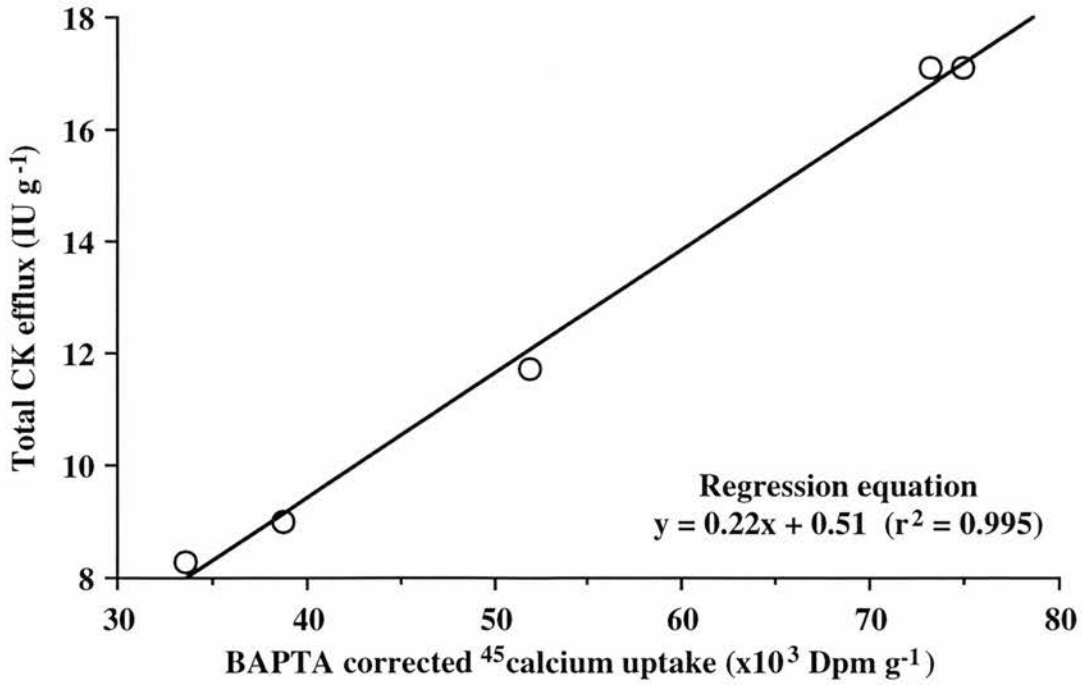

Figure 9.2 Regression analysis of total CK efflux and BAPTA corrected ^{45}calcium uptake in muscles incubated with increasing concentrations (50-200 μmol litre^{-1}) of monensin Na^+ ionophore.

Figure 9.2 shows the linear correlation between total muscle CK efflux and BAPTA corrected ^{45}calcium uptake for the range of monensin concentrations examined. Regression analysis showed a significant positive correlation ($r^2 = 0.995$) between the two variables measured. These findings suggest that increases in intracellular Na+ concentration ($[Na^+]_i$), mediated by monensin ionophore can cause an increase in ^{45}calcium accumulation and subsequent muscle damage as reflected by the loss of CK. However, the same results might be obtained if, despite of its reported specificity for Na^+, monensin was acting as a Ca^{2+} ionophore. In the absence of the measurement of $[Na^+]_i$ it is not possible to say with any certainty, whether monensin exerted its effects through its recognised action as a Na^+ ionophore or through direct Ca^{2+} entry. In order to clarify this point, two additional experiments were undertaken to further clarify the potential role of Na^+ in the muscle damage process.

The effects of elevated extracellular Na^+ concentration (Experiment X)

If monensin exerts its effect on ^{45}calcium uptake through Ca^{2+} entry and not via a process mediated by Na^+ as has been proposed earlier in this chapter, then it should be possible to confirm this by incubating muscles with monensin in medium containing a higher concentration of Na^+. Elevating the incubation medium Na^+ concentration would increase the amount of Na^+ available for translocation into the muscle by monensin but would leave the amount of extracellular Ca^{2+} available for uptake the same. Therefore if there was no direct effect of monensin on Ca^{2+} entry there should be no increase in ^{45}calcium uptake. This hypothesis was tested in **Experiment X**.

Na^+ concentration was increased in the incubation medium by the addition of NaCl to give a final medium Na^+ concentration of 225 mmol litre^{-1} (a 50% increase). Monensin stock solutions were made-up as previously described in the materials and methods section of this chapter. Elevated Na^+ treatment muscles were initially incubated under the same conditions as the control muscles for the 2 x 30 minutes in medium containing a Na^+ concentration at 150 mmol litre^{-1}, then incubated in medium at the higher Na^+ concentration for the remainder of the experiment (3 x 30 minutes). Control muscles were incubated in medium containing Na^+ at 150 mmol litre^{-1} throughout. The effect of raised extracellular Na+ concentration on monensin-induced muscle ^{45}calcium uptake and CK release is shown in **Figure 9.3**. In the control muscles, ^{45}calcium uptakes and total CK losses were the same as those reported in the initial dose response experiment shown in **Figure 9.1**. Incubation at the higher Na^+ concentration in the absence of monensin had no effect on either ^{45}calcium uptake or

total CK loss above that observed for control muscles. In muscles incubated with monensin (100 μmol litre^{-1}) there was a predicted increase in both ^{45}calcium uptake and total CK efflux comparable with values obtained from muscles incubated at the same monensin concentration in **Experiment IX**, representing increases in ^{45}calcium uptake and total CK loss of 56.9% ($p<0.05$) and 2.6-fold ($p<0.001$) respectively. Muscles incubated with monensin in medium at the higher Na^+ concentration demonstrated a significant increase in ^{45}calcium uptake (17.7 %; $p<0.05$) and total CK loss (23.9%; $p<0.05$) compared with muscles incubated only with ionophore.

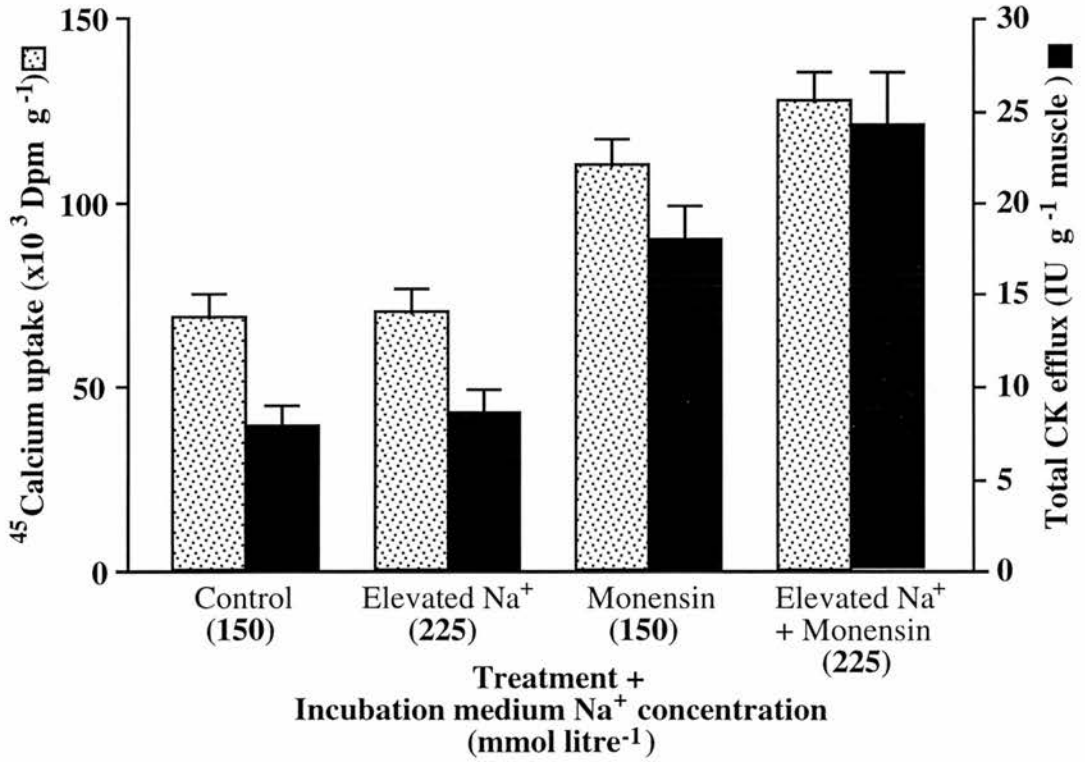

Figure 9.3 Effects of elevated incubation medium Na^+ concentration (225 mmol litre^{-1}) on ^{45}calcium uptake in and total CK efflux from muscles treated with monensin Na^+ ionophore (100 μmol litre^{-1}). Values represent means ± S.D. for 8 muscles per treatment.

The effects of Na^+/K^+ ATPase inhibition (Experiment XI)

In addition to augmenting intracellular Na^+ loading by incubating monensin treated muscles in medium at a higher Na^+ concentration, it is also possible to raise intracellular Na^+ concentration by preventing Na^+ extrusion activity. This can be achieved by directly inhibiting the processes that mediate intracellular Na^+ homeostasis. In virtually all cells, Na^+ concentration is specifically regulated at the level of the cell membrane (plasmalemma) by the action of several intrinsic membrane

exchange proteins (ion-motive ATPases) which extrude Na^+ in favour of the uptake other ions such as K^+, H^+ and Ca^{2+} (Trump and Berezesky, 1984). The primary exchange protein responsible for the maintenance of Na^+ homeostasis is the Na^+/K^+ exchanger (Na^+/K^+ ATPase), which extrudes Na^+ in favour of the uptake of K^+ and accounts for between 70-90% of Na^+ translocation in virtually all cells under normal resting conditions (Smith *et al.*, 1989). It has long been recognised that it is possible to inhibit the action of the Na^+/K^+-ATPase using the cardiac glycoside, ouabain (Bowman and Rand, 1982). Therefore, the objective of this second supplementary experiment was to investigate the effects of ouabain-mediated Na^+/K^+ ATPase inhibition on ^{45}calcium uptake in and CK release from muscles treated with monensin Na^+ ionophore.

Ouabain was dissolved in medium 199 to give a final concentration of 2 mmol litre^{-1}. As with the other experiments described in this chapter, ouabain and monensin treatment muscles were initially incubated under the same conditions as the control muscles for the 2 x 30 minutes, then incubated with inhibitor and/or ionophore for the remainder of the experiment (3 x 30 minutes). Control muscles were incubated in control medium throughout the experiment.

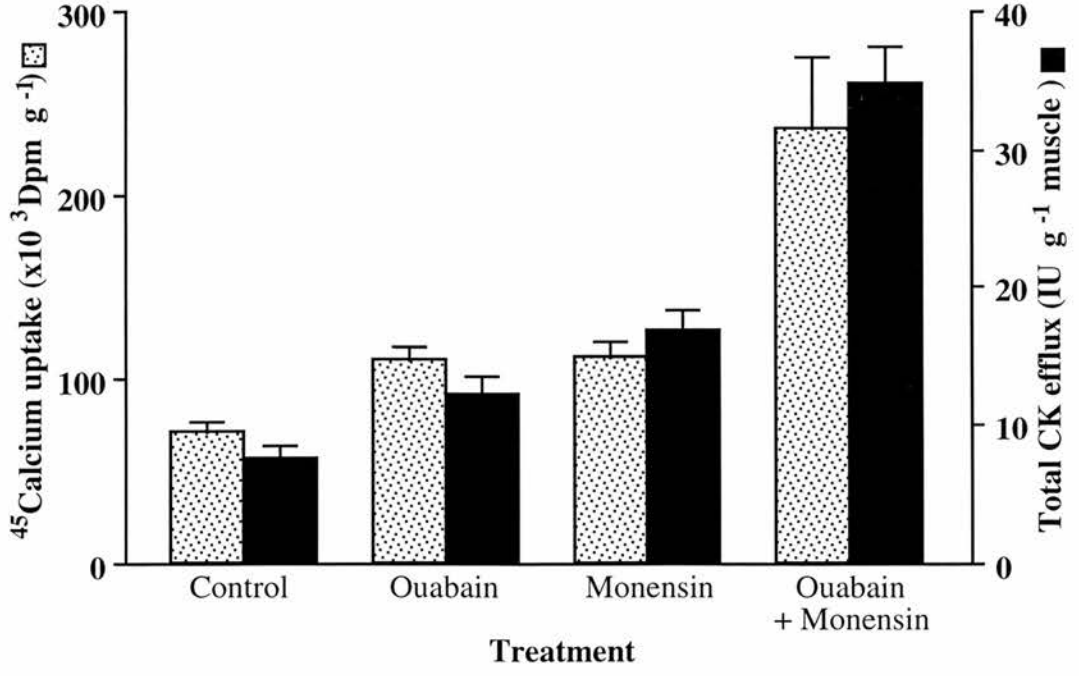

Figure 9.4 Effect of ouabain (2 mmol litre^{-1}) on 45calcium uptake and total CK efflux in muscles treated with monensin Na+ ionophore (100 µmol litre^{-1}). Values represent means ± S.D. for 8 muscles per treatment.

The effects of ouabain on monensin-treated muscles are illustrated in **Figure 9.4.** Muscles incubated only with ouabain (2 mmol litre^{-1}) demonstrated a 56.4% ($p<0.05$) increase in ^{45}calcium uptake and a 1.6-fold ($p<0.001$) increase in total CK release. The combined effects of incubation with monensin and ouabain profoundly increased muscle ^{45}calcium uptake and CK loss 1.9 and 2.3-fold ($p<0.001$) respectively, relative to muscles treated only with monensin. Values for control and monensin treatment ^{45}calcium uptakes and CK losses were consistent with previous experiments in this chapter.

From on the findings of **Experiment X** it would appear that monensin-induced Ca^{2+} uptake into muscle cells was Na^+ mediated as originally proposed, and not caused by any direct ionophoretic action of monensin on Ca^{2+}. This is further supported by the results of **Experiment XI** which clearly showed a increase in ^{45}calcium uptake in muscles, following the inhibition of Na^+ extrusion by ouabain, which was greatly increased in the Na^+ loaded (monensin-treated) muscles. The mechanism by which Na^+ may mediate Ca^{2+} uptake might be as a direct consequence of Na^+/Ca^{2+} exchange. In addition to the role the Na^+/K^+ exchanger plays in the regulation of $[Na^+]_i$ it is also well recognised that, intracellular Na^+ concentration can also be mediated via other membrane-localised exchange proteins (Blaustein, 1974; 1977, Reeves, 1990). The Na^+/Ca^{2+} exchanger has the capacity to translocate Na^+ and Ca^{2+} in opposite directions depending on the prevailing Na^+ gradient, such that in Na^+ loaded cells cytoplasmic Na^+ accumulations are rapidly dissipated by the active outward pumping of Na^+ at the expense of inward Ca^{2+} translocation (Smith *et al.*, 1989; Tani and Neely, 1990).

If Na^+ exerts its effect on ^{45}calcium uptake in muscle via Na^+/Ca^{2+} exchange then inhibition of this process may confirm its involvement in the mechanism of muscle damage. This can be achieved by either directly inhibiting the exchange protein (Hoya and Venosa, 1992, 1995) or preventing exchange by reducing extracellular Ca^{2+} availability for translocation.(Tsien, 1980).

The effects Na^+/Ca^{2+} ATPase inhibition (Experiment XII)

Amiloride (N-amidino-3, 5-diamino-6-chloropyrazine-2-carboxamide) and its derivatives 5-(N, N-Dimethyl) and 5-(N, N-Hexamethylene) amiloride are recognised inhibitors of both Na^+/Ca^{2+} and Na^+/H^+ exchange proteins (Kaczoroski *et al.*, 1985, Smith *et al.*, 1985; Kleymann and Cragoe, 1988). Amiloride has been reported to inhibit Na^+/H^+ and Na^+/Ca^{2+} exchange (with a 50% inhibitory concentration) in the between the ranges of 1-10 µmol litre^{-1} and 1-10 mmol litre^{-1} respectively (Northover, 1992). The objective of this experiment was therefore to examine the effects of

amiloride-mediated Na^+/Ca^{2+} exchange inhibition in Na^+ loaded muscles in which normal Na^+/K^+ exchange was inhibited using ouabain (**See Experiment XI**).

Amiloride was solublised in 5% methanol to give a final concentration of 1 mmol litre^{-1}. Again, as with the other experiments described in this chapter, inhibitor treatment muscles were initially incubated under the same conditions as the control muscles for the 2 x 30 minutes, then incubated with ouabain and/or amiloride for the remainder of the experiment (3 x 30 minutes). Control muscles were incubated in control medium throughout the experiment. The effects of amiloride on ouabain-treated muscles are shown in Figure 9.5. There was no difference in either ^{45}calcium uptake or total CK release in muscles treated only with amiloride (1 mmol litre^{-1}) compared to controls (77.7 x10^3 Dpm g^{-1} and 7.9 IU g^{-1}). Similarly there was no apparent effect of the Na^+/Ca^{2+} exchange inhibitor on ^{45}calcium uptake or total CK loss on muscles also treated with ouabain. Again, values for control and ouabain treatment ^{45}calcium uptakes and CK losses were consistent with previous experiments in this chapter.

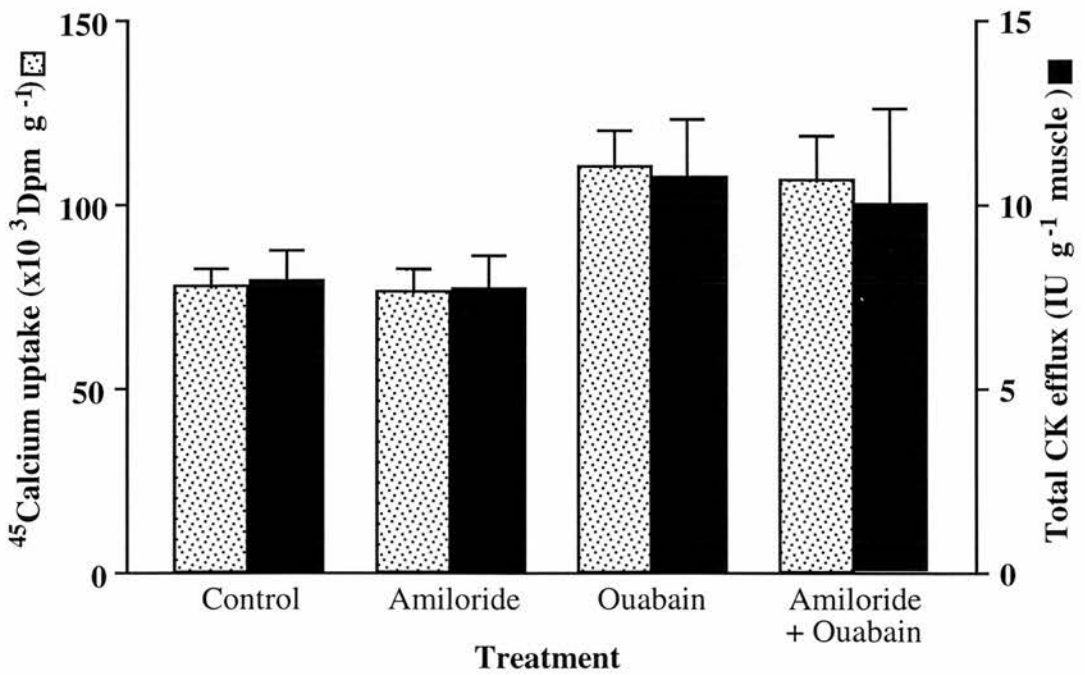

Figure 9.5 Effect of amiloride (1 mmol litre^{-1}) on ^{45}calcium uptake and total CK efflux in muscles treated with ouabain (2 mmol litre^{-1}). Values represent means ± S.D. for 8 muscles per treatment.

The effects of reduced external Ca^{2+} availability (Experiments XIII and XIV)

If the process of ^{45}calcium exchange is involved in the development of muscle damage then it should be also possible to establish any potential role it might play by preventing the translocation of Ca^{2+} for Na^{+} by reducing the amount of external calcium available for exchange. This can be achieved by either incubating muscles in medium made-up without the inclusion of calcium (Ca^{2+} -free medium) or in medium in which the calcium present in it was either complexed or bound. Two experiments were designed to examine the latter of the two aforementioned experimental approaches. Muscles were incubated in medium in which the Ca^{2+} was either complexed with the highly specific calcium chelating agent, BAPTA (**Experiment XIII**) or incubated in medium containing albumin (**Experiment XVI**), the primary Ca^{2+} binding protein found in plasma (Bowman and Rand, 1980; Tsien, 1980).

BAPTA was dissolved in medium 199 to give a final concentration of 5 mmol litre^{-1}. BSA was added (5% w/v) to a separate medium stock. The pH of both incubation solutions was adjusted to 7.4 with 0.1M HCl. As with previous experiments undertaken in this chapter, all muscles were initially incubated under the same conditions as the control muscles for the 2 x 30 minutes, then incubated with BAPTA or BSA, and/or monensin for the remainder of the experiment (3 x 30 minutes).

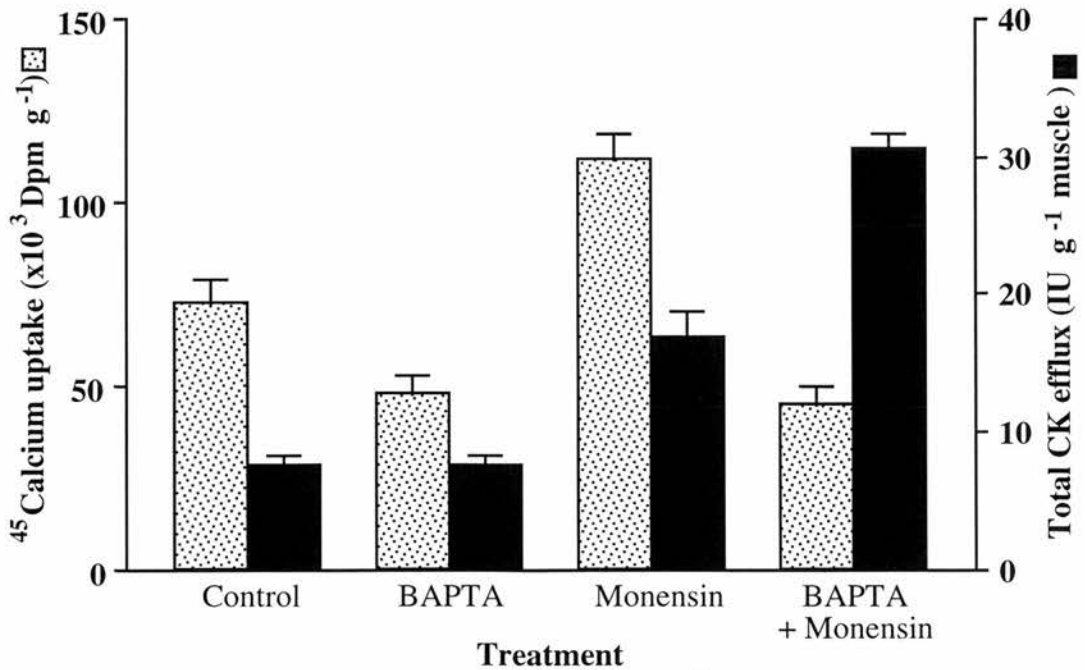

Figure 9.6 Effect of BAPTA (5 mmol litre^{-1}) on ^{45}calcium uptake and total CK efflux in muscles treated with monensin Na^{+} ionophore (100 µmol litre^{-1}). Values represent means ± S.D. for 8 muscles per treatment.

The effects of BAPTA (Experiment XIII)

The effects of BAPTA on monensin-treated muscles are illustrated in **Figure 9.6**. Muscles incubated in medium containing BAPTA (5 mmol litre^{-1}) demonstrated a significant decrease in ^{45}calcium uptake (36.5%; $p<0.05$) compared to control muscles, but had no effect on total CK efflux. Treatment with only monensin induced a increase in ^{45}calcium uptake (54.2 %; $p<0.05$) with a corresponding 2.2-fold increase ($p<0.001$) in total CK loss was consistent with previous results. Muscles incubated in medium containing BAPTA and monensin showed a reduction in ^{45}calcium uptake almost identical to that observed in muscles treated with only BAPTA (37.8%) but exhibited a 84.5% and 4.0-fold greater increase in total CK loss ($p<0.001$) than muscles incubated only with monensin or BAPTA, respectively.

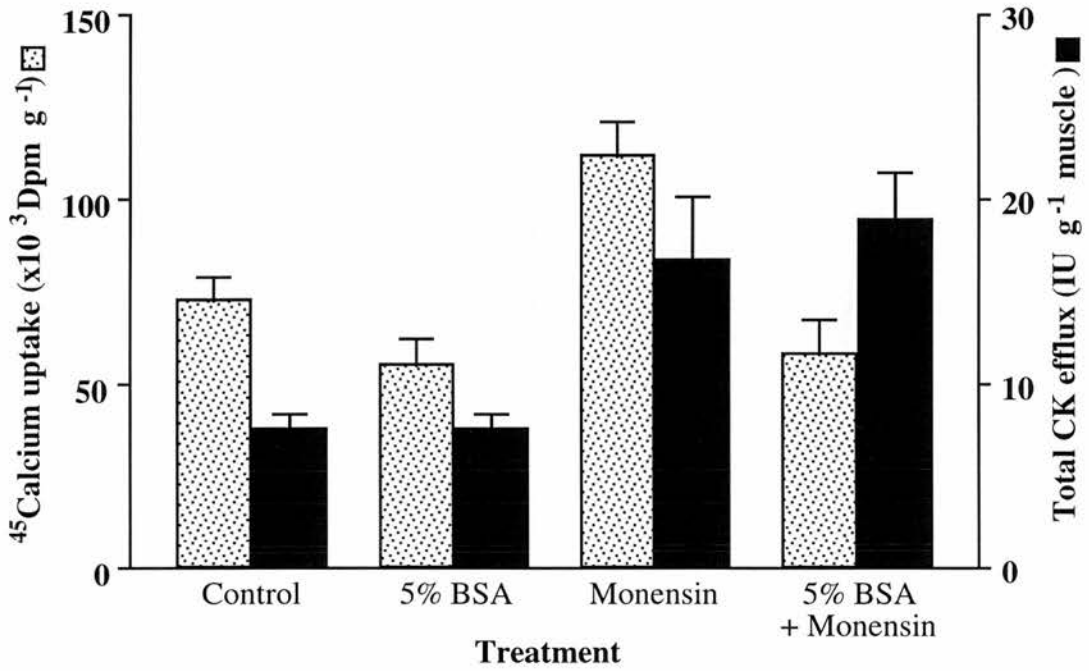

Figure 9.7 Effect of the addition of 5% BSA to incubation medium on ^{45}calcium uptake and total CK efflux in muscles treated with monensin Na^+ ionophore (100 µmol litre^{-1}). Values represent means ± S.D. for 8 muscles per treatment.

The effect of BSA (Experiment XIV)

Muscles incubated in medium containing 5% BSA demonstrated a significant decrease in ^{45}calcium uptake (24.1%; $p<0.05$) compared to control muscles, but had no effect no total CK efflux. Treatment with only monensin induced a increase in ^{45}calcium uptake (53.9%; $p<0.05$) with a corresponding 2.2-fold increase ($p<0.001$) in total CK loss values consistent with previous results. Muscles incubated in medium containing 5% BSA and monensin showed a significant reduction in ^{45}calcium uptake

similar to that observed in muscles treated with only 5% BSA, and exhibited a 33.7% greater increase ($p<0.05$) in total CK loss than muscles incubated only with monensin.

Discussion and conclusions

It is proposed that the primary cause of a potentially damaging influx of Ca^{2+} into cells is an sustained significant increase in intracellular Na^+ concentration (Blaustein, 1974; Piper, 1989, Trump *et al.*, 1989). Therefore the collective aim experiments presented in this chapter was to investigate the possible role of external Na^+ entry on trans-membrane Ca^{2+} flux and its subsequent effect on CK release in chicken skeletal muscle using the sodium ionophore monensin.

The findings of **Experiment IX** clearly show that with increasing concentrations of monensin there was a dose dependent increase in muscle ^{45}calcium uptake and total CK efflux which demonstrated a strong linear relationship ($r^2= 0.995$) over range of monensin concentrations examined (25 -200 μmol litre^{-1}). Similar evidence of dose-dependent increases in cytosolic calcium concentration using monensin have been also reported in studies on isolated rat aorta (Ozaki *et al.*, 1982), thyroid (Ambroz *et al.*, 1990) and sertoli cells (Gorczynska and Handelsman, 1993) over a monensin concentration range of 1 - 100 μmol litre^{-1}. In all of these previous studies, monensin was only used at concentrations (10 - 30 μmol litre^{-1}) which altered cellular Na^+ and Ca^{2+} fluxes within a narrow physiological range (nanomolar) but do not induce any of the characteristics of cell damage (e.g. intracellular enzyme loss) associated with high intracellular calcium concentrations (Nicotera *et al.*, 1992). Studies by Shier and Dubourdieu (1992) have however, reported evidence of cell killing caused by an direct increase in toxic levels of calcium, mediated by monensin-induced Na^+ influx in isolated rat cardiomyocytes. Indirect measurements of the effects of monensin on Ca^{2+} uptake in tissues have also been reported by Brading (1978, 1981) and Herion *et al.* (1992) who have demonstrated Na^+-mediated increases in muscle tone and contraction in isolated rabbit smooth muscle.

Further confirmation of the possible direct effect of Na^+ on Ca^{2+} entry in skeletal muscle was demonstrated in **Experiments X** and **XI**, in which Na^+ mediated increases in ^{45}calcium uptake and CK release were greatly elevated in muscles incubated with monensin in medium containing either an elevated Na^+ concentration (**Experiment X**) or ouabain, an inhibitor of Na^+/K^+ ATPase one of the primary regulators of Na^+ homeostasis in cells (**Experiment XI**).

Muscles incubated with monensin in medium at a higher Na^+ concentration demonstrated a greater increase in ^{45}calcium uptake and CK loss than muscles incubated with monensin in medium containing Na^+ at a normal physiological

concentration (**See Figure 9.3**). These observations presumably reflect the consequences of the effect of a greater increase in Na^+ influx into the muscle cells due to the existence of a greater "driving" gradient for the ion. It is also interesting to note in the same experiment, that in the absence of any ionophore challenge the increase in external Na^+ concentration had no effect on muscle ^{45}calcium uptake and CK release above that observed under control Na^+ conditions.

Similarly, in muscles in which Na^+ extrusion was prevented by inhibiting Na^+/K^+-ATPase activity using ouabain, there was as a significant increase in muscle ^{45}calcium uptake and subsequent CK loss compared to the corresponding control values (**see Experiment XI**). Incubation with ouabain produced comparable ^{45}calcium uptakes to those caused by treatment with monensin, however, there was a noticeable 25.8% greater increase in total CK loss in the monensin treated muscles compared to those only receiving ouabain. Why treatment with monensin should produce this differential effect on CK for the same apparent calcium uptake is not known, but would tend to suggest that the ionophore may be producing other additional localised actions on the muscle different to those produced by primarily preventing Na^+ extrusion. It is clear however that the combined effects of both treatment on ^{45}calcium uptake and CK release were additive (**see Figure 9.4**). The effects of ouabain on external Ca^{2+} uptake are supported to some extent by the findings of Zavecz and Anderson (1992) who have demonstrated evidence of ouabain-mediated contractures in isolated strips of rat diaphragm caused by elevated extracellular Ca^{2+} entry. Elevations in $[Na^+]_i$ caused by the inhibition of the Na^+/K^+ ATPase by ouabain, leading to increases in $[Ca^{2+}]_i$ causing inotropic or toxic effects in isolated rat and guinea pig cardiomyocytes have also been reported (Sheu and Fozzard, 1982, Grinwald, 1987; Levi, 1991; Satoh, 1994).

On the basis of the findings of **Experiments X-XI** it would appear that Ca^{2+} uptake into muscle cells can be mediated by initial increases in $[Na^+]_i$. The most likely mechanism by which Na^+ may mediate the uptake of calcium is believed to involve the process of Na^+/Ca^{2+} exchange (Baker *et al.*, 1967; Allen *et al.*, 1989; Reeves, 1990). The exchanger has the capacity to translocate and in opposite directions depending on the prevailing Na^+ gradient, such that in Na^+ loaded cells, Na^+_i accumulations are rapidly dissipated by the outward pumping of Na^+ at the expense of inward Ca^{2+} translocation (Smith *et al.*, 1989).

Attempts to directly inhibit the Na^+/Ca^{2+} exchange process in muscles incubated with ouabain (**see Experiment XII**) using amiloride were unsuccessful (**see Figure 9.5**). The results of this experiment demonstrated no effect of the putative Na^+/Ca^{2+} exchange inhibitor on either ^{45}calcium uptake or CK release in

muscles in which Na^+ extrusion had been prevented inhibiting Na^+/K^+ activity. In the absence of an effect of amiloride on the two parameters measured it is proposed that the concentration of amiloride used may not have been high enough to promote sufficient inhibition of the Na^+/Ca^{2+} exchanger in this experiment. This suggestion is supported by several other authors who have reported species differences in inhibitor potency and specificity on the Na^+/Ca^{2+} exchange mechanisms (Kaczorowski *et al.*, 1985; Smith *et al.*, 1985; Northover, 1992; Welsh and Lindinger, 1996). Whilst there have been no apparent previous investigations of the effect of amiloride on Na^+/Ca^{2+} exchange in avian skeletal muscle, its inhibitory effects at a physiological level have been reported in amphibian and mammalian skeletal muscle (Vigne *et al.*, 1982; Hoya and Venosa, 1992, 1995; Dorup and Clausen, 1996) using concentrations of 5 mM or higher. Therefore, on the basis of the findings in **Experiment XII**, no conclusions could be drawn about the possible involvement of the Na^+/Ca^{2+} exchanger in the mechanism of skeletal muscle CK release from these results.

However, in addition to investigating the possible role of the Na^+/Ca^{2+} exchange mechanism in the muscle damage process by trying to directly inhibit its action using amiloride, its role was also examined indirectly by reducing external free Ca^{2+} availability for exchange. Reduction of external free Ca^{2+} availability was achieved using the highly specific Ca^{2+} chelator BAPTA (Tsien, 1980) which at the concentration used reduced external free calcium in the incubation medium by >95% (**Experiment XIII**). If the Na^+/Ca^{2+} exchange mechanism was involved in the process of Na^+-mediated Ca^{2+}-induced CK release in chicken skeletal muscle, then in the absence of external Ca^{2+} availability there should a reduction in CK release. The results illustrated in **Figure 9.6** showed however, that there was a paradoxical increase in CK efflux in the Na^+ loaded cells in the virtual absence of external Ca^{2+} entry[1]. Given that the results of previous experiments presented in this thesis have demonstrated unequivocally that the process of CK release from avian skeletal muscle can be mediated by external Ca^{2+} entry (**see Chapters 7** and **8**), it is proposed that in the absence of the adequate Na^+ translocation, the cytosolic increase in Na^+ was perhaps mediating the release of Ca^{2+} from an intracellular source such as the muscle sarcoplasmic reticulum (SR). This hypothesis is supported by the findings of several other *in vitro* studies which have demonstrated increases in Ca^{2+}_i accumulation resulting from its release from SR stores in response to increases in $[Na^+]_i$, in the absence of extracellular Ca^{2+} entry (Leblanc and Hume, 1990; Rios and Pizzaro, 1991; Gorczynska and Handelsman, 1993; Borin *et al.*, 1994). Whilst this hypothesis might explain the results of **Experiment XIII** it does not provide any additional

[1] See Chapter 7

information on the possible involvement of the Na^+/Ca^{2+} exchanger in the CK release process. However there is some evidence from studies on rat ventricular myocytes and human A-431 epidermoid cells that Na^+ mediated increases in $[Ca^{2+}]_i$ may involve both the activation of the reversed mode of the Na^+/Ca^{2+} exchanger and the mobilisation of Ca^{2+} from sarco/endoplasmic reticulum Ca^{2+} stores (Eisner *et al.*, 1989; Kiang *et al.*, 1992; Kiang and Smallridge, 1994). These authors have proposed that increased Na^+ influx activates the reverse mode of the Na^+/Ca^{2+} exchanger thereby leading to an increase in Ca^{2+} entry which in turn promotes the release of Ca^{2+} from intracellular SR stores. It is also possible, as discussed previously, that increased Na^+ influx may stimulate the release of Ca^{2+} from intracellular SR stores directly. It is also recognised that during incubation with monensin there may be an unopposed alkalisation of the cell (Hoya and Venosa, 1992) which in addition to the increase in $[Na^+]_i$ may promote the release of Ca^{2+} from intracellular SR stores (Shoshan *et al.*, 1981). It has been reported by Shoshan *et al.* (1981) that the dissipation of H^+ gradients in isolated skinned skeletal muscle fibres can produce a transient release of Ca^{2+} from the SR. Whilst it is apparent that these proposed mechanism have been investigated and may operate at a physiological level it is not known if they have a role to play in any pathological context.

Furthermore, it has recently been proposed by McCall and Duncan (1995) from findings from *in vitro* studies on isolated skeletal muscle that the removal of external Ca^{2+} using chelators such as EGTA or its derivatives may cause temperature sensitive molecular perturbations that reversibly converts the "CK release mechanism" from its normal state where it is triggered by a large rise in $[Ca^{2+}]_i$ to an activated condition that may be stimulated by only a modest rise in $[Ca^{2+}]_i$.

As a follow-up to **Experiment XIII**, muscles were also incubated in medium containing 5% BSA (**Experiment XIV**). This experiment was designed to examine the effects of elevated $[Na^+]_i$ on muscle ^{45}calcium uptake and CK loss under conditions in which there was a reduction in extracellular free Ca^{2+} availability comparable to that caused by respiratory alkalosis in birds exposed to acute heat stress (**see Chapter 12**). The results of **Experiment XIV** (**see Figure 9.7**) were consistent with the findings of **Experiment XIII** (**see Figure 9.7**) in that they demonstrated what appeared to be a paradoxical increase in total CK loss in monensin treated muscles incubated in medium in which the free Ca^{2+} availability was significantly reduced due to binding to BSA. The extent of the extracellular Ca^{2+} binding influencing the magnitude of the subsequent release of CK. On the basis of these findings is apparent that the mechanism of CK release from avian skeletal muscle may not necessarily rely on a large influx of external Ca^{2+} to produce changes

sarcolemmal integrity that lead to CK loss. These results clearly demonstrate that under conditions of reduced external Ca^{2+} availability the process of CK release can be mediated via an increase in $[Ca^{2+}]_i$ via its release from an intracellular source.

In conclusion these results of the experiments present in this chapter have shown that conditions promoting either increased Na^+ influx and/or a reduced Na^+ extrusion can promote external Ca^{2+} entry and CK release in avian skeletal muscle possibly through the process of Na^+/Ca^{2+} exchange. However, whilst there was good circumstantial evidence to substantiate this claim, no direct proof of its operation was demonstrated.

In addition, it was then subsequently demonstrated that increased Na^+ influx could promote CK release in the apparent absence or reduction in external Ca^{2+} entry an unexpected finding which would tend to contradict the central "Ca^{2+} uptake" hypothesis. These results suggested that the Ca^{2+} required to mediated the CK release process may be mobilised or released from an intracellular source such as the sarcoplasmic reticulum. However it conceivable that under conditions where extracellular free Ca^{2+} entry is permitted that both mechanisms may operate concurrently, as has been shown in other tissues. These hypotheses are examined further in **Chapter 10**.

Chapter 10

The effect of the re-distribution and release of Ca^{2+} from sarcoplasmic reticulum (SR) Ca^{2+} stores on CK release from avian skeletal muscle

Introduction

The cytosolic concentration of Ca^{2+} in skeletal muscle is maintained approximately 10^5 times lower than the free Ca^{2+} concentration of the external environment, due to its cytotoxic effects at high concentration (Martonosi, 1984). The large gradient created across the muscle cell membranes are maintained by active calcium extrusion at the level of the sarcolemma via ATP-dependent Ca^{2+} pumps and the Na^+/Ca^{2+} exchange proteins (Mullins, 1977; Carafoli, 1981; Reuter, 1982; Schatzmann, 1989), and by the sequestration of Ca^{2+} into intracellular stores in the muscle sarcoplasmic reticulum (SR) via ATP-dependent SR Ca^{2+} pumps or "SERCAs"[1] (Pozzan *et al.*, 1994).

In addition to its co-operative role in maintaining low cytosolic Ca^{2+} concentrations, by sequestering and storing Ca^{2+}, it is recognised that the SR also provides the activating Ca^{2+} necessary for muscle contraction. Therefore at any one time, the Ca^{2+} content of the SR reflects the balance between the rate of Ca^{2+} taken up from the sarcoplasm in to the lumen of the SR (via the action of the SERCAs), and the rate of Ca^{2+} release from the SR in to the sarcoplasm through SR Ca^{2+} release channels.

SERCAs in skeletal muscle have been shown to be uniformly distributed throughout the SR (Junker and Sommer, 1979, 1980) and consisting of single poly peptide chains of 100-150 kilo-daltons (kDa) (Møller *et al.*, 1980). It has recently been demonstrated that all SERCA isoforms share the property of being selectively inhibited by thapsigargin, a plant derived sesquiterpene lactone (Thastrup 1990; Thastrup *et al.*, 1990). Thapsigargin has been reported to cause an emptying of SR Ca^{2+} stores by inhibiting SERCA Ca^{2+} uptake, without affecting sarcolemmal Ca^{2+} ATPase transport (Sagara and Inesi, 1991; Sagara *et al*, 1992). For this reason thapsigargin has found much popularity as an agent for manipulating intracellular Ca^{2+} distribution (Bian *et al.*, 1991, Lytton *et al.*, 1991, Janczewski and Lakatta, 1993; Pizzo *et al.*, 1997).

Whilst thapsigargin has been repeatedly shown to affect the mobilisation and re-compartmentalisation of Ca^{2+} from SR Ca^{2+} stores in cardiac and skeletal muscle, at

[1] Sarco/endoplasmic reticulum Ca^{2+}-ATPases

a physiological level, it is not known if inhibition of SERCA Ca^{2+} uptake is sufficient to induce any pathological changes mediated by any Ca^{2+} activated degenerative processes. In skeletal muscle, the discharge or release of Ca^{2+} from SR stores (excluding passive Ca^{2+} diffusion) is primarily mediated via Ca^{2+} release channels (Martonosi, 1984; Tsien and Tsien, 1990). These channels are highly sensitive to the plant alkaloid ryanodine and have been shown, when isolated and purified, to act as a ryanodine receptors (RyR) (Imagawa *et al.*, 1987; Fill and Coronado, 1988; Rios and Pizarro, 1988). The RyR is a 450 kDa tetramer protein consisting of a ryanodine-binding site, a calcium release channel and a foot structure spanning between SR and T-system membranes (Coronado *et al.*, 1994). The activity of the RyR is strongly enhanced by adenine nucleotides, caffeine and by Ca^{2+} itself (Endo, 1977; Meissner and Henderson, 1987; Wyskovsky *et al.*, 1990; Yamaguchi *et al.*, 1997) and inhibited by dantrolene sodium, ruthenium red and tetracaine (Olivares *et al.*, 1993, Jong *et al.*, 1997; Yamaguchi *et al.*, 1997).

Dantrolene sodium has long been used as a skeletal muscle relaxant to treat various forms of muscle spasticity and maligant hyperthermia in both human and veterinary medicine (Foster and Denborough, 1993, Klont *et al.*, 1994; Rosenberg and Fletcher, 1994). Dantrolene has been shown to depress excitation/contraction (EC) coupling in vertebrate skeletal muscle by inhibiting RyR mediated SR Ca^{2+} release (Jong *et al.*, 1997). Like thapsigargin, dantrolene has found widespread acceptance as an agent for manipulating intracellular Ca^{2+}, as its effects are specific to the RyR (Parness and Palnitkar, 1995) and do not interfer with neuromuscular transmission or with the conduction of action potentials.

Virtually all of the *in vitro* studies that have examined the inhibitory effects of dantrolene on SR Ca^{2+} release in skeletal muscle have been undertaken using either mammalian (rat, rabbit and pig) or amphibian (frog) isolated intact single muscle fibres (Allen *et al.*, 1992, Holland *et al.*, 1992, Bakker *et al.*, 1996) or microsomal SR fractions (Foster and Denborough, 1993; Bull and Marengo, 1994; Yamaguchi *et al.*, 1997). Examination of the physiological effects of dantrolene on RyR mediated Ca^{2+} release have been reported in a few studies using isolated chick skeletal muscle preparations (Wali, 1986, Dhillon *et al.*, 1992). There is no evidence in the current literature that the effects of dantrolene have been investigated in avian tissues in relation to any possible amelioration of Ca^{2+} mediated pathology. It has however, recently been shown that dantrolene reduces the elevated release of CK from isolated rat skeletal muscle caused by ethanol, cocaine and high frequency electrical stimulation (Pagala *et al.*, 1997). Whether dantrolene may act to reduce Ca^{2+} mediated CK release in isolated chicken skeletal muscle in the same way is not yet known.

Experimental aims

The aim of the experiments presented in this chapter were two-fold. First, to investigate the effect of the inhibition of SERCA mediated SR Ca^{2+} uptake on ^{45}calcium uptake and CK release using thapsigargin, under conditions in which external calcium entry was permitted, or prevented by treatment with the calcium chelator BAPTA (**Experiment XVI**). Secondly to examine the effect of dantrolene induced inhibition of RyR mediated Ca^{2+} release on ^{45}calcium uptake and CK release in monensin treated muscles (**Experiment XVII**).

Materials and methods

Experiment XVI

Thapsigargin and BAPTA were dissolved in medium 199 to give a final concentrations of 20 μmol litre^{-1} .and 5 mmol litre^{-1} respectively. The pH of the BAPTA containing medium was adjusted to pH 7.4 using 0.1M HCl. The muscles used in this experiment were essentially incubated according to the protocol used in **Experiment VIII**. All muscles were initially incubated under the same conditions as the control muscles for the 2 x 30 minutes, then incubated with thapsigargin or BAPTA or a combination of both for the remainder of the experiment (3 x 30 minutes). Control muscles were incubated in control incubation medium throughout as in all previous experiments.

Experiment XVII

Dantrolene sodium was dissolved in pure methanol to give a final concentration of 250 μmol litre^{-1} when added to incubation medium stock (final methanol concentration 2%). In accordance with previous monensin experiments outlined in **Chapter 9**, the ionophore was solubilized in pure methanol and added to an incubation medium stock at a final monensin concentration of 100 μmol litre^{-1}. All stock solutions (including control) were adjusted to contain 2% methanol by volume. Dantrolene and monensin treatment muscles were initially incubated under the same conditions as the control muscles for 2 x 30 minutes. After this period, treatment muscles were incubated for 3 x 30 minutes in medium containing either dantrolene sodium or monensin or a combination of both drugs. Control muscles were incubated in control medium throughout the duration of the experiment.

All muscles were incubated and processed according to the experimental protocol described in **Chapter 4.** Isotope-labelling of the medium with ^{45}calcium and the methods for the determination of ^{45}calcium uptakes and CK efflux activities were the same as those outlined in **Chapter 7**. Data are presented as means ± S.D.

Levels of significance were determined by analysis of variance or unpaired Students t-test.

Results

The effects of SERCA inhibition (Experiment XIII)

The effects of thapsigargin on BAPTA-treated muscles are illustrated in **Figure 10.1.** Muscles incubated in medium containing BAPTA demonstrated a significant decrease in ^{45}calcium uptake (35.3%; $p<0.05$) compared to control muscles, but had no effect on total CK efflux above that observed for controls. These findings were consistent with results from previous experiments (**see Chapter 9; Experiment XIII**) using BAPTA at this concentration. Treatment with only thapsigargin caused a 36.0% increase in ^{45}calcium uptake ($p<0.05$) compared to controls, and was associated with a 2.0-fold greater total CK loss ($p<0.001$). Muscles incubated in medium containing thapsigargin and BAPTA demonstrated a reduction in ^{45}calcium uptake comparable to that observed in muscles treated with only BAPTA (36.6%, $p<0.05$) but exhibited a 2.7-fold greater increase ($p<0.001$) in total CK release. When compared with muscles only treated with thapsigargin, the same muscle treatment group demonstrated a 2.2-fold less uptake of ^{45}calcium but showed a 32.8% greater total loss of CK.

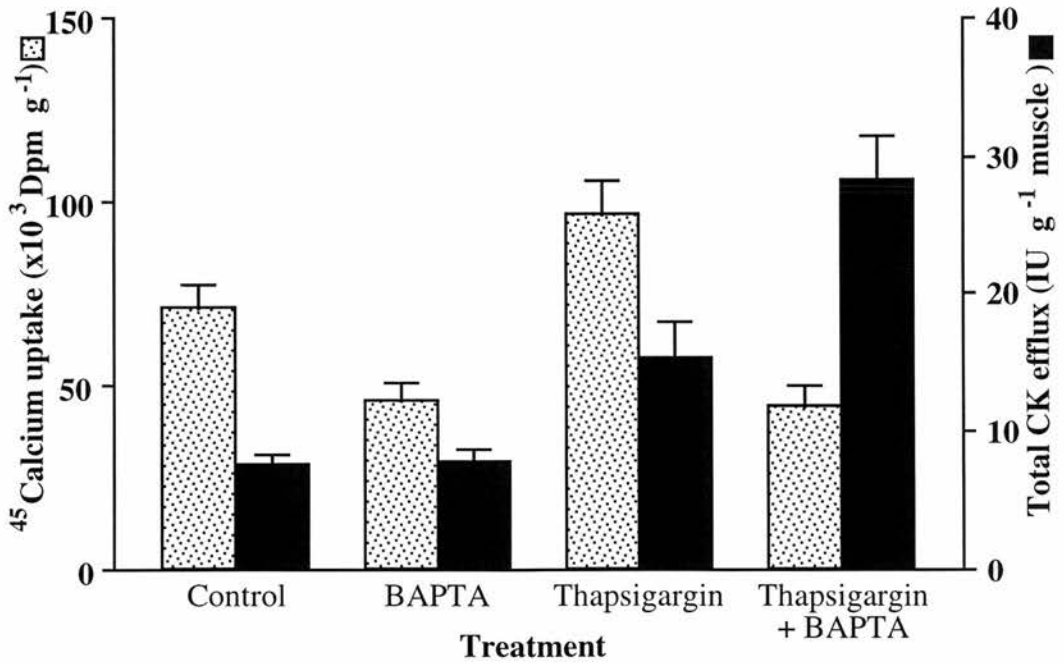

Figure 10.1 Effect of thapsigargin (20 μmol litre^{-1}) on ^{45}calcium uptake and total CK efflux in muscles incubated with or without BAPTA (5 mmol litre^{-1}). Values represent means ± S.D. for 8 muscles per treatment.

The effects of RyR Ca^{2+} release inhibition (Experiment XVI)

The effects of dantrolene sodium on monensin-treated muscles are illustrated in **Figure 10.2**. ^{45}Calcium uptake and total CK loss from muscles incubated with dantrolene sodium was essentially the same as for controls. Muscles incubated with monensin demonstrated a predictable increase in both ^{45}calcium uptake and total CK loss, reflecting increases in both parameters of 46.3% ($p<0.05$) and 2.2-fold ($p<0.001$) respectively. These figures are comparable with previous values obtained in experiments reported in **Chapter 9**, using monensin at the same concentration. In muscles incubated with dantrolene and monensin there was a 15.6% reduction in muscle ^{45}calcium uptake compared with muscles treated only with monensin, although this reduction was not significant at $p<0.05$. Total CK loss was however significantly reduced by 32.2% ($p<0.05$).

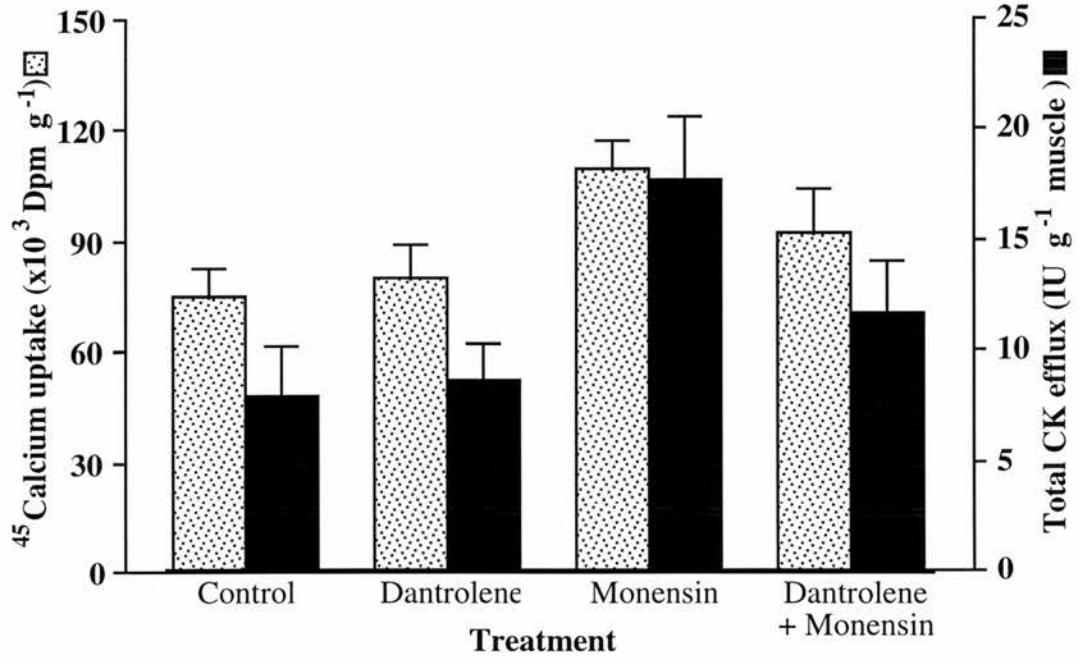

Figure 10.2 Effect of dantrolene sodium (100 µmol litre^{-1}) on ^{45}calcium uptake and total CK efflux in muscles treated with monensin Na^+ ionophore (100 µmol litre^{-1}). Values represent means ± S.D. for 8 muscles per treatment.

Discussion and conclusions

The objectives of the two experiments reported in this chapter were to first examine whether the re-distribution and mobilisation of Ca^{2+} from intracellular SR Ca^{2+} stores under conditions of reduced extracellular calcium entry could cause an increase in the release of CK. Secondly, investigate the possible role of the SR

ryanodine-sensitive release channel (RyR) as mediator of Ca^{2+} release under metabolically stressful conditions.

The results of **Experiment XV** show that incubation with the SERCA inhibitor thapsigargin, in the absence of external Ca^{2+} entry, produced an significant increase in CK release from isolated chicken skeletal muscle. This would suggest, contrary to the main hypothesis of the thesis, that the entry of external Ca^{2+} may not necessarily mediate the release of CK, and that Ca^{2+} induced muscle CK loss can be mediated by the release of intracellular Ca^{2+} from SR stores. These observations are supported in part, by the findings of Xuan *et al.* (1992), who demonstrated sustained increases in intracellular Ca^{2+} concentration in rat aortic smooth muscle cells treated with thapsigargin and incubated in Ca^{2+} free buffer containing 1 mM EGTA.

It is apparent however, from the results illustrated in **Figure 10.1** that in muscles incubated only with thapsigargin under conditions of normal external Ca^{2+} availability that there was stimulation of an increase in external Ca^{2+} uptake. Similar observations have been reported in other *in vitro* studies where thapsigargin was shown to inhibit SERCA activity and promote external calcium entry in isolated rabbit skeletal muscle (Sagara and Inesi, 1991: Sagara *et al.*, 1992), rat aortic smooth muscle (Xuan *et al.*, 1992) and rat parotid acinar cells (Takemura *et al.*, 1989). This has prompted suggestions that the mobilisation of Ca^{2+} from SR stores may be coupled to the activation of external Ca^{2+} entry, so called "capacitative Ca^{2+} entry" (Putney, 1990). The precise nature of such a Ca^{2+} entry mechanism is not known, but is believed involve either receptor (ROC), voltage (VOC) or storage operated (SOC) L-type Ca^{2+} channels (Tsien and Tsien, 1990).

Whether in thapsigargin treated muscles, such a precise homeostatic mechanism operates to facilitate the relatively high levels of external Ca^{2+} entry associated with CK release is not clear. It would seem more likely that in these muscles, the entry of external Ca^{2+} occurs as a secondary consequence of events mediated by the initial release of Ca^{2+} from the SR, possibly though Ca^{2+} mediated alterations in sarcolemmal permeability.

In summary, **Experiment XV** demonstrated that thapsigargin mediated inhibition of SERCA activity in isolated chicken skeletal muscle promoted a significant increase in CK loss, presumably through a combination of SR mediated Ca^{2+} release and recompartmentalisation and external Ca^{2+} uptake. However, the effects of thapsigargin on muscle CK release were exacerbated in the absence of external Ca^{2+} entry, thus prompting the suggestion that the activation of external Ca^{2+} entry may in its self be mediated by the initial release of Ca^{2+} from SR stores.

Having demonstrated that it was possible to stimulate the release of CK from isolated skeletal muscle by re-compartmentalising the Ca^{2+} from SR stores, the second of the experiments undertaken investigated the possible role of the SR ryanodine-sensitive Ca^{2+} release channel (RyR) as mediator of Ca^{2+} release under metabolically stressful conditions. The process of Ca^{2+} release from skeletal muscle SR *in vitro* has been shown to be regulated by the gating properties of the RyR Ca^{2+} release channels and to a lesser extent, the Ca^{2+} gradient across the SR membrane (Volpe and Simon, 1990). In **Experiment XVI**, the incubation of muscles with dantrolene sodium reduced monensin-induced increases in CK efflux by approximately 35% (**see Figure 10.2**). It may be suggested that the mechanism that mediates CK efflux involves the release of Ca^{2+} from the muscle SR via the dantrolene inhibitable RyR Ca^{2+} release channel. Similar effects of dantrolene on toxin mediated SR Ca^{2+} release and subsequent CK release are supported to some extent by the findings of Ameladevi *et al.* (1995) and Pagala *et al.* (1997) who demonstrated evidence of dantrolene-mediated reductions in CK release from ethanol and cocaine treated isolated rat skeletal muscles.

Thus whilst monensin as an ionophore may induce changes in sarcolemmal integrity and muscle damage through several mechanisms including the entry of extracellular Ca^{2+} (possibly via Na^{+}/Ca^{2+} exchange, **see Chapter 9**), it is apparent that a major component of the monensin effect may be mediated by the release of Ca^{2+} from intracellular SR stores, via ryanodine-sensitive Ca^{2+} channels. The inhibition of Ca^{2+} release associated CK efflux by dantrolene confirms the role of this avenue of Ca^{2+} release in the process of cell damage associated with monensin ionophore treatment. Whether, the same mechanisms mediate CK release from broiler skeletal muscle under conditions of stress exposure *in vivo* is not yet known. This hypothesis was tested in **Chapter 12**.

Chapter 11

The effect of dietary supplementation with monensin on the release of creatine kinase from chicken skeletal muscle *in vivo*

Introduction

Monensin is a polyether carboxylic-ionophore antibiotic which has long been employed in the animal production industry for its anti-coccidial activity (Shumard and Callender, 1968). The safety margin of this drug is relatively small and toxicosis caused by accidental over dosage or differential host sensitivity is not uncommon. Toxicosis associated with monensin feed medication has been widely reported in many species including cattle (Collins and McCrea, 1978), horses (Matsuoka *et al.*, 1996) sheep (Van Ryssen, 1991) pigs (Van Vleet *et al.*, 1983a,b; Anadon and Martinez-Arranaga, 1991), and poultry (Howell *et al.*, 1980; Hanrahan *et al.*, 1981; Wages and Ficken, 1988; Ficken *et al*, 1989, Dowling, 1992; Baird *et al.*, 1997). The primary symptoms of this toxic syndrome are cardiac and skeletal muscle dysfunction and weakness, which it is believed occur as a consequence of the ionophoretic action of monensin in these tissues. Studies by van Vleet *et al* (1983, a, b) have demonstrated that monensin induced myopathy in pigs may be caused by ionophore mediated increases in intracellular calcium (Ca^{2+}) concentration, as determined by histological examination. Associated with these pathological changes were marked increases in serum creatine kinase (CK) and aspartate aminotransferase (AST) activity. Similar serum enzyme responses to monensin treatment have been reported in broiler chickens (Horowitz *et al.*, 1988) and in turkey breeder hens (Ficken *et al.*, 1989). Van Vleet *et al.* (1983, a, b) have proposed that monensin causes pathological changes in pig skeletal muscle by producing net increases in muscle Na^+ concentration which promotes increases in cellular Ca^{2+} concentrations leading to a cell calcium overload. This overload then precipitates a series of degradative alterations in the cell culminating in membrane damage and ultimately necrosis. This hypothesis is supported by the *in vitro* findings reported in this thesis (**see Chapter 9**) which have demonstrated a dose dependent relationship between monensin concentration, extracellular calcium uptake and muscle damage, as reflected by the loss of CK.

In the modern broiler chicken genetic selection for rapid growth rate may have resulted in an increased susceptibility to myopathy (Mitchell and Sandercock; Sandercock *et al.*, 1995). Whilst it is acknowledged that accidental overdose with monensin may induce muscle damage in broilers as reflected by CK release (Horowitz

et al., 1988), it is not known if at anti-coccidial therapeutic doses there is any influence upon calcium homeostasis and muscle membrane integrity in the current rapidly growing bird.

Experimental aims

The present study was designed to examine and compare the effect of a therapeutic (100 mg kg^{-1}) and a known myotoxic dose of monensin (200 mg kg^{-1}) administered as a dietary supplement as in commercial practice (Dowling, 1992)

Materials and methods

Subjects

Thirty six, 14 day-old female commercial broiler chicks were used in the present study. Birds were initially divided into three groups of 12 and placed in one of three floor pens on litter with *ad libitum* access to food and water and maintained at 21°C/50% RH. A photoperiod of 14 h-light: 10 h-dark was maintained throughout the duration of the experiment. The birds were grown on from 14 to 42 d (commercial slaughter age).

Monensin supplementation

From 21d until the end of the experiment the birds received dietary monensin supplementation (Elancoban® 100 premix, Elanco Animal Health, Basingstoke UK.) at concentration of 0 (control), 100 or 200 mg kg^{-1} of feed. Monensin premix was combined with standard broiler feed ingredients[2] and pelleted at 75°C. Approximately 3% of the monensin activity is lost during the pelleting process (Elanco Anticoccidials Technical Handbook, 1996).

Body weight measurement and blood sampling

Bird body weight measurements were taken as it has been demonstrated that at high dosage, monensin can cause suppression of appetite in broiler chickens which lead to reductions in body weight gain (Bartov and Jensen, 1980). Body weight measurements and blood samples were taken from the birds at 14, 28 and 42 days of age, corresponding to durations of monensin supplementation of 0, 7 and 21 days. Bird body weight measurements were obtained using a top loading animal balance (Sartorius Limited, Surrey, UK). Blood samples (2.5 ml) were taken from the brachial vein and transferred into heparinised blood tubes (Teklab) and immediately placed on ice. Following whole blood analyses, plasma samples were prepared by

[1] See appendix I

centrifugation of the blood at 1500g for 5 minutes, then immediately frozen at -20°C pending analysis (**see Chapter 2**).

Results

Body weights

No differences in body weight were observed in any of the three dietary treatment groups prior to monensin medication at 14 days of age (**see Figure 11.1**). At 28 days of age, after receiving monensin for 7 days, there was a significant decrease (6.7%, $p<0.05$) in body weight in the 200 mg kg^{-1} monensin treated birds compared to controls. No difference in body weight was observed between the controls and the birds receiving 100 mg kg^{-1} monensin. At 42 days of age, after 21 days of monensin treatment, there was an consistent reduction (6.4%, $p<0.05$) in body weight in the birds receiving the highest dietary monensin concentration (200 mg kg^{-1}) compared to controls. No significant difference in body weight was observed between the controls and the birds receiving the recommended 100 mg kg^{-1} prophylactic dose of monensin despite some evidence of a small increase. A 9.5% difference in body weight was observed between the two monensin supplemented groups at this age.

Plasma CK activity

Plasma CK activities were the same in all three treatment groups at 14 days of age prior to dietary monensin supplementation (**see Figure 11.2**). Significant increases in plasma CK activity (65.0%, $p<0.001$) were observed in the birds receiving 200 mg kg^{-1} monensin at 28 days of age, after 7 days of monensin supplementation. No difference was observed between the birds receiving 100 mg kg^{-1} monensin and the controls at this age. Significant differences in plasma CK activity were observed in all three treatment groups at 42 days after 21 days of monensin supplementation. Compared to controls there was a (20.5%, $p<0.01$) and (43.5%; $p<0.001$) increase in plasma CK activity in the 100 and 200 mg kg^{-1} monensin treated groups respectively. There was also a 19.1 % difference in plasma CK activity ($p=0.02$) between the two groups of monensin treated birds.

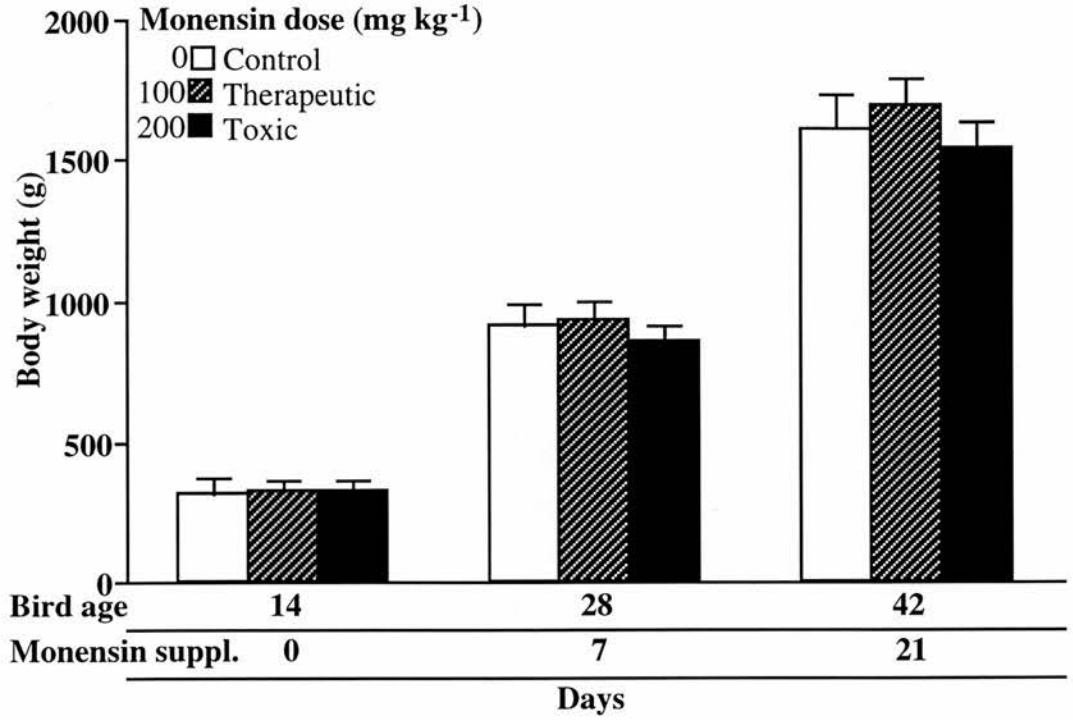

Figure 11.1 Effect of dietary monensin supplementation at 0, 100 and 200 mg kg^{-1} on broiler chicken body weight at 14, 28 and 42 days of age. Birds received monensin over a 21 d period. Supplementation was introduced at 21 days and was continued to 42 days. Values represent means ± one S.D., n=12 birds per treatment.

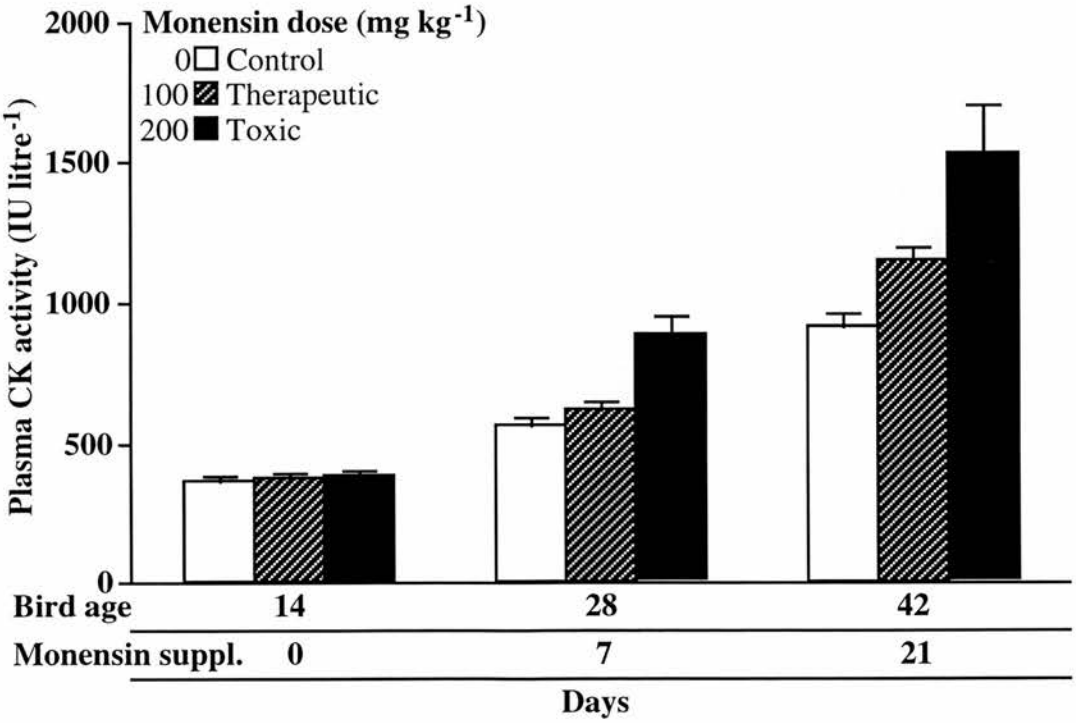

Figure 11.2 Effect of dietary monensin supplementation at 0, 100 and 200 mg kg^{-1} on plasma CK activity in the broiler chicken at 14, 28 and 42 days of age. Birds received monensin over a 21 d period. Supplementation was introduced at 21 days and was continued up to 42 days. Values represent means ± one S.D., n=12 birds per treatment.

Discussion and conclusions

The aim of this experiment was to determine if there were physio-biochemical effects of monensin at therapeutic doses in broiler chicken skeletal muscle. The results of this experiment are consistent with those previously reported by Horowitz *et al.* (1988) demonstrating dose dependent increases in plasma CK activity in broiler chickens receiving dietary monensin supplementation. Comparisons between the two studies of the magnitude of the increases in plasma CK activity at the two doses of monensin used is not possible due to differences in the starting age and duration of monensin supplementation. However, these authors reported profound increases (>2000 IU litre^{-1}) in plasma CK activity in 56 day old (8 wk) in broiler chickens receiving dietary monensin supplementation at 200 mg kg^{-1} from 35 days of age. The increases in plasma CK activity observed in the control birds in the present study are consistent with age dependent increases in plasma CK previously reported by Mitchell and Sandercock (1995).

Whilst no attempt was made to measure the effective concentration of monensin available to act on the muscles of the chickens used in this study, monensin residues have been measured in chicken skeletal muscle at therapeutic doses (Donoho *et al.*, 1982, Donoho, 1984, Atef *et al.*, 1990). Tissue concentrations of monensin of 0.84 and 0.70 μg g^{-1} were reported by Atef and co-workers (1993) for breast and thigh muscle respectively in 8 week old female broiler chickens receiving 14 days monensin supplementation at 120 mg kg^{-1}. These monensin values were determined in homogenised tissues, therefore estimation of the relative distribution of the drug in the intra and extracellular compartments was not ascertained. However based on calculations assuming equal distribution throughout the extracellular space it is suggested that the monensin concentrations present in the extracellular fluid (ECF) is in the micro-molar range (Dr M. Mitchell *pers. comm.*).

The depressive effects of monensin on bird body weight were consistent with other previous studies in poultry (Bartov and Jensen, 1980; Horowitz *et al.*, 1988; Braunius, 1985, Oyawoye and Kruger, 1990). The reduction in body weight caused by monensin in domestic fowl has been attributed to a depression in feed intake caused by the appetite-suppressing (anorectic) action of the drug (Oyawoye and Kruger, 1990). The effects of monensin on appetite suppression are believed to be similar in action to those produced by phenylpropanolamine.

In conclusion the results of the present experiment confirm that dietary monensin supplementation produces a myopathy in broiler chickens. The myopathy is associated with an increase plasma CK activity that is consistent with experimentally

induced skeletal muscle damage and CK release caused using monensin *in vitro*. These results support the use of monensin as a suitable pharmacological agent for investigating the mechanisms of CK release from isolated chicken skeletal muscle *in vitro*, in order to ultimately understand the mechanisms that induce muscle damage in broiler chickens under conditions of stress *in vivo*. The results of this experiments show that monensin is toxic even at therapeutic doses. It is probable that the observed myopathy induced by the ionophore at these concentration is mediated by disturbances in intracellular Ca^{2+} homeostasis (**see Chapter 9**). These findings may be important from a bird health perspective, as whilst it has been reported by several authors that at these doses of ionphore there may be no overt signs of myopathy as defined by histological examination (Hanrahan *et al.*, 1981;Vanderkop *et al.*, 1989), it is possible that at a sub-clinical level muscle function may be disturbed. This may affect resistance to other stress induced myopathies (Mitchell and Sandercock, 1995) and have implications for meat quality and animal welfare. As growth rate increases and problems with Ca^{2+} homeostasis and sarcolemmal integrity increases the risk of monensin induced myopathy may be exacerbated. This has important commercial implications.

Chapter 12

The effect of dantrolene sodium on skeletal muscle creatine kinase (CK) release in broiler chickens exposed to acute heat stress.

Introduction

Previous experiments described in **Chapter 3** of this thesis, and other studies undertaken in these laboratories (Mitchell *et al.*, 1992; Mitchell and Sandercock, 1995) have demonstrated the induction of skeletal muscle damage and subsequent CK release in broiler chickens following exposure to an acute heat stress challenge (2 h 32°C/80% RH).

An investigation of the mechanisms that might mediate CK release from this tissue was undertaken using an *in vitro* isolated skeletal muscle preparation (**see Chapters 4-10**). From these studies it was established that factors promoting disturbances in intracellular calcium (Ca^{2+}) regulation, which we believe cause sustained elevations in sarcoplasmic Ca^{2+} concentration induced Ca^{2+}-dependent increases in phospholipase A_2 activity (and other degradative processes) produced alterations in muscle cell membrane permeability leading to enhanced loss of CK (**see Chapters 7 and 8**). From these *in vitro* experiments it was also established that increases in sarcoplasmic Ca^{2+} concentration could be mediated through both the entry of extracellular Ca^{2+} via Na^{+}/Ca^{2+} exchange (**see Chapter 9**) and by its release from SR Ca^{2+} stores via ryanodine-sensitive (RyR) Ca^{2+} release channels (**see Chapter 10**). The results of the experiments further proposed that the entry of external Ca^{2+} into skeletal muscle cells may be secondary to the release of Ca^{2+} from SR stores, occurring as a result of initial SR mediated alterations in sarcolemmal integrity.

On the basis of these findings it is proposed that stress induced skeletal muscle damage and CK release in the broiler chicken *in vivo*, may be caused by increases in muscle sarcoplasmic free Ca^{2+} concentration mediated by SR RyR Ca^{2+} release channels. If these mechanisms operate during "stress" to cause the release of CK from chicken skeletal muscle, then their effects should be inhibited by the action of dantrolene.

Dantrolene sodium is widely used clinically as a muscle relaxant to relieve chronic muscle spasticity associated with multiple sclerosis, cerebral palsy, stroke and spinal cord injury (Waldman, 1994). Dantrolene has also been successfully used in humans and pigs to treat malignant hyperthermia (MH), an inherited hypermetabolic disorder in which defects in the SR ryanodine receptor (RyR) Ca^{2+} release channel

cause abnormal and sustained elevations in sarcoplasmic free calcium concentrations in skeletal muscle cells (MacLennan *et al.*, 1990, Fujii *et al*, 1991; Rosenberg *et al.*, 1992; MacLennan, 1992; MacLennan and Philips, 1992). The clinical state of MH is characterised by uncontrolled muscle contracture, greatly elevated muscle metabolism, hyperthermia, lactacidosis, hyperkalaemia and disruption of muscle sarcolemmal integrity and elevated muscle enzyme release into the circulation (O'Brien *et al.*, 1990; Maclennan, 1992; Rosenberg *et al.*, 1992, Antognini, 1995). MH in pigs has been shown to be triggered by a number of factors such as halothane anaesthetic administration, physical activity and "stress" (O'Brien, 1987; Gallant and Goettl, 1989; Bjurstrom *et al.*, 1995). Whilst there have been no reports of MH in avians, stress induced muscle damage (as reflected by increased plasma CK activities) has been demonstrated in broiler chickens in response to exposure to transportation (Mitchell *et al.*, 1992) and high environmental temperatures (Ostrowski-Meissner, 1981, **see Chapter 3**). Currently, it is not known if the myopathic consequences of exposure to these stressful conditions may be mediated by the release of Ca^{2+} via the SR ryanodine sensitive (RyR) Ca^{2+} release channel.

Experimental aims

The aim of the present study therefore was to examine the effects of dantrolene sodium on the release of CK from skeletal muscle *in vivo*, in chickens subjected to a standardised period of acute heat stress exposure (2h; 32°C/80%RH). In this heat stress study, a wide range of biochemical responses were determined in addition to the measurement of plasma CK activity and rectal temperature. This allowed us not only to determine the degree of hyperthermia induced, but establish to what extent the birds were attempting to thermoregulate and what consequences this had on other aspects of whole animal physiology. These measurements included blood pCO_2, pH, free calcium (Ca^{2+}), sodium and potassium as well as total plasma calcium and protein.

Materials and methods

Subjects

The birds used in this study were six-week old female broiler chickens approximately 1.9-2.2 kg in body weight. From two weeks of age until one week before the beginning of the experiment, the birds were caged in groups of six to eight birds in standard battery units at 21°C/ 50% RH with a photoperiod of 14 h-light:10 h-dark. The birds were fed *ad libitum* with a commercial broiler diet and had free access

to water. One week prior to experimentation, 32 birds were placed in a floor pen containing wood shavings, in a controlled climate chamber and maintained under the same environmental conditions as previously described. This procedure was undertaken to allow the birds to adjust to the novel housing conditions of the climate chamber, and to recover from any stress incurred due to handling and re-locating prior to the actual experiment (Lefebvre *et al.*, 1992; Mitchell and Carlisle, unpublished data).

Dantrolene administration

Dantrolene sodium was solublised in a solution of 5% mannitol (275 mM) and adjusted to pH 8.0 with 0.4M sodium hydroxide (NaOH) at 20°C to a give a final dantrolene concentration of 1 mg ml^{-1} (Lopez *et al.*, 1987). Dantrolene solution was prepared in an aluminium foil covered flask to prevent possible photodegradation (Fawcett *et al.*, 1994) at least 2 h prior to injection to ensure complete dissolution of the drug. Control vehicle solution was prepared in exactly the same way but without the addition of dantrolene sodium.

Food and water were withdrawn 3 hours prior to treatment on the day of the experiment. 16 birds were randomly selected for injection with 2.5 mg kg^{-1} dantrolene sodium solution (D). The remaining 16 birds received an equivalent body weight-dependent volume of dantrolene vehicle solution (V). Blood samples (2.5 ml) were taken immediately prior to injection from the brachial vein of the left wing of all the birds. Rectal temperature measurements were also taken prior to drug injection. The birds were injected in the brachial vein of the opposite wing with a single bolus dose of either D or V. The injection of dantrolene was administered 2 hours prior to exposure to heat stress to allow time for the incorporation of the drug into the target tissues (Klont *et al.*, 1994).

Heat stress protocol

After this period of time, 8 birds from each of the two treatment groups were transferred equally in to 2 commercial poultry transport containers and placed in a controlled climate chamber at 32°C/80% RH for a period of 2 hours (heat stress; HS). The remaining birds from each treatment group were also transferred into 2 commercial poultry transport containers and placed in a controlled climate chamber at 21°C/50% RH for the same duration (control temperature; C). Container micro-environment was monitored at 1 minute intervals using TinyTalk® II temperature and humidity loggers (RS Components, UK).

Blood samples and deep body temperatures were taken immediately after the heat stress exposure (0 h). Deep body temperature was measured by the insertion of an electronic temperature probe (RS Components, UK) 2.5 cm into the rectum. The birds were returned to their original floor pen to recover, with free access to food and water. Additional blood samples and rectal temperature measurements were taken 6h and 24 h post-heat stress as pervious work in these laboratories by Neil and Mitchell (unpublished observations) demonstrated a peak in plasma CK activity at 6h in broilers subjected to the same standard HS challenge, and which had returned to pre-HS levels by 24 h.

All blood sample collection was according to the protocol described in the general materials and methods section of this thesis (**see Chapter 2**). Briefly, 2.5 ml blood samples were taken and transferred into heparinised blood tubes (Teklab) and immediately placed on ice. Following whole blood analyses, plasma samples were prepared by centrifugation of the blood at 1500g for 5 minutes, then immediately frozen at -20°C pending analysis.

Whole blood measurements

The measurement of whole blood constituents such as carbon dioxide (as pCO_2), pH, unbound calcium (Ca^{2+}), sodium (Na^+) and potassium (K^+) was performed using clinical analysers (**See Chapter 2**). All measurements were determined on chilled bloods within approximately 1 minute of withdrawal using clinical analysers (CIBA-Corning; Models 238, 614 and 634, Halstead, UK) with a body temperature and pH correction (Hocking *et al.*, 1994). The analysers were set-up in an array to facilitate the rapid measurement of these blood constituents. The average time taken to process a sample through all three analysers was less than 1 minute. The rate limiting factor for successive measurements was the time taken to withdraw the blood sample.

Plasma analyses

Plasma creatine kinase (CK) activities, total plasma calcium and protein concentrations were determined using commercially available kits, modified for use in an automated plate reading spectrophotometer and adapted for use with avian plasma (**See Chapter 2**).

Statistical analyses

Where appropriate the data are presented as means ± one standard deviation (S.D.). Levels of significance were determined by analysis of variance or unpaired Students t-test.

Results

The 32 birds used in this experiment were divided into 4 treatment groups. The birds were initially divided into 2 groups of 16, and injected with either dantrolene sodium solution (D) or dantrolene vehicle (V) and then sub-divided within treatment to receive 2 h exposure to either control "thermoneutral" (C) or heat stress (HS) conditions. The possible combinations of injection+treatment giving rise to the following permutations: vehicle+control (VC), vehicle+heat stress (VHS), dantrolene+control (DC) and dantrolene+heat stress (DHS).

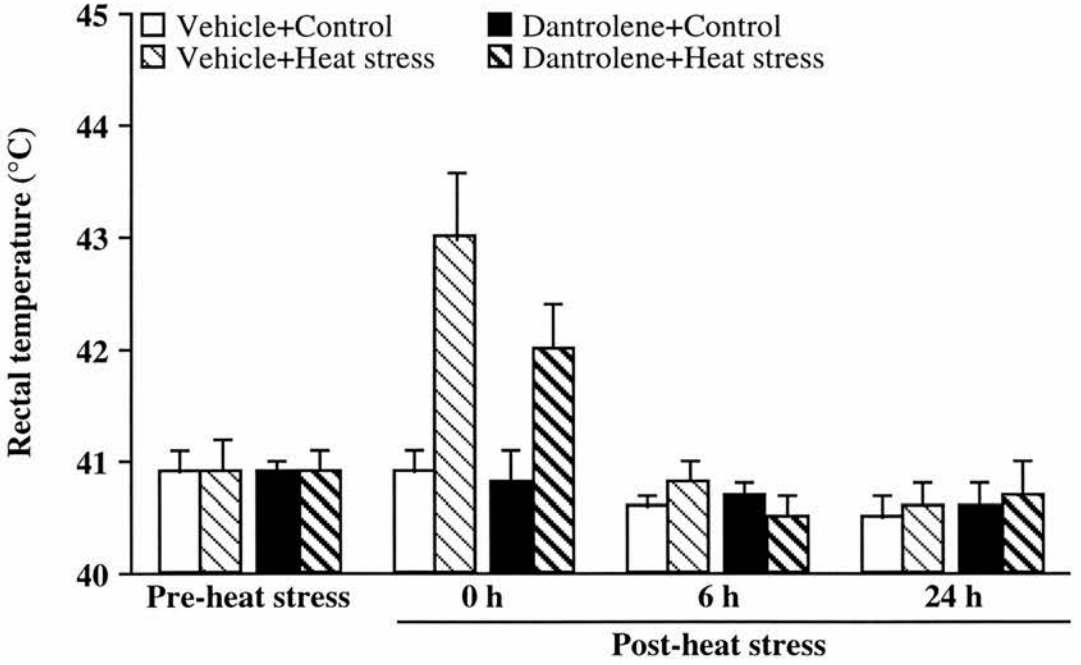

Figure 12.1 Effect of acute heat stress (HS) exposure (2h; 32°C/80% RH) on deep body temperature in broiler chickens injected with 2.5 mg kg^{-1} dantrolene sodium (D) or vehicle (V) Control environment birds (C) were kept at 21°C/50% RH. Values are presented as means ± SD for 8 birds per treatment.

Rectal temperature

Exposure to a high thermal load (32°C/80% RH) induced significant increases in the deep body temperature in both VHS and DHS birds (**see Figure 12.1**). Rectal temperatures increased by 2.4°C ($p<0.001$) and 1.3°C ($p<0.05$) respectively, reflecting a 84.6% greater increase ($p<0.001$) in deep body temperature in the VHS group. Post HS rectal temperatures had returned to pre-heat stress values in both groups by 6h. Deep body temperature measurements did not alter in either of the two treatment groups held under control conditions for 2 hours, or throughout the duration

of the study. Similarly, there was no difference in the pre-HS rectal temperature measurements between any of the 4 treatment groups. Average container temperatures and relative humidities in the control and heat stress groups were 31.6°C/76% RH and 21.2°C/43% RH respectively.

Plasma CK activity

Plasma CK activity was significantly elevated in the VHS birds but not in the DHS birds following HS challenge (**See Figure 12.2**). A 64.5% increase in CK activity was observed immediately post-HS (0 h) in the VHS birds, which increased three hours later to a value 70.8% greater that pre-HS activity. Plasma CK activity in this group had returned to VC levels by 24 h. A non-significant increase in CK activity was observed in the DHS birds compared with DC birds (10.8%, p=0.26) which roughly paralleled the profile of VC plasma CK activity. In the DC birds there was a noticeable reduction in plasma CK activity (28.1%) compared with VC birds although this observed decrease just failed to reach significance at a 95% confidence interval (p=0.061). No difference in pre-HS plasma CK activity was observed between any of the 4 treatment groups, mean CK activity being 518 ±112 IU litre^{-1}.

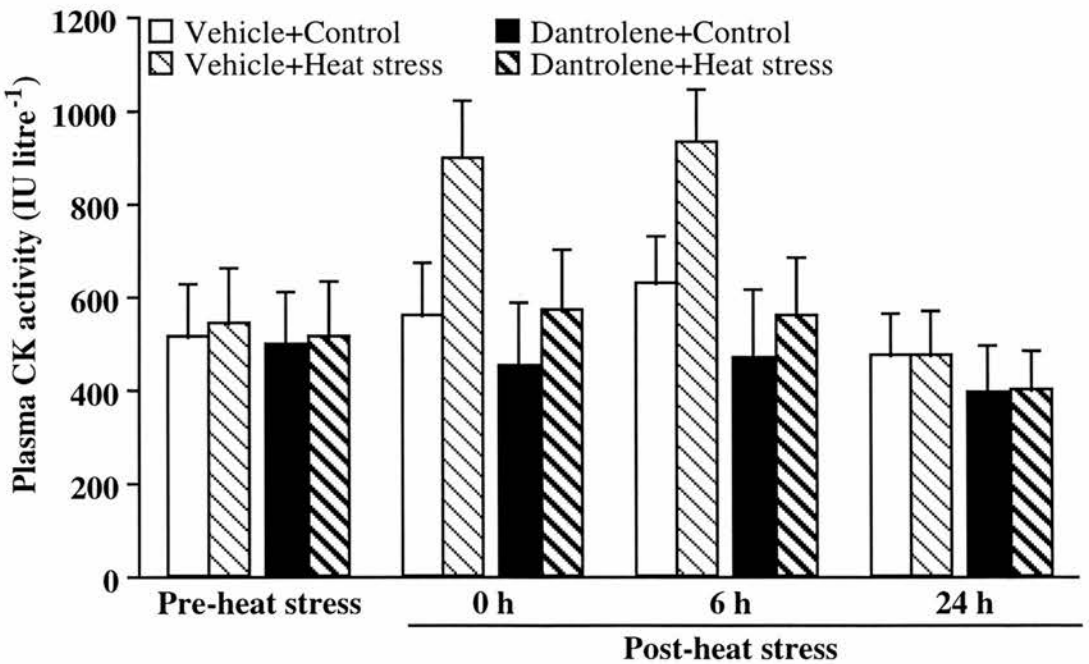

Figure 12.2 Effect of acute heat stress (HS) exposure (2h; 32°C/80% RH) on plasma creatine kinase (CK) activity in broiler chickens injected with 2.5 mg kg^{-1} dantrolene sodium (D) or vehicle (V). Control environment birds (C) were kept at 21°C/50% RH. Values are presented as means ± SD for 8 birds per treatment.

pCO_2 and pH

Comparable significant decreases in venous blood pCO_2 (hypocapnea) were observed in the VHS (32.0%; $p<0.001$) and DHS (34.1%; $p<0.001$) birds immediately following exposure to a high thermal load (**see Figure 12.3**). Slight decreases in pCO_2 were also observed in the VC (9.8%) and DC (15.0%) but failed to reach significance at $p<0.05$. By 6 h post-HS, blood pCO_2 values had return to pre-HS levels in both of the vehicle injected treatment groups, with the VHS showing classic signs of over-compensation. The pCO_2 values of the DC and DHS birds at 6 h and 24 h post-HS were similar to those observed for DC immediately post-HS (0 h). These values remained relatively constant, and were significantly lower than the either of the vehicle injected treatment groups throughout the post HS phase of the study; DC (14.0%; $p<0.05$), DHS ($p<0.01$). No difference in pre-HS pCO_2 was observed between any of the 4 treatment groups.

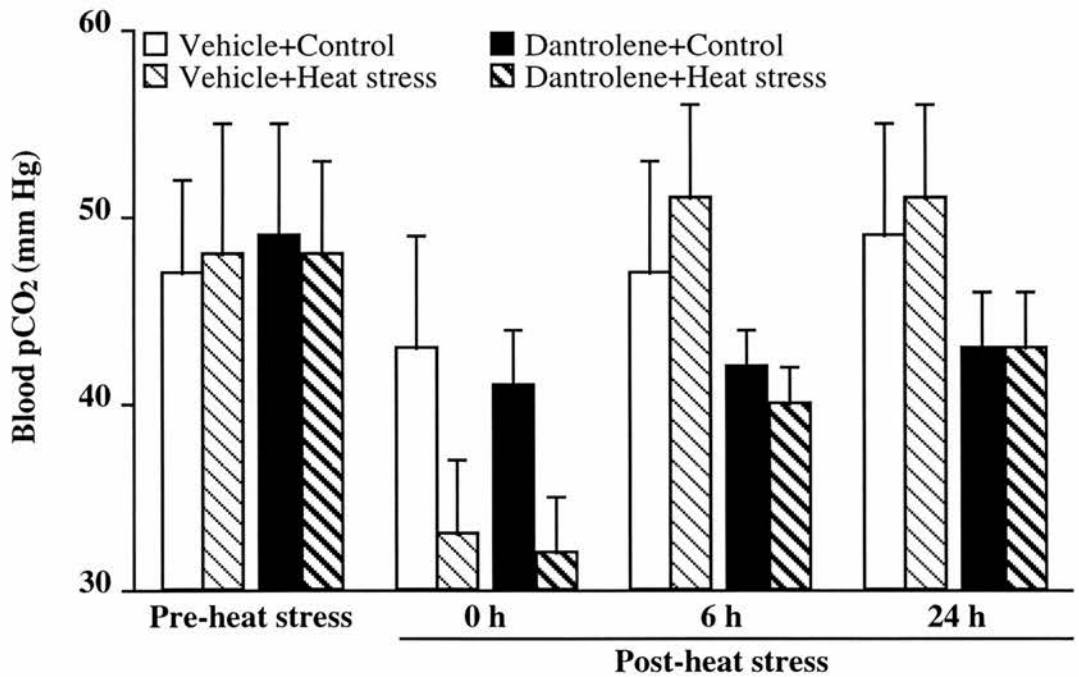

Figure 12.3 Effect of acute heat stress (HS) exposure (2h; 32°C/80% RH) on venous blood pCO_2 in broiler chickens injected with 2.5 mg kg^{-1} dantrolene sodium (D) or vehicle (V). Control environment birds (C) were kept at 21°C/50% RH. Values are presented as means ± SD for 8 birds per treatment.

Associated with the HS induced hypocapnea were significant changes in blood pH (**see Figure 12.4**). The reduction in blood pCO_2 resulted in a marked increase in blood pH (respiratory alkalosis). Exposure to a high thermal load induced comparable significant increases in the blood pH ($p<0.01$) in both VHS (+0.15 units)

and DHS (+0.13 units) birds (0 h). A significant increase in blood pH was also observed at 0 h, in the DC birds (0.10 units, $p<0.05$), but not in VC blood pH, which was unaltered throughout the whole of the experimental period. By 6 h post HS, blood pH in VHS had returned to pre-HS levels, whereas the pH of both of the dantrolene injected treatment groups remained higher than their vehicle only treated counterparts; DC (+0.08 units; $p<0.05$), DHS (+0.11 units; $p<0.001$). Essentially the same differences in blood pH were still apparent in the vehicle and dantrolene injected birds at 24 h post HS.

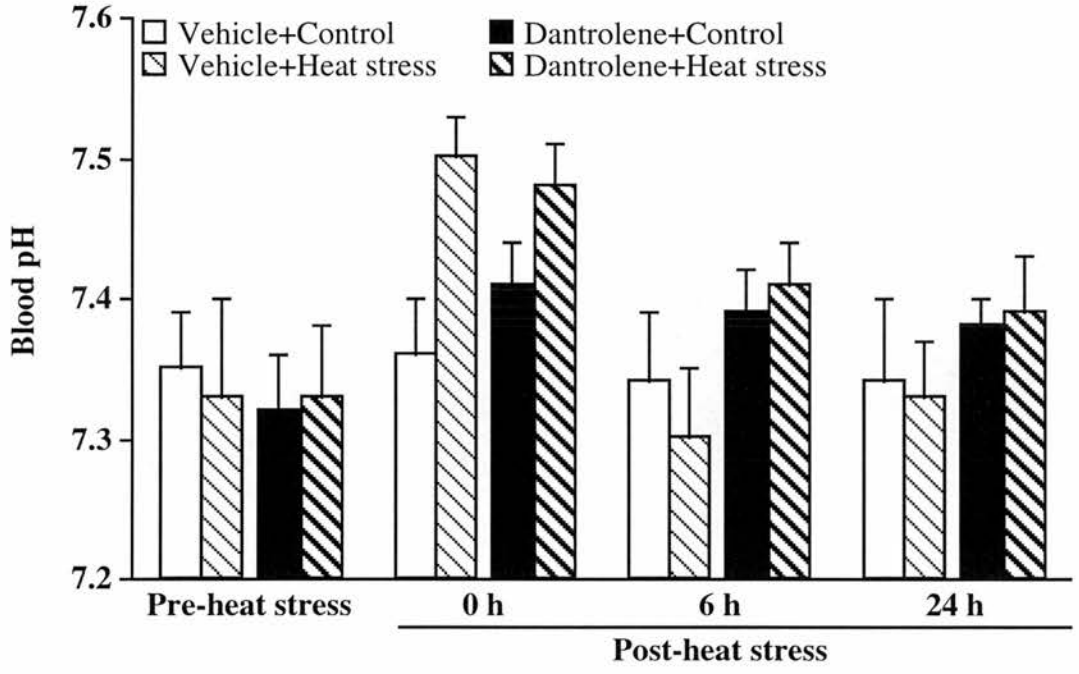

Figure 12.4 Effect of acute heat stress (HS) exposure (2h; 32°C/80% RH) on venous blood pH in broiler chickens injected with 2.5 mg kg^{-1} dantrolene sodium (D) or vehicle (V). Control environment birds (C) were kept at 21°C/50% RH. Values are presented as means ± SD for 8 birds per treatment.

Total and free calcium concentrations

No change in total plasma calcium concentration (TPCC) was observed immediately following HS exposure (0 h) in any of the 4 treatment groups (**see Figure 12.5**). Elevations in TPCC were subsequently observed 6 h post-HS in both groups of dantrolene injected birds. These increases were significant in the DHS birds ($p=0.04$), but not in the DC treated birds ($p=0.10$). The observed elevations in TPCC 24 h post HS in the two dantrolene injected groups were not significant at $p<0.05$. No changes in TPCC were seen in either of the vehicle injected groups over the duration of the study.

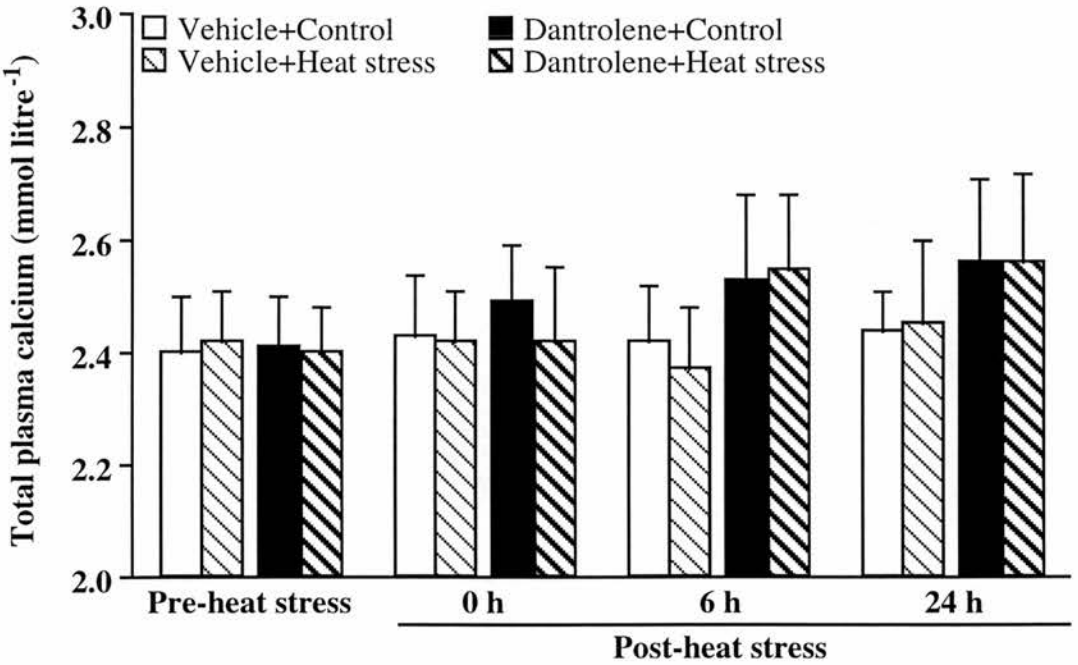

Figure 12.5 Effect of acute heat stress (HS) exposure (2h; 32°C/80% RH) on total plasma calcium concentration (TPCC) in broiler chickens injected with 2.5 mg kg^{-1} dantrolene sodium (D) or vehicle (V). Control environment birds (C) were kept at 21°C/50% RH. Values are presented as means ± SD for 8 birds per treatment.

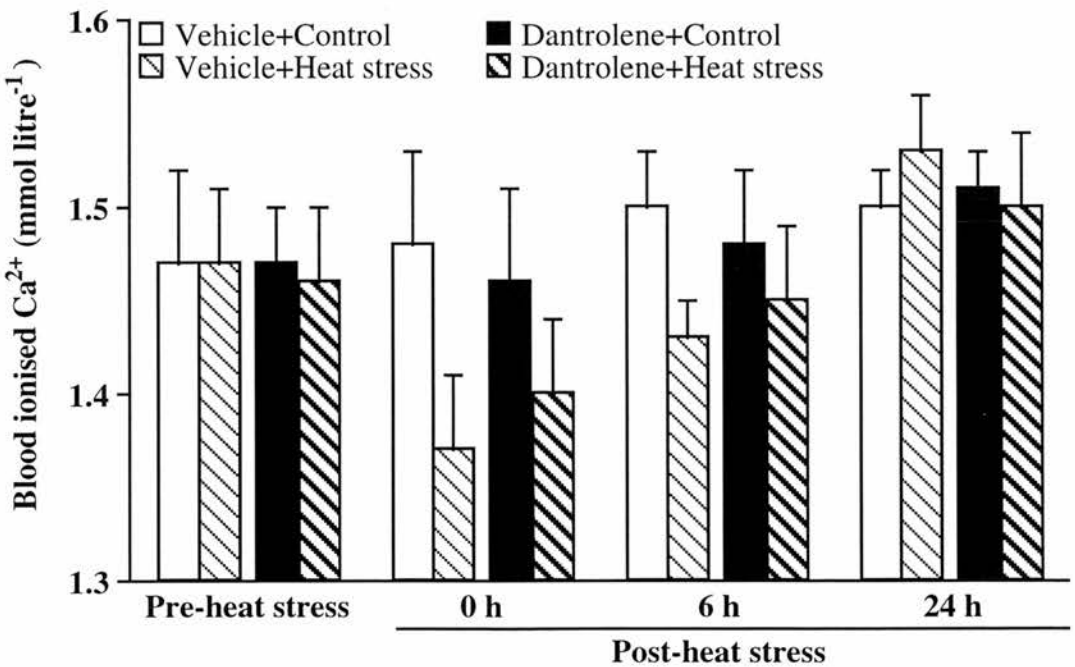

Figure 12.6 Effect of acute heat stress (HS) exposure (2h; 32°C/80% RH) on plasma free calcium (Ca^{2+}) concentration (PFCC) in broiler chickens injected with 2.5 mg kg^{-1} dantrolene sodium (D) or vehicle (V). Control environment birds (C) were kept at 21°C/50% RH. Values are presented as means ± SD for 8 birds per treatment.

Reductions in plasma free calcium (Ca^{2+}) concentration (PFCC) were observed in the VHS (-0.12 mmol litre^{-1}; $p<0.001$) and DHS (-0.09 mmol litre^{-1}; $p=0.001$) birds immediately following exposure to HS (**see Figure 12.6**). The reductions in PFCC were still apparent 6 h post-HS in the two HS exposure groups, but were only significant in the VHS birds (-0.07 mmol litre^{-1}; $p<0.01$). No difference in PFCC was observed between the 4 treatment groups 24 h post HS.

Sodium and potassium concentrations

No differences in plasma sodium (Na^+) concentration were observed in any of the 4 treatment groups immediately following HS exposure (0 h) nor at any of the subsequent post HS time intervals measured (**data not shown**). All plasma Na^+ concentrations were comparable with pre-HS values reflecting mean plasma sodium Na^+ concentration of 155 ±3 mmol litre^{-1}.

Significant decreases plasma potassium (K^+) concentration were observed in the VHS (9.6%; $p<0.001$) and DHS (8.9%; $p<0.001$) birds immediately following exposure to a high thermal load (**see Figure 12.7**). Slight decreases were also observed in the VC and DC but failed to reach significance at $p<0.05$. Strikingly different changes in plasma K^+ concentrations were observed in the VHS and DHS treated birds 6 and 24 h post HS. At 6 h post-HS, plasma K^+ concentration in the VHS birds had changed dramatically from an initial decrease at 0 h post-HS to a 10.2% increase ($p<0.001$; $\Delta K^+ = +0.82$ mmol litre^{-1}). This elevation in plasma K^+ was still apparent at 24 h post HS and had increased further (11.5% ; $p<0.001$) relative to VC. Conversely, plasma K^+ concentrations in the DHS birds remained decreased at 6 h post HS (7.0%; $p<0.001$) increasing only slightly by 24 h post HS. Comparison of VHS and DHS treated birds at 6 and 24 h post HS revealed absolute differences of 0.91 ($p<0.001$) and 0.88 ($p<0.001$) mmol litre^{-1} in plasma K^+ concentration respectively.

Protein concentration

No differences in total plasma protein concentration were observed in any of the 4 treatment groups immediately following HS exposure (0 h) nor at any of the subsequent post HS time intervals measured (**data not shown**). All plasma protein concentrations were comparable with pre-HS values reflecting mean plasma protein concentration of 65.4 ±2.6 mmol litre^{-1}.

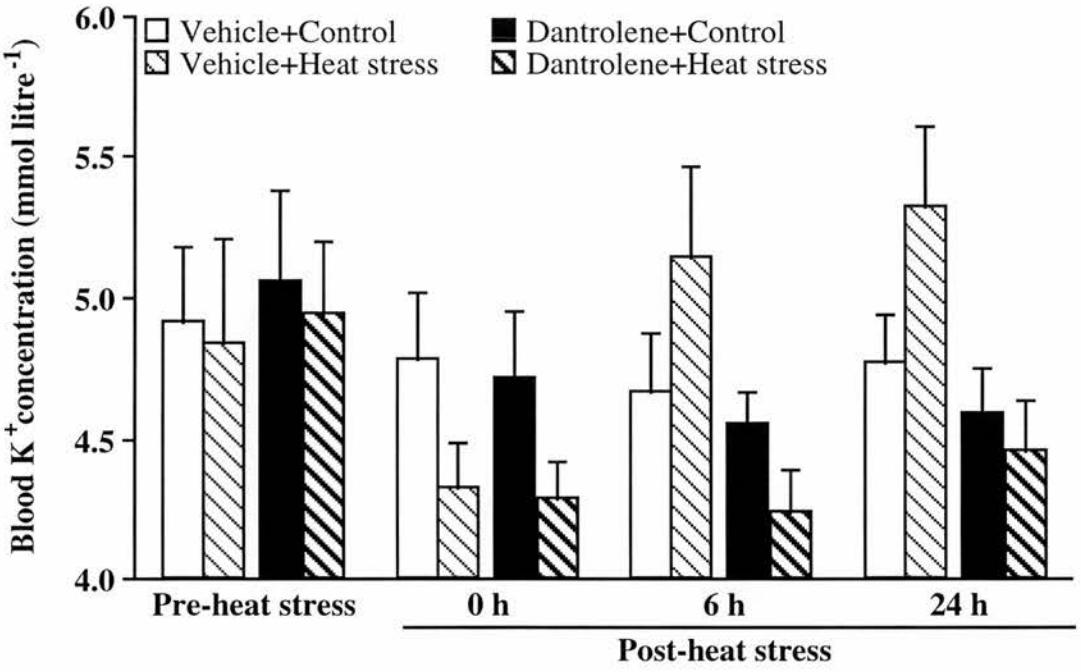

Figure 11.7 Effect of acute heat stress exposure (2h; 32°C/80% RH) on blood K^+ concentration in broiler chickens injected (i.v.) with 2.5 mg kg^{-1} dantrolene sodium (D) or vehicle (V). Control birds (C) were kept at 21°C/50% RH. Values are presented as means ± S.D for 8 birds per treatment.

Discussion and conclusion

The primary aim of the present study was to examine the effects of dantrolene sodium on the release of CK from skeletal muscle *in vivo*, in chickens subjected to a standardised period of acute heat stress (HS) exposure. In addition, plasma acid-base values and plasma metabolite and electrolyte concentrations were determined to establish if any observed changes in these parameters due to exposure to acute HS might be connected with the development of skeletal muscle damage and subsequent CK loss *in vivo*.

The exposure to acute HS (2 h; 32°C/80% RH) induced a hyperthermia in both dantrolene sodium (D) and vehicle (V) injected birds. The degree of hyperthermia attained in the VHS birds was +1.1°C greater than in the DHS birds. The deep body temperature obtained in the VHS birds were similar to values previously reported in **Experiment II** of this thesis (**see Chapter 3**) and with other studies undertaken in these laboratories using the same standardised HS protocol (Mitchell *et al.*, 1990, 1992; Sandercock and Mitchell, 1996). The apparent body temperature-reducing effect of dantrolene sodium on HS-induced increases in deep body temperature have not previously been demonstrated in broiler chickens subjected to this or any other type of stressor. The body temperature-reducing effects of dantrolene sodium have

however previously been reported in studies in MH susceptible humans and pigs (Britt, 1977; Gronert, 1980; Ørding, 1988; MacLennan and Philips, 1992), where its effects were attributable to the attenuation of hypermetabolic heat production in skeletal muscle caused by SR RyR Ca^{2+} cycling (MacLennan and Philips, 1992; Block, 1994; Wedel *et al.*, 1995). Dantrolene has also been shown to attenuate tension independent heat production in isolated fast and slow twitch muscles of non-MH susceptible mice (Wendt and Barclay, 1980). These authors have proposed that since tension-independent heat is the thermal representation of the processes involved in excitation-contraction (EC) coupling, then dantrolene interferes with the normal sequence of events in EC coupling through the inhibition of SR mediated Ca^{2+} release. More recently, Block (1994) has shown that in fish muscles selected for heat generation, muscle derived thermogenesis can be achieved without contraction a process called excitation-thermogenic (ET) coupling. Whether such a thermogenic mechanism occurs in avian skeletal muscle is not yet known. However there is evidence of a paradoxical increase in metabolic heat production in broiler chickens during exposure to acute heat stress (Sandercock *et al.*, 1995). It has been proposed that the increase in heat production observed in this study may be due to muscle hypermetabolism (as reflected by high CK loss) coupled with a possible Q_{10} effect.

Increases in the plasma CK activity of vehicle (V) injected birds subjected to acute HS exposure were consistent with a marked degree of post HS skeletal muscle damage (**see Figure 12.3**). The CK responses were immediate and sustained for up to 6 h post HS and returned to pre HS levels by 24 h. The magnitude of elevations in plasma CK activity in the VHS immediately following HS exposure were consistent with values obtained in **Experiment II** (**see Chapter 3**) and with previously cited studies undertaken by Mitchell *et al.* (1990, 1992) and Sandercock and Mitchell (1996). Conversely in the dantrolene injected (DHS) birds, there was virtually no increase in plasma CK activity. Unlike the VHS treated group there appeared to be an almost complete inhibition of HS induced skeletal muscle membrane damage. The pattern of DHS post HS CK plasma activity was comparable with that of the VC birds. To date, there have been no other reports of dantrolene mediated reductions in HS induced skeletal muscle damage in the broiler chickens. These results were however consistent with several previous mammalian studies in which dantrolene treatment prevented elevations in plasma and serum CK activities. Bjurstom *et al.* (1995) demonstrated that serum levels of CK did not increase in "stress" susceptible (MH) pigs subjected to experimental restraint stress when injected intravenously with dantrolene (5 mg/kg). Dantrolene was shown to reduce skeletal muscle CK release in MH-positive patients (Antognini, 1995). Interestingly, these reductions in plasma CK

were not observed in patients undergoing common surgical procedures in which tissue damage was incurred. Dantrolene treatment was also shown to significantly reduce plasma CK activity associated with rhabdomyolysis in patients suffering with neuroleptic malignant syndrome or "NMS" (Jauss *et al.*, 1996; Laurence *et al.*, 1996; Kornhuber and Weller, 1996). *In vitro* studies by Pagala *et al.* (1997) have also demonstrated dantrolene reduces enhanced leakage of CK caused by ethanol, cocaine and chronic stimulation in isolated fast and slow muscles of the rat. Increases in plasma CK activity in rabbits caused by repetitive blood sampling were not found to be significantly reduced after treatment with 1.0 mg/kg dantrolene (Lefebvre *et al.*, 1992). In view of the demonstration of the effect of dantrolene on plasma CK activity in the present study, it is proposed that heat stress induced skeletal muscle damage in the broiler chicken may be caused by heat stress stimulated Ca^{2+} release from skeletal muscle SR through ryanodine-sensitive (RyR) Ca^{2+} channels.

Heat stress induced changes in blood pCO_2 and pH were consistent with previous heat stress studies in broiler chickens (Ait-Boulahsen, 1989, 1995; Sandercock *et al.*, 1995) and in laying hens (Koeklebeck and Odom, 1994, 1995). The degree of hypocapnea produced by thermal panting was approximately the same in both dantrolene and vehicle injected birds subjected to acute heat stress (**see Figure 12.3**). These results would suggest therefore that thermoregulatory effort expended by both treatment groups in an attempt to minimise increases in deep body temperature was similar. Indeed concomitant elevations in blood pH of equal magnitude would tend to support this hypothesis (**see Figure 12.4**). Whilst blood pCO_2 had returned to normal pre-heat stress levels 6 h after heat stress in the vehicle injected birds, pCO_2 levels in the dantrolene injected birds remained consistent and significantly lower (14-18%) up to 24 h after heat stress and were comparable with VC post-heat stress pCO_2 levels. These trends were mirrored in the pH measurements of dantrolene injected birds where their blood pH values remained consistently higher than the vehicle only injected birds. Why dantrolene treatment should cause a sustained reduction in plasma pCO_2 is not clear. There are two possible mechanisms by which this phenomenon could be achieved. First, by a sustained hyperventilated condition which would eliminate CO_2 leading to a rise in plasma pH (respiratory alkalosis) or secondly, excess base production (metabolic alkalosis). The first of these two explanations is unlikely as one might expect the actions of a muscle relaxant to induce a hypoventilation and thus promote an increase plasma pCO_2. In the absence of any measurement of base status it can not be said for sure if excess base production is a factor. It may also be possible that through its action, dantrolene may affect muscle acid production, as one its reported effects *in vivo* is a reduction in lactacidosis

seen in MH susceptible individuals resulting from the stimulation of muscle hypermetabolism (O'Brien *et al.*, 1990; MacLennan, 1992). Although this analysis was performed on venous, and not arterial blood samples in the present study, this is an accepted method of the assessment of pCO_2 and pH status (Hocking *et al.*, 1994).

Neither exposure to acute heat stress nor injection with dantrolene caused any significant change in total plasma calcium concentration (TPCC) immediately post heat stress, although there was some evidence of an increase in TPCC in the dantrolene injected birds 6 and 24 h later. This increase, although non-significant at $p<0.05$, is difficult to explain but presumably reflects an effect of the dantrolene on calcium metabolism. The rise in TPCC caused by dantrolene may be due to an effect on calcium absorption from the gut or increased mobilisation from bone. These results are in agreement with Koelkebeck and Odom (1995) who reported no change in total plasma calcium concentrations in 50 week-old laying hens exposed to acute heat stress. The reduction in plasma free calcium concentration (PFCC) in the two groups of birds subjected to high thermal load is cosistent with the results of previous heat stress studies in domestic fowl (Odom *et al.*, 1986; Ait-Boulahsen, 1989; Bogin *et al.*, 1996). These observations reflect the consequences of an increase in plasma pH caused by respiratory hypocapnea in the heat stressed panting bird. The degree of binding of calcium in the plasma is dependent on pH. The binding affinity of the primary plasma binding protein, albumin is increased by an elevation in plasma pH thus lowering free calcium (Ca^{2+}) availability. The alkalosis that lowers the PFCC has also been shown to produce contractures in isolated rat muscle due to the stimulation of SR calcium release (Ren *et al.*, 1993). The findings of Ren *et al.*, (1993) may offer a possible mechanism for how heat stress induced skeletal muscle damage in chickens may mediated *in vivo*. In addition, reductions in plasma free calcium may also affect the integrity of the plasma membranes of excitable tissues such as skeletal muscle, where a reduction in PFCC can reduce the stabilising action of Ca^{2+} on the membranes (Bowman and Rand, 1982), which as discussed in **Chapter 9** may alter the CK release process.

The reduction in plasma K^+ concentration observed in the two groups of chickens (**see Figure 12.8**) exposed to high thermal load was consistent with previous findings of Huston, (1978) and Ait-Boulahsen *et al.* (1989). This decrease may be explained by a K^+ shift between muscle and extracellular fluid (ECF) during acute hyperventilation (Kaufman and Papper, 1983). The reversal in plasma K^+ status in the VHS birds from 6 h post heat stress is indicative of cellular damage with the subsequent leakage of K^+ out of cells (Edens, 1977). This type of acute episodic increase in plasma K^+ concentration is usually associated with loss of K^+ from skeletal

muscle and as such is consistent with the profile of plasma CK activities demonstrated in **Figure 12.2**. The fact that plasma K^+ concentrations in the dantrolene treated remained lower than normal would further support this suggestion. The sustained reduction in plasma K^+ concentrations observed in the dantrolene injected birds may also be a function of plasma pH as prolonged alkalosis leads to the loss of K^+ in the urine (Oster *et al.*, 1978). In the absence of any changes in plasma protein and Na^+ concentrations (**data not shown**) it is unlikely that these effects can be explained by haemodynamic changes, which it has been reported accompanies heat stress in mammals (Parker *et al.*, 1971). Studies by Koike *et al.* (1983) have shown that chickens differ from mammals in this respect in that they have a much greater ability to maintain vascular volume through a higher conservation of Na^+ balance. No changes in plasma Na^+ were reported by Ait-Boulahsen *et al.* (1989) in 7 week-old broiler chickens exposed to acute HS (41°C/35% RH). Similarly, no changes in plasma Na^+ were observed in laying hens under the same type of thermal conditions (Arad *et al.*, 1983; Koelkebeck and Odom, 1995). The absence of an effect of acute heat stress on plasma protein concentration was consistent with previous studies undertaken in these laboratories by Mitchell (unpublished data) using the same standardised heat stress protocol, and with studies in laying hens by Koelkebeck and Odom (1995). These results however, did not agree with the findings of Ostrowski-Meissner (1981) who reported a decrease in total serum protein in 7 week-old cockerels exposed to 15 min thermal stress at 42°C/60%RH. Sex differences, and differences in the magnitude and duration of the heat stress challenge may account for the disparity in findings. The observation of a lack of effect of acute heat stress on plasma volume (hydration state) in the present study are indirectly supported by the findings of Ait-Boulahsen *et al.*(1989) who showed no changes in plasma osmolarity in acutely heat stressed fowl (see above).

In conclusion, the results of the present study show that injection with dantrolene sodium prior to acute heat stress exposure almost completely inhibits the increase plasma CK activity normally induced in broiler chickens by exposure to high thermal loads. These findings suggest that the mechanism of HS induced skeletal muscle damage involves stimulation of muscle SR Ca^{2+} release though the dantrolene inhibitable ryanodine (RyR) Ca^{2+} release channels. Transient disturbances in blood acid-base status induced by thermal panting, causing alkalosis and a reduction in plasma free Ca^{2+} availability may be responsible for triggering the intracellular Ca^{2+} release.

Chapter 13

General Discussion

The aim of this thesis was to investigate the causes and mechanisms of stress-induced increases in plasma CK activity in broiler chickens following exposure to high thermal loads. The experimental results have been discussed in detail in the previous chapters. The aim of this concluding chapter is to provide a general discussion of these findings and their possible implications.

In **Chapter 3** it was found that nearly all (>99%) of the total plasma CK activity found in the plasma of broiler chickens "under normal conditions" was of skeletal muscle origin (MM-CK), as determined by isoenzyme separation chromatography and that its activity increased in response to an episode of acute heat stress (**see Experiment II**). Thus confirming that the increase in CK activity reflected a possible detrimental consequence of stress on broiler chicken skeletal muscle. Whilst it is acknowledged that other researchers examining the effects of heat stress on domestic fowl have demonstrated increases in plasma CK activity (Ostrowski-Meissner, 1981; Bogin *et al.*, 1996) and implied that this observation may reflect skeletal muscle damage, it is believed that through characterising the plasma isoenzyme response, the work presented in **Chapter 3** is the first to actually confirm this in the fowl. Furthermore, it is clear that the anion exchange technique described in this thesis can efficiently separate the isoenzyme activities in mixtures of tissue extracts and plasma and can give quantitative recoveries (**see Experiment I**). The technique may therefore be used to provide distinct isoenzyme profiles in plasma which indicate the tissue origin of the CK activity. These profiles may prove invaluable in the assessment of tissue dysfunction and damage in a range of avian pathological conditions such as recognised muscle dystrophies and myopathies (Tripp and Schmitz, 1982, Cardona *et al.*, 1992, 1993; Baird *et al.*, 1997), cardiovascular diseases (Maxwell *et al.*, 1990; 1993) and lesions of the central nervous system (Bhaiyat *et al.*, 1995; Fuhrmann and Sallimann, 1995a, b).

The findings presented in **Chapter 3** therefore provided the justification for developing a suitable in vitro skeletal muscle preparation (*m tensor patigialis*) with which to elucidate the mechanisms of CK release in broiler chickens during acute stress (**see Chapter 4**). The technique of incubating isolated tissues has long proved useful in the investigation of many aspects of cellular physiology and biochemistry. In developing a skeletal muscle preparation and incubation system it was recognised that optimum tissue viability was critical, especially when investigating possible pathological mechanisms *in vitro*. The results of the first experiments suggest that

tissue viability in the preparation was very good, with respect to CK release, thus vindicating its use in these studies (**Experiment III**). Isolated chicken skeletal muscle preparations have been previously developed by other research groups (Klasing and Jarrell, 1984, 1985; Baracos *et al.*, 1989) to investigate physiological processes in this tissue (e.g. protein turnover), however there is limited evidence of their use in the study of pathological processes (Dawson, 1966; McLoughlin *et al.*, 1991).

In **Chapter 6** it was found that incubating skeletal muscles in the presence of 2,4 DNP, which inhibits oxidatively-derived ATP availability by uncoupling the process of oxidative phosphorylation in mitochondria, produced a significant elevation in CK release (**Experiment VI**). Prompting the suggestion that conditions which might produced impaired ATP availability *in vivo*, either through synthesis inhibition or excessive utilisation of ATP may promote the development of skeletal muscle damage in the broiler chicken during heat stress. These proposals are supported by the work of Jones *et al.*(1993) and Jackson *et al.*(1984) who have extensively examined the role of ATP availability and CK release in isolated mouse skeletal muscle. Unlike the research undertaken by these previous authors which examined the effects of the inhibition of ATP availability in muscle preparations comprised of differing relative proportions of myofibre type (i.e. *extensor digitorum longus*; type IIB, and *soleus*; type I), the investigations of these effects were restricted to a type IIB representative muscle only. Attempts were made at the out-set of the project to identify a suitable predominantly oxidatively functioning skeletal muscle in the broiler chicken but were unsuccessful. The validity of using an oxidative metabolism inhibitor on muscle that predominantly relies on glycolytic metabolism may be questioned. With the benefit of hindsight the use of glycolytic metabolism inhibitors such as iodoacetic acid or potassium cyanide (Jackson *et al.*, 1984) may have been more appropriate.

Perhaps the most significant finding presented in this thesis is the profound effect of an increase in cellular calcium ion (Ca^{2+}) content on the release of CK from skeletal muscle (**see Chapter 7**). The ubiquitous role of Ca^{2+} in many key physiological processes is widely recognised, however, its role in the development of cell injury or pathology is also becoming increasingly more apparent. The findings of the effects of 4Br-A23187 calcium ionophore on muscle CK release (**see Experiment VII**) are consistent with those of previous studies on mouse isolated skeletal muscle by Duncan and Jackson, (1987) and Phoenix *et al.*(1989). On the basis of these findings it is proposed that CK loss occurs as a consequence of Ca^{2+}-mediated alterations in muscle cell membrane integrity, possibly through the action of

some Ca^{2+} activated degradative process. In order to further examine this hypothesis, muscles were incubated in the presence of a novel phospholipase A_2 (PLA_2) inhibitor. The findings of **Chapter 8** demonstrated that incubation with the PLA_2 inhibitor significantly reduced the release of CK following an ionophore-mediated increase in intracellular Ca^{2+} concentration (**see Experiment VIII**). On the basis of these findings it is proposed that following sustained elevations in cytosolic calcium concentration ($[Ca^{2+}]_i$), muscle cell membrane hydrolysis is accelerated via increased Ca^{2+}-mediated PLA_2 activity. This may represent the decisive cellular end-point to a pathway that facilitates the release of CK from muscle cytosol into the extracellular environment. Attenuation of the loss of intracellular constituents using several well recognised inhibitors of PLA_2 activity (p-BPB and mepacrine) has been previously been demonstrated in several cell and tissue types, including skeletal muscle (Rice *et al.*, 1990; Ownby *et al.*, 1993), however it is believed that the results of the work presented in **Chapter 8** are the first demonstrate of the effects of Ro-31-4493/001 on skeletal muscle enzyme release in avian tissue. Whilst it has been shown in this thesis that Ca^{2+}-mediated alterations in cell membrane permeability increase the loss of CK to the extracellular environment, it is recognised that other intracellular Ca^{2+}-induced changes may also occur in skeletal muscle such as alterations to cytoskeletal organisation (Publicover *et al.*, 1978; Reddy *et al.*, 1983; Furuno and Goldberg, 1986; Goll *et al.*, 1991) and mitochondrial function (Wrogeman and Pena, 1976). It has been proposed that large increases in muscle intracellular calcium concentration may also produce different manifestations of skeletal muscle damage in addition to its deleterious effects on sarcolemmal membrane integrity (Helliwell *et al.*, 1994; Kuipers, 1994). These other Ca^{2+}-induced forms of skeletal muscle damage include sarcomeric ultrastructural disruption (e.g. Z-line dissolution), mitochondrial Ca^{2+} loading, and mitochondrial dysfunction and nuclear chromatin condensation and DNA fragmentation (Publicover *et al.*, 1978; Belcastro *et al.*, 1988; Bullard *et al.*, 1990; Trump and Berezesky, 1992; Nicotera *et al.*, 1992). It is evident that Ca^{2+} plays an important role in developing skeletal muscle damage as demonstrated by this and other research which has revealed some of the biochemical mechanisms whereby intracellular Ca^{2+} overload may activate several degradative processes. On the basis of the convincing *in vitro* findings of **Chapters 7** and **8**, it is tentatively proposed that the mechanism of CK release from the skeletal muscle of broiler chickens exposed to acute heat stress may be mediated in part by factors that promote sustained increases in $[Ca^{2+}]_i$ and ensuing PLA_2 activity. It is easy to envisage how changes in $[Ca^{2+}]_i$ induced by endogenous ionophore treatment or detrimental pharmacological manipulation might produce elevations in $[Ca^{2+}]_i$ in isolated skeletal muscle.

However, it is less apparent what mechanisms might mediate such relatively large changes *in vivo*.

All cells regulate $[Ca^{2+}]_i$ very closely, maintaining basal cytoplasmic levels some 10^{-5}-fold below extracellular concentrations by having a low plasma membrane permeability for the cation and actively extruding and buffering its intracellular concentration (Ashley, 1995). The primary routes of extracellular Ca^{2+} entry into skeletal muscle are via high-affinity, low capacity voltage-gated L-type Ca^{2+} channels (VOCs) which are depolarisation dependent, or mechanically-operated (MOCs) or tonically-activated (TACs) Ca^{2+} channels (Turner *et al.*, 1988; Franco and Lansman, 1990; Tsien and Tsien, 1990). The amount of calcium entry mediated by these gated mechanism is tightly regulated in skeletal muscle and functionally coupled to key physiological processes such as excitation-contraction coupling and maintaining active muscle tone (Tsien and Tsien, 1990). In order to control the close regulation of Ca^{2+} these processes operate a positive Ca^{2+} feedback mechanism, which permits the spatial propagation of information and ensures the cell responds appropriately when a localised stimulus is applied. This prevents an inappropriate accumulation of Ca^{2+} in the cells which is energetically costly, and which may a precipitate the development of cellular damage via secondary Ca^{2+}-activated degradative processes. It therefore seems unlikely that direct stimulation of Ca^{2+} entry via these mechanisms would produce increases in $[Ca^{2+}]_i$ of sufficient magnitude to induce CK loss from skeletal muscle, that have been demonstrated in this thesis. For this reason some other mechanism must be invoked. In the absence of gross mechanical tissue damage (e.g. tear or crush injury) it has long been proposed that the inability to maintain total cellular ion balance may be the primary cause of cellular perturbation. (Trump and Ginn, 1969, Trump and Arstila, 1975; Trump and Berezesky, 1989). In particular, increases in intracellular sodium (Na^+) concentration have often been regarded as the principal determinant of cellular ionic imbalance in excitable tissues such as skeletal muscle (Piper, 1989). It has been demonstrated *in vivo*, that the initial cause of Ca^{2+} intrusion into mammalian cardiac muscle cells following the onset of ischaemia and energy depletion is a loss of cellular Na^+ balance (Grinwald, 1982; Kleber and Wilde, 1986; Piper, 1989, Tani, 1990). These Na^+ induced changes in Ca^{2+} uptake were associated with ultrastructural alterations in plasma cell membranes and contractile apparatus (Tani, 1990). This possible common causal mechanism was subsequently investigated in the experiments presented in **Chapter 9**. *In vitro* manipulation of intracellular sodium concentration was achieved using the Na^+ ionophore monensin, which it is recognised can produce skeletal muscle damage (myopathy) in domestic fowl when administered in feed as an anti-coccidial agent (Dowling, 1992). A finding

that was confirmed in this thesis in **Chapter 11**. Concentration-dependent increases in Ca^{2+} uptake and CK release were observed in muscle incubated with the Na^+ ionophore. These results suggesting that by inducing increases in $[Na^+]_i$ it was possible to produce elevations in intracellular Ca^{2+} sufficient to mediate subsequent CK release (**see Experiment IX**). These findings were supported by studies by Shier and Dubourdieu (1992) who also reported concentration-dependent increases in cell killing (as determined by vital dye exclusion) caused by a direct increase in cytosolic Ca^{2+} concentration, mediated by monensin induced Na^+ influx into rat cardiac muscle cells. These findings of **Experiment IX** were further supported by two other experiments which demonstrated augmented increases in Ca^{2+} accumulation and CK release in monensin treated muscles exposed to elevated external Na^+ concentrations (**Experiment X**) and ouabain, an inhibitor of Na^+/K^+-ATPase, the primary mechanism of cellular Na^+ extrusion (**Experiment XI**). On the basis of these findings it was hypothesised that a potential mechanism by which Na^+ might mediate increases in Ca^{2+} uptake in skeletal muscle was via a process of Na^+/Ca^{2+} exchange (Allen *et al.*, 1989). The exchange mechanism can mediate Ca^{2+} fluxes in either direction across cell membranes, depending on the prevailing electrochemical gradients for Na^+ and Ca^{2+} (Reeves, 1991). This system provides a high capacity transport route for Ca^{2+} entry that is distinct from membrane Ca^{2+} channels (Caroni and Carafoli, 1983, Eisner and Lederer, 1985). Exchanger activity in muscle cells under normal conditions may be latent at basal Na^+ and Ca^{2+} concentrations (Smith *et al.*, 1989). The Na^+/Ca^{2+} exchange protein uses the electrochemical gradient for Na^+ into the cell to "pump' out Ca^{2+} with a stoichiometry of 3 Na^+ to 1 Ca^{2+}. Although it has been demonstrated that the exchanger is activated by normal depolarisation, this by itself does not translocate sufficient Ca^{2+} to activate Ca^{2+} induced SR Ca^{2+} release at normal intracellular Na^+ concentration (Lederer *et al.*, 1990). The normal physiological role of the exchange system is to pump Ca^{2+} out of the cell (forward-mode) using the inwardly-directed electrochemical gradient for Na^+ maintained by the Na^+/K^+-ATPase as an energy source (Reeves, 1991). It is suggested however that in monensin-mediated Na^+ loaded cells there is an associated alteration in the sarcolemmal potential difference which crosses the reversal potential for the exchanger, thus promoting the uptake of Ca^{2+} into the cell (reverse-mode). Whilst there have been demonstrations at a physiological level of the effects of monensin on Na^+/Ca^{2+} exchange mediated Ca^{2+} flux in skeletal muscle (Hoya and Venosa, 1992, 1995), its potential involvement in a pathological context had not previously been explored. It is proposed that findings of the aforementioned experiments in **Chapter 9** may be the first to implicate the Na^+/Ca^{2+} exchange mechanism in this process, in

avian skeletal muscle. It is acknowledged however, that several investigators have previously reported sarcolemmal damage and cellular enzyme loss associated with excessive Ca^{2+} entry, mediated via Na^+/Ca^{2+} exchange during reperfusion following ischaemia (Grinwald, 1982; Tani, and Neely, 1988; Pike *et al.*, 1988) and during reoxygenation after hypoxia (Murphy *et al.*, 1988) in isolated cardiac muscle cells. Implication of the Na^+/Ca^{2+} exchange mechanism in the damage process in these studies was based on nuclear magnetic resonance (NMR) and ion selective micro-electrode measurements of elevations in cytosolic free Na^+ concentration. These findings prompted a suggestion by Tani (1990) that if the $[Na^+]_i$ was important for Ca^{2+} overload in muscle cells then the elevation in Na^+ must be sustained in order facilitate sufficient Ca^{2+} ingress through the Na^+/Ca^{2+} exchange mechanism. What Na^+ concentration or potential threshold is required to promote Na^+/Ca^{2+} exchange-dependent Ca^{2+}-mediated skeletal muscle membrane degradation is not yet known, and would be difficult to determine from the results obtained in **Chapter 9** in the absence of any measurement of intracellular Na^+ concentration. Although, ten-fold increases in cytosolic Ca^{2+} uptake producing enhanced intracellular enzyme loss have been reported by Tani (1990), following a 4-fold rise in $[Na^+]_i$ (~80 μmol g^{-1} wet weight) in isolated rat cardiomyocytes after 30 minutes ischaemia + reoxygenation. It is clear however from several of the findings of this thesis and from what has been previously demonstrated in other studies on cardiac tissue, that factors that impair cellular Na^+ balance such as metabolic inhibition, hypoxia, ischaemia and re-oxygenation, exercise or treatment with Na^+ selective ionophore anti-biotics can produce elevations in cytosolic Ca^{2+} accumulation that are sufficient to promote intracellular enzyme efflux.

In order to confirm the involvement of the Na^+/Ca^{2+} exchanger in the mechanism of CK release from skeletal muscle, muscles were incubated in the presence of amiloride (Kleyman and Cragoe, 1988), a reported inhibitor of the Na^+/Ca^{2+} exchange protein (**see Experiment XII**). Unfortunately, the results of this experiment showed the inhibitor had no difference in effect on either Ca^{2+} uptake or CK release compared to either control muscles or muscles in which Na^+ extrusion had been prevented using ouabain (**see Figure 9.5**). In the absence of an effect of amiloride on the two parameters measured it is proposed that the concentration of inhibitor used in this experiment may not have been high enough to promote inhibition of exchanger mechanism in this tissue or amiloride may not be effective in chicken skeletal muscle. These suggestions are supported to some extent by several reports which provide evidence of species and tissue differences in inhibitor potency and specificity on the Na^+/Ca^{2+} exchange mechanism (Kaczorowski *et al.*, 1989; Smith *et*

al., 1989; Northover, 1992; Welsh and Lindinger, 1996). Whilst there have been no apparent previous investigations of the effect of amiloride on Na^+/Ca^{2+} exchange in avian skeletal muscle, its inhibitory effects at a physiological level have been reported in amphibian and mammalian skeletal muscle (Vigne *et al.*, 1982; ; Hoya and Venosa, 1992, 1995; Dorup and Clausen, 1996) using amiloride concentrations of 5 mM or higher. Therefore, on the basis of the findings in **Experiment XII**, no firm conclusions could be drawn about the possible involvement of the Na^+/Ca^{2+} exchanger in the mechanism of skeletal muscle CK release by trying to directly inhibit its action using amiloride. However, it was thought that in addition to investigating the Na^+/Ca^{2+} exchange mechanism using amiloride inhibition, its role could also be examined indirectly, in Na^+ loaded muscle cells, by reducing external free Ca^{2+} availability for translocation via this mechanism. If according to the proposed hypothesis the Na^+/Ca^{2+} exchange mechanism was involved in the process of Na^+-mediated Ca^{2+}-induced CK release in chicken skeletal muscle, then in the absence of external Ca^{2+} availability there should a reduction in CK release. However, in muscles incubated in medium in which the free Ca^{2+} availability had been significantly reduced (>95%) using BAPTA (**see Experiment XIII**) was an apparent paradoxical increase in CK release in the Na^+ loaded muscle cells in the absence of external Ca^{2+} entry. From the findings of **Chapters 7** and **8** it had demonstrated unequivocally that the process of CK release from avian skeletal muscle was Ca^{2+}-mediated, therefore it was proposed that in the absence of the adequate Na^+ translocation and/or extrusion, the cytosolic increase in Na^+ was perhaps mediating the release of Ca^{2+} from an intracellular source such as the muscle sarcoplasmic reticulum (SR). This hypothesis was supported by the findings of several other *in vitro* studies which demonstrated increases in $Ca^{2+}{}_i$ accumulation resulting from its release from SR stores in response to increases in $[Na^+]_i$, in the absence of extracellular Ca^{2+} entry (Leblanc and Hume, 1990; Rios and Pizzaro, 1991; Gorczynska and Handelsman, 1993; Borin *et al.*, 1994). Again, whilst this hypothesis explained the results of **Experiment XIII** it did not provide any additional information as to the possible involvement of the Na^+/Ca^{2+} exchanger in the CK release process. On the basis of these findings it is apparent that the mechanism of CK release from avian skeletal muscle may not necessarily rely on a large influx of external Ca^{2+} to produce changes sarcolemmal integrity that lead to CK loss and that under conditions of reduced external Ca^{2+} availability the process of CK release can be mediated via an increase in $[Ca^{2+}]_i$ via its release from an intracellular source. These newly proposed hypotheses were investigated **in Chapter 10** under conditions of normal external calcium availability.

The results of **Experiment XV** showed that incubation with the SERCA inhibitor thapsigargin, in the absence of external Ca^{2+} entry, produced an significant increase in CK release from isolated chicken skeletal muscle. This would suggest that the entry of external Ca^{2+} may not necessarily mediate the release of CK, and that Ca^{2+} induced muscle CK loss can be mediated by the release of intracellular Ca^{2+} from intracellular SR stores. It is apparent however, from the results illustrated in **Figure 10.1** that in muscles incubated only with thapsigargin under conditions of normal external Ca^{2+} availability that there was evidence of a stimulation of an increase in external Ca^{2+} uptake. An observation that has also been reported in other *in vitro* studies where thapsigargin inhibition of SERCA activity promoted external calcium entry at a physiological level and in other tissues including mammalian skeletal muscle (Sagara and Inesi, 1991: Sagara *et al.*, 1992; Xuan *et al.*, 1992). On the basis of these studies it has been suggested that the release of Ca^{2+} from SR stores may be coupled to the activation of external Ca^{2+} entry, via a capacitative Ca^{2+} entry process (Putney, 1990). The precise nature of which is yet known, but is believed involve membrane localised Ca^{2+} channels. Whether in thapsigargin treated muscles, such a precise homeostatic mechanism operates to facilitate the relatively high levels of external Ca^{2+} entry associated with myopathy is not clear. Although it would seem more likely that the relatively high level of external Ca^{2+} entry may occur as a secondary consequence of events mediated by the initial release of Ca^{2+} from the SR, possibly though Ca^{2+} mediated alterations in sarcolemmal permeability. The results of **Experiment XIV** demonstrated that thapsigargin mediated inhibition of SERCA activity in isolated chicken skeletal muscle promoted a significant increase in CK loss, possibly through a combination of SR mediated Ca^{2+} release and external Ca^{2+} uptake. The effects of thapsigargin on muscle CK release were exacerbated in the absence of external Ca^{2+} entry, thus prompting the suggestion that the activation of external Ca^{2+} entry may in its self be mediated by the initial release of Ca^{2+} from SR stores.

Having demonstrated it was possible to stimulate the release of CK from isolated skeletal muscle by re-compartmentalising the Ca^{2+} from SR stores, the second of the experiments undertaken investigated the possible role of the SR ryanodine-sensitive Ca^{2+} release channel (RyR) as a mediator of SR Ca^{2+} release under metabolically stressful conditions. The process of Ca^{2+} release from skeletal muscle SR *in vitro* has been shown to be regulated by the gating properties of the RyR Ca^{2+} release channels and to a lesser extent, the Ca^{2+} gradient across the SR membrane (Volpe and Simon, 1990). In **Experiment XVI**, the incubation of muscles with dantrolene sodium reduced monensin-induced increases in CK efflux by approximately 35% (**see Figure 10.2**). These results not only confirmed that the

release of CK from chicken skeletal muscle could be mediated by triggering the mobilisation of Ca^{2+} from SR Ca^{2+} stores, they demonstrated that the mechanism through which this process was occurring was via the ryanodine-sensitive Ca^{2+} release channels (RyR) of the SR. These findings were supported by several previous *in vitro* demonstrations of the inhibitory effects of dantrolene on skeletal muscle RyR channel Ca^{2+} release and subsequent cellular damage in studies on isolated mammalian and porcine skeletal muscle (Allen *et al.*, 1992, Klont *et al.*, 1994; Pagala *et al.*, 1997). It is believed that the results of **Experiment XVI** are the first to demonstrate these effects in avian skeletal muscle.

In conclusion, from the results of the *in vitro* investigations presented in **Chapters 4-10** it was therefore established that factors promoting disturbances in intracellular calcium (Ca^{2+}) regulation, leading to sustained elevations in sarcoplasmic free Ca^{2+} concentration, induced Ca^{2+}-dependent increases phospholipase A_2 activity which subsequently produced alterations in muscle cell membrane permeability leading to enhanced loss of CK (**see Chapters 7 and 8**). It was demonstrated that increases in sarcoplasmic Ca^{2+} concentration could be mediated through both the entry of extracellular Ca^{2+} via the process Na^{+}/Ca^{2+} exchange (**see Chapter 9**) and by Ca^{2+} release from SR stores via ryanodine-sensitive (RyR) Ca^{2+} release channels (**see Chapter 10**). These results further suggested that the process of external Ca^{2+} entry may occur as a secondary consequence of Ca^{2+} mediated alterations in sarcolemmal integrity cause by the initial stimulation of Ca^{2+} release from SR Ca^{2+} stores. In the light of these findings it was then suggested that stress induced skeletal muscle damage and CK release in the broiler chicken *in vivo*, may be caused by increases in muscle sarcoplasmic free Ca^{2+} concentration mediated by SR RyR Ca^{2+} release channels. If these proposed mechanisms are involved in the release of CK from chicken skeletal muscle following exposure to acute heat stress then their effects should be inhibited by the action of dantrolene in vivo.

The results of **Experiment XVIII** clearly demonstrated the absence of an increase in plasma CK activity in dantrolene treated birds exposed to an episode of acute heat stress. This compared with an 85% increase in plasma CK activity in the birds not receiving dantrolene but exposed to the same environmental conditions. These results corroborated the *in vitro* findings of **Chapter 10** which had suggested that the mechanism of heat stress induced skeletal muscle CK release may be mediated by the stimulation of muscle SR Ca^{2+} release via ryanodine-sensitive (RyR) Ca^{2+} release channels. In addition, the results of **Experiment XVIII** also demonstrated that the transient disturbances in blood acid-base status induced by thermal panting, causing alkalosis and a reduction in plasma free Ca^{2+} availability may be responsible

for triggering intracellular SR Ca^{2+} release. A finding which is supported by the earlier *in vitro* findings presented in **Chapter 9** (**see Experiment XIV**) which demonstrated substantial increases in the amount of CK released from metabolically stressed muscle under conditions of reduced external calcium availability.

In summary it is therefore proposed that acute heat stress induces Ca^{2+} release from the SR in chicken skeletal muscle via the ryanodine-sensitive channels which in combination with the entry of external Ca^{2+} possibly through the process of Na^{+}/Ca^{2+} exchange activates PLA_2 mediated alterations in sarcolemmal permeability which facilitates the intracellular loss of CK.

Possible Implications of Research

Animal Welfare

Given that the findings of this research have demonstrated the biochemical and physiological mechanisms of a tissue pathology caused by the exposure to an acute stress situation, it is not unreasonable to assume that they may have implication for animal welfare. In the light of rationalising these findings and their implications in a animal welfare context it may be argued that because the birds are slaughtered shortly after being subjected to the stressful ordeal of being transported that the post stress exposure manifestations of are of little consequence. It should however be recognised that in understanding the basis and mechanisms of a physiological stress response as has been described for CK release in broiler chicken skeletal muscle in this thesis, allows the determination of the extent of stress imposed upon that animal. Historically, stress responses have been considered to be either adaptive or protective and should thus prevent or minimise the detrimental effects of the stressor upon that animal. This objective may frequently not be achieved. The two major physiological systems mediating the stress response are the sympatho-adrenal system described as producing the "Fight or flight" reaction (Cannon, 1932) and hypothalamo-adrenocortical axis involved in the "General Adaptation Syndrome" (Selye, 1936). Other stress responses may be mediated through the more recently identified endocrine functions of the immune system (Blalock and Smith, 1985). The success of adaptive responses and the resultant effects upon the animal may be categorised in terms of the adequacy of the compensation in the face of a challenge tending to disturb a controlled physiological variable, these are: adequate compensation (successful compensation), inadequate compensation (perturbations in controlled variables) and decompensation (pathological failure of compensatory mechanisms or direct deleterious effects of compensation)(Kettlewell and Mitchell, 1996). Physiological changes may be

measured in all three type of response. Alterations in the slope of a plot of a stress variable such as CK activity against the magnitude of the stressor or a marked step change may indicate transition from one category to another. Such observation can then be used to determine the severity of the stress imposed (**see Figure 13.1**)

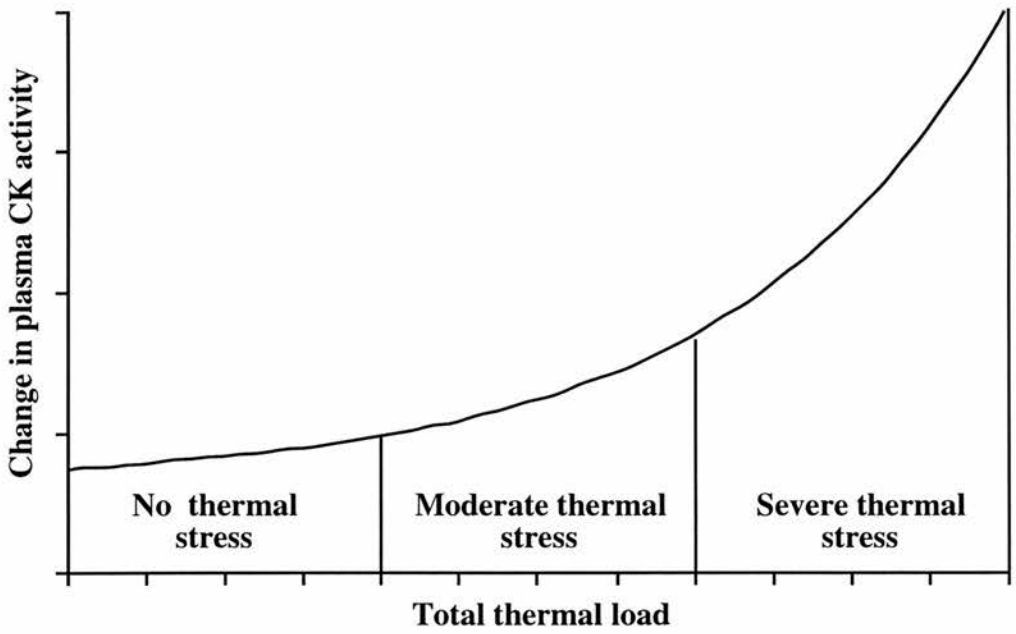

Figure 13.1 Hypothetical change in physiological parameter (plasma CK activity) in response to a thermal load (Adapted from Figure 1. Kettlewell and Mitchell, 1996).

It is therefore clear that the evaluation of stress by the magnitude and form of responses such as changes in plasma CK activity and other physiological stress indices (Mitchell *et al.*, 1992) may have relevant implications for animal welfare. Particularly if in a wider context these measurements are coupled with simultaneous measurements of behavioural responses (Duggan, 1996) and environmental conditions (Kettlewell, 1996). Collective assessment of these various factors may then provide the scientific basis for recommendations for improvements in transportation practices.

Meat Quality

In addition to its apparent animal welfare implications, it is also recognised that the major reported findings of this research may have further implications for meat quality. Whilst it is recognised that problems with meat quality can be created during post-mortem storage its is also acknowledged that detrimental changes in meat quality are influenced by ante-mortem factors which lead to changes in the biochemistry and morphology of the muscle (Backstrom and Kauffman, 1996; Sosnicki and Wilson, 1996). These factors include nutrition, disease or pathology, environment and

management, exposure to stress and differences in age sex and genotype (Ngoki *et al.*, 1982; Ming-Tsao *et al.*, 1991; Sosnicki and Wilson, 1991; Henckel, 1996; Rémignon *et al.*, 1996; Troeger, 1995; Santos *et al.*, 1997). It has been shown in certain pig breeds, particularly those selected for rapid growth rate and high meat yield that exposure to a range of stressful stimuli such as handling, tethering, physical activity and extreme environmental conditions, can result in profound skeletal muscle damage and reduced post-mortem meat quality (MacLennan and Phillips, 1992). This phenomenon has been described as "Porcine Stress Syndrome" (PSS) and is a source of major economic losses to the pig industry and is associated with a predisposition towards the production of what is described as Pale Soft Exudative meat (PSE) (Bendall, 1973; Arey, 1991; Backstrom and Kauffman, 1996). PSE meat is characterised by its pale streaky colour, soft texture and poor water holding capacity. The biochemical basis of this phenomenon is believed to be caused by large increases in sarcoplasmic Ca^{2+} concentration which occur as a consequence of impaired intracellular homeostasis following exposure to stress (Klont *et al.*, 1994; Marten, 1997). Increases in Ca^{2+} normally activate several non-lysomal Ca^{2+}-dependent proteolytic processes, the main one of which, the calpains are responsible for post-mortem proteolysis that results in meat tenderisation (Koohmaraie, 1994). It has been suggested that accelerated post-mortem proteolysis produced by ante-mortem increases in Ca^{2+} concentration may be an influential factor in the development of PSE meat in pigs (Bendall, 1973, Koohmaraie, 1996). A rapid decline in pH post-mortem caused by rapid glycolysis and ~P, and disruptions to the muscle sarcolemma have also been implicated in the cause of this phenomenon (Schwalege *et al.*, 1996a, b).

Whilst there have as yet been no reports of an analogous "stress syndrome" in broiler chickens, a PSE type condition has been reported in turkeys (Barbut, 1993, 1996, 1997; McCurdy *et al.*, 1996; Pietrzak *et al.*, 1996). Again the aetiology of this condition in these heavily selected animals is believed be similar to that observed in pigs (Barbut, 1996). It is clear that the proposed biochemical mechanisms that produce ante-mortem muscle damage in broiler chickens during stress may be very similar to those that produce the stress related PSE syndrome in pigs and may therefore also mediate the induction of undesirable properties or characteristics in poultry meat.

Future Research

The work presented in this thesis demonstrates that exposure to acute heat stress causes skeletal muscle membrane damage as reflected by the intracellular loss of the skeletal muscle form of CK (MM-CK). Through *in vitro* investigation of the mechanisms that might cause the enhanced release of this enzyme it was established that CK loss occurred as a consequence of Ca^{2+}-mediated alterations in muscle membrane permeability, probably through the action of Ca^{2+}-activated phospholipase A_2. Subsequent *in vitro* experiments demonstrated that the Ca^{2+} responsible for promoting disturbances in membrane permeability was that released from SR localised ryanodine-sensitive channels (RyR) in response to the stress stimuli. The findings of the work presented in this thesis have obvious implications for animal welfare as well as bearing on other production issues such as meat quality. Work is already continuing in areas of interest, which have been directly brought about from the findings of the work in this thesis. Future work will mainly be focused on two complimentary areas of research which will investigate the effect of genotype (i.e. selection for rapid growth rate and how this relates to other less intensively selected genotypes), as well as to continuing to develop an understanding of the biochemical and molecular mechanisms involved in the damage process. Future research will include:

1) Genotype comparisons: "stress-susceptibility"

This research will investigate differences in genotype response to stressful stimuli. *In vivo* this would involve exposure to acute heat stress. The comparison will also be extended to *in vitro* preparations. Work has already been undertaken in this area comparing the physiological responses of the commercial rapidly growing broiler chicken (see Chapter 3) to its genetic predecessor (relaxed line). These birds had been subjected to the same selection regime as the current commercial broiler line until 1974 where upon selection was "relaxed". These birds were random mated to provide a stable genetic control population (Sandercock *et al.*, 1995).

2) Using a modification of the developed isolated muscle preparation investigate the functional properties of broiler skeletal muscle when forced to work, either under tension or load. Again comparisons with muscles taken from relaxed line broilers may reveal any differences in the ability of the muscles to work. This would have major implications for bird welfare if it were shown that any differences were detrimental.

3) The isolation of SR microsomal membranes to be able to quantify and characterise the functional properties of ryanodine-sensitive Ca^{2+}channels in the skeletal muscles of rapidly growing broiler chickens. Following the successful isolation and characterisation of the channel, comparison could be made with channels from either relaxed line birds or traditional and non-selected breeds (e.g. white leghorn and red jungle fowl).

The results of these experiments could yield valuable information about the effect of intensive selection for growth rate and meat yield on the physiology of broiler skeletal muscle. This information could be incorporated into existing selection programs to help improve bird well being as well as productivity.

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Appendices

Appendix I

Standard broiler diet formulation (g/kg)

Wheat (250), maize (330), Soya meal (300), vegetable oil (28), fish meal (21), meat and bone meal (46), limestone (20), salt (2), vitamin and mineral supplement* (5)

* Provided per kg diet:

Retinol (688 µg), cholecalciferol (15 µg), α-tocopherol (25 mg), menadione (1.3 mg), riboflavin (4 mg), nicotinic acid (28 mg), pantothenic acid (10 mg), biotin (50 µg), zinc (50 mg), copper (3.6mg), iodide (400 µg), iron (80 mg), manganese (100 mg), selenium (150 µg)

Appendix II

Formulation of medium 199 with Hanks salts (concentration mg litre^{-1})

Inorganic salts

$CaCl_2.2H_2O$ (264.9), $Fe(NO_3)_3.9H_2O$ (0.72), KCl (400.0), $MgSO_4.7H_2O$ (200.0), NaCl (6800.0), NaH_2CO_3 (2200.0), $NaH_2PO_4.2H_2O$ (158.3)

Amino acids

DL-alanine (50.0), L-arginine.HCL (70.0), DL-aspartic acid (60.0), L-cysteine.HCl.H_2O (0.11), L-cysteine (20.0), DL-glutamic acid (133.4), glycine (50.0), L-histidine.HCl.H_2O (21.9), L-hydroxyproline (10.0), DL-isoleucine (40.0), DL-leucine (120.0), L-lysine.HCl (70.0), DL-methionine (30.0), DL-phenylalanine (50.0), L-proline (40.0), DL-serine (50.0), DL-threonine (60.0), DL-tryptophan (20.0), L-tyrosine (40.0), DL-valine (50.0)

Vitamins

L-ascorbic acid (0.05), DL-tocopherol phosphate (0.01), biotin (0.01), calciferol (0.10), D-pantothenate (0.01), choline chloride (0.50), folic acid (0.01), myo-inositol (0.05), menadione (0.01), nicotinic acid (0.025), nicotinamide (0.025), P-aminobenzoic acid (0.05), pyridoxal. HCl (0.025), pyroxidine. HCl (0.025), riboflavin (0.01), thiamin. HCl (0.01), vitamin A acetate (0.14)

Other ingredients

adenine sulphate (10.0), ATP di-sodium salt (1.0), 5' AMP (0.20), cholesterol (0.20), 2-deoxy-D-ribose (0.50), glucose (1000.0), glutathione, (0.05), guanine.HCl (0.30), Hypoxanthine (0.30), phenol red (10.0), D-ribose (0.50), sodium acetate (50.0), thymine (0.30), tween 80 (20.0), Uracil (0.30), xanthine (0.30).

Appendix III

Calculation for plasma CK activity (Equation 1)

$$\text{CK activity (IU litre}^{-1}) = \frac{\Delta A/\text{min} \times 1000_a \times V_T \times 1000_b}{(6.3 \times 10^3) \times L_P \times V_S}$$

Where:

$\Delta A/min$	=	change in absorbance per minute
1000_a	=	converts ml to litre
VT	=	total volume (ml)
1000_b	=	converts mmol to µmol
6.3×10^3	=	molar extinction coefficient for NADPH at 340 nm
LP	=	light path (cm)
VS	=	sample volume (ml)

Appendix IV

In vitro muscle ^{45}calcium uptake and CK efflux calculations

In order to relate ^{45}calcium isotope uptake in the muscles to CK efflux activities in the incubation medium it was necessary to express both parameters in a standardised common unit of measurement. Muscle ^{45}calcium activities and CK effluxes were calculated and expressed in terms of activity per gram of muscle per minute of incubation. All values were calculated using the following equations.

Calculation of muscle ^{45}calcium activity (Equation 2)

$$Ax = \frac{(CA \times nC)+(TA \times nT)}{nC+nT} \quad \text{(i)}$$

$$AQ = (AH/AX) \times 10^5 \quad \text{(ii)}$$

$$^{45}\text{Calcium activity (Dpm g}^{-1}\text{ min}^{-1}\text{)} = (AQ/MW)/IT \quad \text{(iii)}$$

Where:

Ax	=	Average of total medium ^{45}calcium activity (Dpm)
CA	=	Control medium ^{45}calcium activity (Dpm)
TA	=	Treatment medium ^{45}calcium activity (Dpm)
nC	=	Number of incubations in control medium
nT	=	Number of incubations in treatment medium
AQ	=	^{45}Calcium activity estimated per 10^5 Dpm added (Dpm)
AH	=	^{45}Calcium activity in muscle homogenate (Dpm)
Mw	=	Muscle weight (g)
IT	=	Total incubation time (minutes)

Quantification of muscle ^{45}calcium activity was essentially derived from a calculation based the ratio of radio-labelled calcium taken up by the muscle and the total amount of ^{45}calcium activity present in the incubation medium (i). For a valid comparison of ^{45}calcium activity across experiments done at different times, all muscle ^{45}calcium activities were calculated based on activity estimates per 10^5 Dpm added (ii) and subsequently corrected for muscle weight and incubation time (iii).

Calculation of muscle CK efflux activity (Equation 3)

$$\text{CK Activity (mU g}^{-1}\text{ min}^{-1}\text{)} = \left(\frac{((\Delta A/\text{min} \times 5144.7)/1000) \times IV}{MW \times IT}\right) \times 1000$$

Where:

ΔA/min	=	Change in absorbance per minute
5144.7	=	Multiplication factor (derived from **Equation 1**)
1000	=	Converts litre to ml
IV	=	Incubation volume (ml)
Mw	=	Muscle weight (g)
IT	=	Incubation period (min)
1000	=	Converts U to mU

Two GWBASIC programs were created for the calculation of both ^{45}calcium and CK efflux activities (**see Appendices IV** and **V**).

Appendix V

The following listing is a GWBASIC program for calculating the uptake of radio-labelled ^{45}calcium into skeletal muscle incubated *in vitro* expressed as radio-active disintegrations per minute per g of muscle per minute of incubation (Dpm g^{-1} min^{-1}).

```
10 DIM MN(10)
20 LET TT=150
30 DIM MW(20)
40 DIM MD(20)
50 DIM Z(10)
60 DIM Q(10)
70 CLS
80 PRINT:PRINT:PRINT:
90 PRINT "CALCULATION FOR 45CALCIUM UPTAKE IN MUSCLES"
100 PRINT
110 INPUT "EXPERIMENT TITLE";E$
120 PRINT
130 INPUT "EXPERIMENT TREATMENT";J$
140 PRINT
150 INPUT "DATE OF EXPERIMENT";D$
160 PRINT
170 INPUT "NO.MUSCLES USED PER TREATMENT";MN
180 REM MAXIMUM OF 10 MUSCLES
190 PRINT
200 INPUT "CONTROL MEDIUM ACTIVITY";CM
210 PRINT
220 INPUT "TREATMENT MEDIUM ACTIVITY";TM
230 PRINT
240 REM LOOP FOR MUSCLE WEIGHTS
250 FOR A=1 TO MN
260 PRINT "MUSCLE WEIGHT (g)";A;: INPUT MW(A)
270 PRINT
280 NEXT A
290 REM INPUT MD (B)
300 FOR A=1 TO MN
310 REM LOOP FOR MUSCLE DPM ACTIVITY
320 PRINT "MUSCLE ACTIVITY (Dpm)";A;:INPUT MD(A)
330 PRINT
340 REM INPUT MD (B)
350 LET MC(A)=((CM*2)+(TM*3))/5
360 LET Q(A)=(MD(A)/MC(A))*100000
370 LET V(A)=(Q(A)/(MW(A))/TT
380 REM TT = TOTAL INCUBATION TIME
390 PRINT " ACTIVITY (Dpm/g/min)";:PRINT USING "########";V(A)
400 PRINT
410 NEXT A
420 REM CLS
430 REM PRINTER ROUTINE
440 LPRINT:LPRINT:LPRINT
450 LPRINT
460 LPRINT "CALCULATION OF MUSCLE 45CALCIUM ACTIVITY (Dpm/g/min)"
470 LPRINT "     (Estimated per 1x10^5 Dpm added)"
480 LPRINT
490 LPRINT
500 LPRINT "       EXPERIMENT TITLE        ";E$
510 LPRINT
520 LPRINT "       EXPERIMENT TREATMENT    ";J$
530 LPRINT
540 LPRINT "       DATE OF EXPERIMENT      ";D$
```

```
550 LPRINT
560 LPRINT "       CONTROL MEDIUM ACTIVITY (Dpm)        ";     CM
570 LPRINT "       TREATMENT MEDIUM ACTIVITY (Dpm)      ";     TM
580 LPRINT
590 LPRINT "       MUSCLE      "; "    MUSCLE "; "        CALCIUM      "
600 LPRINT "       WEIGHT (g) "; "    (Dpm)   "; "      (Dpm/g/min)    "
610 LPRINT
620 FOR A=1 TO MN
630 LPRINT USING "       #######.#    ";     MW(A), MD(A), V(A)
640 NEXT A
650 LPRINT
660 LPRINT
670 CLS.
```

Appendix VI

The following listing is a GWBASIC program for calculating the Creatine kinase (CK) activity released from skeletal muscle incubated *in vitro* expressed in milli -International Enzyme Units of activity released per g of muscle per minute of incubation (mU g^{-1} min^{-1}).

```
10 DIM MW(10)
20 DIM OD(20,20)
30 DIM MD(20,20)
40 CLS
50 PRINT:PRINT:PRINT:
60 PRINT "CALCULATION OF CREATINE KINASE (CK) RELEASE (mU g-1 min-1)"
70 PRINT
80 INPUT "EXPERIMENT TITLE";E$
90 PRINT
100 INPUT "EXPERIMENT TREATMENT";J$
110 PRINT
120 INPUT "DATE OF EXPERIMENT";D$
130 PRINT
140 INPUT "NO.INCUBATION PERIODS          ";IP
150 REM MAXIMUM OF 8
160 PRINT
170 INPUT "NO.MUSCLES USED PER TREATMENT  ";MN
180 REM MAXIMUM OF 10 MUSCLES
190 PRINT
200 REM LOOP FOR MUSCLE WEIGHTS
210 FOR A=1 TO MN
220 PRINT "MUSCLE WEIGHT (g)";A;: INPUT MW(A)
230 REM INPUT MD (A)
240 NEXT A
250 FOR A=1 TO MN
260 REM LOOP TIME PERIOD
270 FOR B=1 TO IP
280 PRINT "INCUBATION PERIOD";B
290 PRINT
300 REM MUSCLE NUMBER LOOP
350 FOR A=1 TO MN
360 PRINT " OPTICAL DENSITY (O.D.)";A;INPUT OD (B,A)
370 CK(B,A)=(OD(B,A)*5144.7*2.5)/IP*MW(A))/2.5
380 PRINT " CK ACTIVITY (mU g-1 min-1)"; CK (B,A)
390 PRINT
400 NEXT A
410 NEXT B
420 REM CLS
430 REM PRINT ROUTINE
```

```
440 PRINT
460 PRINT "CK EFFLUX ACTIVITIES (mU g-1 min-1)    "
470 PRINT
480 INPUT "DATE                                   ";D$
490 PRINT
500 PRINT "MUSCLE WEIGHT (g)","OPTICAL DENSITY","CK (mU g-1 min-1)"
510 FOR B=1 TO TP
520 PRINT
530 PRINT "INCUBATION PERIOD";B
540 PRINT
550 FOR A=1 TO MN
560 PRINT
570 PRINT MW(A), OD(B,A), CK(B,A)
580 PRINT
590 NEXT A
600 NEXT B
610 PRINTER ROUTINE
620 LPRINT:LPRINT:LPRINT
630 LPRINT "CK EFFLUX ACTIVITIES (mU g-1 min-1)  ""
640 LPRINT
650 LPRINT "      EXPERIMENT TITLE                ";E$
660 LPRINT
670 LPRINT "      EXPERIMENT TREATMENT            ";J$
680 LPRINT
690 LPRINT "      DATE OF EXPERIMENT              ";D$
700 LPRINT
710 LPRINT "      INCUBATION PERIOD (min)";IP
720 LPRINT
730 LPRINT "      MUSCLE     "; "   OPTICAL "; " CK ACTIVITY      "
740 LPRINT "      WEIGHT (g) "; "   DENSITY "; " mU g-1 min-1     "
750 LPRINT
760 FOR B=1 TO IP
770 LPRINT "      TIME PERIOD ";B
780 LPRINT
790 FOR A=1 TO MN
800 LPRINT USING "      ####.#      ";    MW(A), OD(B,A), CK(B,A)
810 NEXT A
820 LPRINT
830 NEXT B
840 CLS.
```

Publications Arising From Thesis

Sandercock D.A. and Mitchell M.A. (1996) Dose dependent myopathy in monensin supplemented broiler chickens: effects of acute heat stress *British Poutry Science* **37**, 92-94.

Sandercock D.A. and Mitchell M.A. (1995) The possible roles of Na^+ and Ca^{2+} overload in the mechanism of monensin-induced myotoxicity in isolated avian (gallus domesticus) *Journal of Physiology* **485**, 41P-42P.

Mitchell M.A. and **Sandercock D.A.** (1995a) Creatine kinase isoenzyme profiles in the plasma of the domestic fowl (gallus domesticus) *Research in Veterinary Science* **59**, 30-34.

Mitchell M.A. and **Sandercock D.A.** (1995b) Increased hyperthermia induced skeletal muscle damage in fast growing broiler chickens *Poutry Science* **74** (supplement 1), 74.