BIOCHEMICAL AND PHYSICAL STUDIES
ON MALIGNANT HYPERPYREXIA

by

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A thesis submitted for the degree of
Doctor of Philosophy of the
Australian National University
March 1980
STATEMENT

The investigations described in this thesis are my own original work.

John S. Sullivan
March 1980
DEDICATION

This is dedicated to my family, and
most especially to Cushla.
ACKNOWLEDGEMENTS

I am greatly indebted to my supervisor Dr Michael Denborough for his assistance and encouragement over the last three years. I would also like to thank Dr David Fenn, Dr Russell Close, Dr Graeme Cox, Dr Ian Young, Dr Neville Ardlie and Mr Louis James for their many helpful suggestions and criticisms. Assistance from the library, photography and workshop staffs is also gratefully acknowledged.

Thanks are also due to my departmental colleagues, including Graham Galloway, Ken Hopkinson, Carmel Boatwright and Joe Jakubowski for their help and friendship. The individual contributions of Dr Brian Creese, Dr Howard Mitchell, Mr Bryan Crocker and Ms Jenny Grigg are especially appreciated as their continued interest in this work ensured its completion.

Finally I would to thank Ms Wendy Adcock for typing this thesis and a special thanks to Cushla and Barbara for their encouragement and assistance during the preparation of this thesis.

The investigations described in this thesis were carried out during the tenure of an A.N.U. Ph.D. Scholarship in the Department of Clinical Science, John Curtin School of Medical Research. Assistance from this scholarship is gratefully acknowledged.
1. A multi-disciplinary approach was adopted in which biochemical, biophysical and pharmacological techniques were used to further characterise the nature of the abnormality predisposing to malignant hyperpyrexia (MH).

2. Chemical characteristics of density gradient fractionated skeletal muscle microsomal membrane preparations from both normal and malignant hyperpyrexic swine failed to reveal consistent differences in either lipid or protein content.

3. $^1$H NMR studies of the effects of halothane on normal and MH microsomal membrane preparations indicated that the anaesthetic "fluidises" such membranes, but these effects were similar in both muscle types.

4. Halothane-membrane interactions were also studied in a model membrane system using $^{19}$F and $^{31}$P NMR spectroscopy. Such an approach permitted the monitoring of halothane and phospholipid environments in the same sample.

5. The temperature dependence of halothane-, caffeine-, succinylcholine- and KCl-induced contractures was investigated in isolated normal and MH skeletal muscle preparations. Drug-induced hypercontractility was abolished at 25°C suggesting that the abnormal drug-induced release of calcium which occurs at 37°C is blocked at 25°C.

6. High concentrations (> 200 μM) of the calcium ion antagonist TMB-8 contracted MH skeletal muscle preparations in vitro, and this response was abolished by E-C uncoupling treatments (i.e. dantrolene sodium, $D_2$O - Ringer or glycerol-induced detubulation).
7. In MH porcine skeletal muscle low concentrations of TMB-8 (100 μM) potentiated contractile responses to halothane, caffeine and succinylcholine but partially blocked KCl-induced contractures. The qualitative similarity between the potentiating effects of TMB-8 on halothane-, caffeine- and succinylcholine-induced contractures suggests a common site of action for these drugs and it is proposed that this site is in fact the triad.

8. 3% halothane, 2 mM caffeine, 1 mM succinylcholine and 80 mM KCl failed to contract normal porcine muscle either in the absence or presence of TMB-8 (100 μM to 1 mM) or over the 20°C to 45°C temperature range.

9. Platelet aggregation was abnormal in a number of MH susceptible individuals suggesting that in at least some individuals susceptibility to MH reflects a generalised defect in a variety of cell types. The variable occurrence of such platelet abnormalities suggests that platelet function tests should not be used in isolation when attempting to identify MH susceptibility.

10. The relevance of these studies to the understanding of the underlying abnormality predisposing to MH is discussed.
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ABBREVIATIONS

ACI acceptor control index
ADP adenosine diphosphate
AK adenylate kinase
AMP adenosine monophosphate
ATP adenosine triphosphate
ATPase adenosine triphosphatase
cAMP cyclic adenosine monophosphate
cGMP cyclic guanosine monophosphate
CP creatine phosphate
CPK creatine phosphokinase
D₂O deuterium oxide
DPL L-a-dipalmitoylphosphatidylcholine
E-C excitation-contraction
EGTA ethylene glycol-bis-(β-aminoethylether)-N,N\textquotesingle-tetraacetate
EPC L-a-phosphatidylcholine
KCI potassium chloride
MDP methylene diphosphonic acid
MEP motor end plate
MH malignant hyperpyrexia
NMR \( (\text{H}, \text{F}, \text{P}) \) nuclear magnetic resonance \( (\text{H}, \text{F}, \text{P}) \)
OI osteogenesis imperfecta
PPP platelet poor plasma
PRP platelet rich plasma
PSE pale, soft exudative
PSS porcine stress syndrome
RCI respiratory control index
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<td>RD</td>
<td>reagent diluent</td>
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<tr>
<td>RMP</td>
<td>resting membrane potential</td>
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<td>S.D.</td>
<td>standard deviation</td>
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<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<td>S.E.M.</td>
<td>standard error of the mean</td>
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<td>SR</td>
<td>sarcoplasmic reticulum</td>
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<td>TEMED</td>
<td>N,N,N',N'-tetraethylmethylenediamine</td>
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<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
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<td>TMB-8</td>
<td>8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate</td>
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<td>TMS</td>
<td>tetramethylsilane</td>
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CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

Since the first description of malignant hyperpyrexia (MH) by Denborough and Lovell in 1960, the occurrence of familial anaesthetic deaths has received considerable attention. The demonstration of the hereditary nature of the syndrome and the concurrent elucidation of the genetic pattern of inheritance (Denborough et al., 1962) confirmed the significance of the initial report. Subsequent work in Canada (Britt and Kalow, 1970), in England (Ellis et al., 1972) and in South Africa (Isaacs and Barlow, 1970) has documented its global distribution.

The syndrome occurs in susceptible individuals on exposure to an appropriate trigger, with the general anaesthetic halothane and the muscle relaxant succinylcholine being the most widely incriminated agents in clinical practice (Britt et al., 1977). The usual rate of occurrence is between 1:10,000 to 1:15,000 (Williams, 1976) and once initiated mortality rates may be as high as 70% (Britt and Kalow, 1970). The clinical features of the syndrome consist of (1) tachycardia; (2) pronounced metabolic acidosis; (3) alterations in serum electrolyte balance; (4) muscular rigidity; (5) hyperpyrexia; (6) vascular constriction and (7) an elevation in body core temperature.

A relationship between MH and an underlying disease of the skeletal musculature was first suggested in a perceptive editorial in the British Medical Journal in 1968. This proposed that there was a causal relationship between the administration of succinylcholine, altered metabolic rates in skeletal muscle tissues and the expression of the syndrome, thus focusing on the skeletal musculature as the site of the abnormality in human MH.
Elevations in the resting levels of serum creatine phosphokinase (CPK) provided the first direct evidence associating MH susceptibility with an underlying myopathy (Denborough et al., 1970a; Isaacs and Barlow, 1970) and subsequent observations (Denborough et al., 1970b) indicated that halothane anaesthesia markedly elevated CPK levels in an individual susceptible to MH.

Two myopathies have been described which predispose to MH (King et al., 1972). The first occurs in young boys and is associated with obvious physical deformities (King and Denborough, 1973), while the second, and more common, is inherited as an autosomal dominant trait and the myopathy is usually subclinical (Denborough et al., 1970a; Isaacs and Barlow, 1970).

The in vitro identification of contractile hypersensitivity to caffeine in human MH muscle (Kalow et al., 1970) demonstrated an abnormality in the metabolism of calcium in affected muscle and established a useful diagnostic test with which to assess MH susceptibility. The failure to obtain abnormal contractures on exposure to halothane (Kalow et al., 1970), contrasted with later reports which showed that halothane contractility was of diagnostic use in identifying MH susceptible muscle (Ellis et al., 1972; Moulds and Denborough, 1972). The unreliability of any single in vitro contracture test to accurately assess human susceptibility to MH prompted the evaluation of drug-induced contractures to a variety of agents, including halothane, caffeine, succinydlcholine and potassium chloride (Moulds and Denborough, 1974a), and this approach provides unequivocal identification of affected individuals. The ability of MH muscle to give an increased contractile response to a variety of agents (Moulds and Denborough, 1974a) reinforces the view that affected muscle is characterised by an abnormality in the processes regulating calcium ion distribution. The exact site of the skeletal muscle abnormality in human
MH is unknown.

The discovery of an experimental animal model (Hall et al., 1966; Harrison et al., 1968) in the late 1960s provided a useful system for studying the biochemical basis of MH. The manifestations of porcine MH are similar to those described in human MH (Williams, 1976; Denborough, 1980) thus suggesting that the underlying mechanisms predisposing to MH are common to both. The in vivo effectiveness of the volatile anaesthetic halothane (Gronert and Theye, 1976a) and the muscle relaxant succinylcholine (Gronert and Theye, 1976b) in inducing MH in susceptible swine has centred investigations to elucidate its aetiology on the skeletal musculature (Campion and Topel, 1975). Elevations in the serum CPK levels in resting (Hall et al., 1970) and post-halothane challenged swine (Woolf et al., 1970) are consistent with observations made in susceptible humans (Isaacs and Barlow, 1970; Denborough et al., 1970a, b) and further underline the similarities between human and porcine MH. The most conclusive evidence highlighting common underlying mechanisms predisposing to MH comes from the in vitro demonstration of drug-induced hypercontractility in isolated human (Moulds and Denborough 1974a) and porcine (Okumura et al., 1979) MH skeletal muscle. The similarity between the contractile responses to a variety of agents in the human (Moulds and Denborough, 1974a) and porcine (Okumura et al., 1979) forms of the syndrome confirms the usefulness of the pig as an animal model for studying MH.

Biochemical studies in susceptible swine have shown that a number of chemical changes (Berman et al., 1970) precede the development of both muscular rigidity and hyperpyrexia. These changes include rapid rises in oxygen consumption and carbon dioxide production, metabolic acidosis and gross changes in serum electrolyte levels (Berman et al., 1970).
Biochemical information on the development and expression of the syndrome in susceptible humans is rather limited and the availability of an animal model, in which the syndrome can be readily induced, explains why so much more is known about porcine MH than human MH. The syndrome has been reported in the Landrace, Yorkshire, Pietrain and Poland China breeds (Campion and Topel, 1975) although the rate of incidence varies considerably from breed to breed (Lucke et al., 1979). The mode of inheritance of the syndrome in swine is complex and genetic mechanisms ranging from a single autosomal dominant gene with incomplete penetrance and/or variable expression (Woolf et al., 1970) to two dominant genes acting in concert (Williams et al., 1975) have been proposed. The confusion as to the exact mode of inheritance of porcine MH stems from the multiplicity of tests used to assess susceptibility (these include the "halothane test" (Eikelenboom et al., 1974), the identification of blood types (Rasmusen et al., 1976) and the in vitro assessment of skeletal muscle contractility to a variety of drugs (Okumura et al., 1979) and from inherent biological variability, which results in a wide spectrum of MH sensitivity (Lucke et al., 1979).

MH represents one of a number of stress related syndromes which have been described in certain breeds of swine; the others include the porcine stress syndrome (PSS) and the pale soft exudative (PSE) pork syndrome. It seems that MH, PSS and PSE may all be different manifestations of a common underlying abnormality (Nelson, 1973; Denborough, 1980) but such an abnormality remains to be identified. The in vivo assessment of stress susceptibility in swine is generally carried out by the "halothane test" (Eikelenboom et al., 1974; Campion et al., 1974, 1979) further emphasising the relationship between PSS and porcine MH (Williams et al., 1975). PSE pork is a post mortem characteristic which is predisposed by abnormal skeletal muscle metabolism in affected swine. The relationship
of the PSE pork syndrome to porcine MH has been demonstrated by the propensity of MH skeletal muscle to develop PSE characteristics post-mortem (Nelson et al., 1974). Breed distribution and a common underlying myopathy are common features of the 3 stress related syndromes in swine (Nelson, 1973) and expression of any of the syndromes may be induced by a multiplicity of common stressors including exercise, excitement, fighting, high environmental temperatures and the administration of certain drugs (Nelson et al., 1974).

1.2 SUMMARY

Malignant hyperpyrexia is a dangerous complication of anaesthesia which occurs in individuals who have an underlying skeletal muscle disease. In humans two predisposing myopathies have been described, one is recessively inherited and associated with various physical abnormalities while the second is dominantly inherited and is usually subclinical. Similar abnormal anaesthetic reactions have been described in swine, suggesting that the human and porcine forms of MH are related disorders. Porcine MH, PSS and PSE appear to be different manifestations of a common underlying skeletal muscle abnormality.

1.3 PROJECT AIMS

In order to more fully document functional and/or structural abnormalities which reflect MH susceptibility, a multidisciplinary approach will be adopted. A variety of chemical, biochemical and pharmacological techniques will be used to achieve this aim.

A detailed biochemical characterisation of muscle microsomal membranes will be carried out using normal and MH porcine skeletal muscle
in an attempt to correlate the functional abnormality in MH with a membrane associated lipid or protein abnormality. H\textsuperscript{1}, F\textsuperscript{19} and P\textsuperscript{31} nuclear magnetic resonance (NMR) spectroscopy provide a non-perturbing technique with which to study structure-function relationships and these methods will be used to study membrane function in MH. Some aspects of halothane-membrane interactions will also be investigated in artificial lipid membranes with the aim of providing more detailed information on the nature of this interaction and how membrane organisation affects this relationship.

Pharmacological studies will include the in vitro assessment of drug-induced contracture as a function of temperature, and the effects of the calcium antagonist TMB-8 on drug-induced contractures in normal and MH porcine skeletal muscle.

The functional similarities with skeletal muscle that platelets display, and their ready availability has prompted a study of platelet function in MH individuals, with the aim of developing a simple reliable technique with which to assess MH susceptibility in humans. Aggregatory responses to a number of drugs will be assessed in some detail in normal and MH humans in whom susceptibility will be simultaneously assessed by muscle biopsy and subsequent pharmacological testing.
CHAPTER 2

STRUCTURE AND FUNCTION OF NORMAL AND MALIGNANT
HYPERPYREXIC SKELETAL MUSCLE

2.1 INTRODUCTION

In MH the evidence favouring skeletal muscle as the principle site of the abnormality is extensive (Campion and Topel, 1975; Britt, 1977, 1979; Lucke et al., 1979; Denborough, 1980) and it is generally held that initiation of the syndrome occurs as a consequent expression of a structural and/or functional lesion in this tissue in response to a specific trigger. The muscular rigidity and the elevations in the blood levels of the muscle associated CPK enzyme, which occur during the development of the syndrome (Chapter 1) provide supportive evidence for a skeletal muscle abnormality but it was the in vitro demonstration of drug-induced contractile hypersensitivity which provided the most conclusive evidence indicative of MH skeletal muscle dysfunction (Kalow et al., 1970; Ellis et al., 1972; Moulds and Denborough, 1972). Prior to discussing the nature and extent of the proposed skeletal muscle lesions a description of the structure and function of skeletal muscle will be presented.

2.2 SKELETAL MUSCLE STRUCTURE AND FUNCTION

The organisational detail in skeletal muscle ongoing from macroscopic to microscopic structures is rather simply shown in Figures 1, 2 and 3. Intact muscle is composed of bundles of individual cells or fibres called fascicles. Each individual fibre and bundle is embedded in a connective tissue matrix called the endomysium and perimysium, respectively. The complete muscle is in turn sheathed in
an additional connective tissue matrix termed the epimysium. The geometric specifications of the individual fibres may vary considerably but each fibre is intact along the whole length of the muscle and individual fibres may be up to 30 cms in length. The fibres are physically anchored to tendons by the terminal sections of the fibre and the tendons are in turn attached to adjacent bones. Each cell is mononucleated with the nucleus located close to the plasma membrane. The plasma membrane or sarcolemma is a specialised membrane and is characterised by a limited region which is pharmacologically responsive to the chemical transmitter acetylcholine. This specific region, the motor end plate (MEP) represents the locus of interaction between nerve and muscle, and is the functional site for neuromuscular transmission. Sensitivity to acetylcholine differentiates the MEP from the remainder of the sarcolemma while the reverse is true for responses to directly applied electrical current as the MEP is electrically inexcitable (Bowman et al., 1969).

Mammalian foetal skeletal muscle fibres are polyneurally innervated but this pattern disappears within a few months of birth, at which stage each fibre is connected to only one motoneurone (Vrbová et al., 1978). In vivo, skeletal muscle contractility is controlled by the motoneurone, where neural stimulation induces the release of acetylcholine, which in turn mediates depolarisation of the MEP. The resting sarcolemma is electrophysiologically asymmetric, with the interior maintained approximately 90 mvolts more negative than the outer surface. This potential difference is a consequence of the maintenance of steep energy dependent ionic gradients (i.e. high potassium and low sodium inside and the reciprocal outside). The receptor-mediated interaction between acetylcholine and the MEP depolarises the end plate and results in an increase in ionic permeability. This localised depolarisation means that the potentials in the end plate region and the remainder of the sarcolemma differ, and this
constitutes the initial step in the translation and amplification of the neural impulse to the remainder of the sarcolemma. On reaching a critical value depolarisation becomes self perpetuating and it spreads radially from the MEP. Thus, initiation and propagation of the action potential occur rapidly and passes around the muscle fibre membrane within milliseconds. The MEP possesses an active acetylcholinesterase which rapidly hydrolyses the released acetylcholine and on repolarisation the MEP is ready to respond to a further release of acetylcholine.

The requirement for some process to facilitate the propagation of the action potential into the muscle fibre was postulated in 1948 (Hill, 1948) and experimentally verified a number of years later (Huxley and Taylor, 1958). Utilising electrophysiological techniques Huxley and Taylor (1958) showed that the postulated process was in fact the invaginations of the T-tubules and that this is the pathway along which the action potential is conducted into the interior of the fibre. T-tubular continuity with the extracellular fluid, and the extent to which the system penetrates the muscle fibre, was demonstrated by Huxley (1964), who showed that ferritin (which under normal conditions does not cross cell membranes) could specifically localise within the T-tubular membrane system. Two functions are fulfilled by the T-tubular network. These are, (1) the propagation of the sarcolemma initiated action potential along the length of the tubule and (2) to communicate this activation signal to the sarcoplasmic reticulum (SR) through the specialised junctional regions where the two membrane systems interact (Costantin, 1975). Little is known about the nature of the activation signal that is transmitted from the T-tubule to the terminal cisternal sections of the SR although a number of mechanisms have been postulated (Endo, 1977). Voltage controlled charge movement has been proposed to be the primary process modulating the release of calcium from the terminal cisternae and
at least two voltage controlled mechanisms are compatible with this scheme (Adrian, 1978). In the first a direct electrical coupling between the T-tubular and terminal cisternal membranes has been proposed, with the release of activator calcium being voltage regulated (Adrian, 1978). An alternative scheme suggests that the eventual release of activator calcium from the terminal cisternae is controlled by the prior release of T-tubular trigger calcium and that this initial release of trigger calcium is the voltage controlled event (Endo, 1977). The latter mechanism, more simply referred to as calcium-induced calcium release, has received indirect experimental support in skinned fibre preparations where high extracellular calcium causes appreciable release of bound SR calcium (Endo, 1977). Such results were obtained with unphysiologically high calcium levels and its importance in controlling normal skeletal muscle calcium release has been proposed to be minimal (Endo, 1977). The electron microscopic demonstration of "feet" apparently spanning the 12 nm gap between the T-tubular and terminal cisternal membranes (Franzini-Armstrong, 1975) provided a possible structure by which electrical continuity could be maintained. Indeed Schneider and Chandler (1973) have presented evidence correlating the number of these "feet" to their estimate of the number of moving charge groups per unit area of membrane.

Although evidence favouring the involvement of such "feet" in the coupling of T-tubular excitation to SR calcium release is circumstantial, (Schneider and Chandler, 1973), a number of observations are pertinent to this relationship; (1) the "feet" originate from the terminal cisternae (Franzini-Armstrong, 1975), (2) they occur only at the T-tubular-SR junctions (Franzini-Armstrong, 1975), (3) their number increases after a potassium chloride-induced contracture (Eisenberg et al., 1979) and in
fatigued muscle (Eisenberg and Gilai, 1979) and (4) there is a correlation between their localised occurrence (Franzini-Armstrong, 1975) and the release of calcium, which is known to occur from the terminal cisternae (Winegrad, 1970). Despite the aforementioned relationships between the presence of the SR associated "feet" and the transmission of the contractile activation signal from the T-tubule to the terminal cisternae, the mechanisms underlying excitation-contraction (E-C) coupling largely remain unknown and this step represents the least characterised process in the skeletal muscle contraction-relaxation cycle.

Irrespective of the nature of the activation signal that is conveyed to the terminal cisternae, it is important to note that this signal does not initiate an all or none response and that the amount of SR calcium released is a function of the extent of T-tubular depolarisation (Costantin, 1975).

With the release of calcium from the terminal cisternae, fibre contractility is controlled by two opposing processes; (1) calcium re-uptake by the longitudinal SR, which results in relaxation and (2) the regulatory interaction of calcium with the troponin-C protein which is a prerequisite for the mechano-coupling of actin to myosin. Prior to elaborating on these processes it is first appropriate to outline the morphological features of the myofibrillar elements within the sarcomere and more importantly how their co-ordinated interaction results in the generation of muscle tension.

Electron microscopy of intact fibres and biochemical analysis of the individual myofibrillar protein constituents have collectively provided an almost complete picture of the functioning of the contractile apparatus. Each sarcomere stretches from adjacent Z lines and is composed of thick and thin filaments. These filaments overlap at certain
stages giving the sarcomere a distinctly banded appearance. Thus the I, H, and A bands constitute thin filaments, thick filaments and a region where both overlap, respectively (Figures 1 and 2). The thin filaments consist of a double helix of tropomyosin along which 7 G-actin molecules are aligned per tropomyosin molecule. An additional protein, troponin, is also localised on the thin filaments and this constitutes a tri-subunit complex consisting of; (1) troponin-C, the calcium binding segment, (2) troponin-T, the segment responsible for the troponin-tropomyosin interaction and (3) troponin-I the inhibitory subunit which in resting muscle physically blocks the interaction of actin with myosin (Murray and Weber, 1974).

Electrophoretic analysis has shown that the thick filaments contain three proteins, but a specific function has been ascribed only to the predominant myosin protein. The specific interaction of myosin with an actin molecule in the thin filament is the process upon which muscular contraction is based.

The release of calcium from the terminal cisternae raises intracellular calcium levels from less than $10^{-7}$ M to greater than $5 \times 10^{-6}$ M and the affinity for calcium displayed by troponin-C is such that under these conditions 2 calcium ions are bound per subunit. This regulatory binding of calcium by troponin-C is somehow "felt" (Perry, 1979) by the adjacent troponin-I protein and the conformational change which results effectively frees the myosin binding site on the G-actin molecule. It is important to note that the interaction between the troponin-I protein and actin occurs only every seventh actin molecule and in the absence of a sufficiently high level of calcium, this interaction effectively blocks the myosin binding site on all actin molecules (Perry, 1979). Prior interaction of the myosin head group with ATP results in an energised
intermediate which in the presence of calcium can interact specifically with actin. The myosin head then undergoes an energy consuming conformational change which alters the angular relationship of the cross bridge to the axis of the thin filament, thus resulting in the movement of the thin filament along the thick filament. In the presence of calcium and with a sufficient supply of ATP, actomyosin cross bridge formation takes place recurrently with a gradual decrease in the length of the H band. Tregear and Marston (1979) have outlined current ideas on the events associated with cross bridge formation and the description here represents a gross over-simplification of the kinetic events associated with actomyosin formation.

As long as the level of stimulation exceeds the contractile threshold for the muscle, tension is maintained. On the cessation of stimulation (either neural or drug induced) the surface membrane system is repolarised and the calcium concentrations return to resting levels as a consequence of the actively mediated uptake of calcium into the longitudinal sections of the SR. Calcium localisation during the contractile recovery phase occurs initially in the longitudinal sections of the SR but within seconds this calcium is transported to the terminal cisternal regions of the SR (Winegrad, 1970). Autoradiography has elegantly demonstrated this functional specialisation of discrete regions of the SR and has in addition shown that the release and re-uptake of calcium are continuous processes which occur in both resting and stimulated muscle (Winegrad, 1970). Thus in stimulated muscle the rate of calcium release greatly exceeds the re-uptake capacity of the muscle and contraction results. Figures 2 and 3 show how the different skeletal muscle membrane systems have developed to fulfil their specific roles in the contraction-relaxation cycle.
Figure 2.1: Organisation of mammalian skeletal muscle

Schematic outline of the organisational detail in skeletal muscle on going from macroscopic to microscopic structures. (From Bloom and Fawcett, 1968).
Figure 2.2: **Sarcomere organisation at the molecular level**

This represents a schematic outline of myofibrillar and membrane organisation in individual sarcomeres. The sarcomere constitutes the regions between adjacent Z lines. (From Bloom and Fawcett, 1968).
Myofibrils
A band
Triad of the reticulum
Z line
Sarcolemma
Transverse tubule
Sarcoplasmic reticulum
Mitochondrion
Transverse tubule
Terminal cisternae
Sarcotubules
Figure 2.3: A three-dimensional reconstruction of mammalian skeletal muscles

The spatial relationships of the individual membrane systems elegantly highlight the relationship of structure to function in skeletal muscle. The functional continuity between the sarcolemmal, T-tubular and SR membrane systems is essential for muscle function. (From Eisenberg et al., 1974).
Over the last five years a great volume of work has appeared on
the many aspects of muscle function, and extensive reviews have appeared
on activation (Costantin, 1975; Podolsky, 1975; Adrian, 1978; Caputo,
1978), calcium release (Endo, 1977), contractile protein functioning
(Tregear and Marston, 1979; Mannherz and Goody, 1976; Tada et al., 1978)
and the energetics of muscle contraction (Curtin and Woledge, 1978;
Homsher and Kean, 1978). The previous section has drawn peripherally from
these reviews and the presentation represents a simplistic outline of
events which characterise the contraction-relaxation cycle in skeletal
muscle fibres.

Thus having reviewed skeletal muscle structure as it relates to
function, the next section examines the evidence favouring
skeletal muscle abnormalities in MH.

2.3 SKELETAL MUSCLE ABNORMALITIES IN MALIGNANT HYPERPYREXIA

2.3.1 Muscular denervation and malignant hyperpyrexia

The enhanced contractility displayed by MH skeletal muscle in
response to specific drugs (Moulds and Denborough, 1974; Okumura et al.,
1979) suggests similarities with denervated muscle (Moulds, 1977, 1978)
as contractile supersensitivity is a reported feature of such muscles in
response to succinylcholine (Thesleff, 1974) and to caffeine (Gutman and
Sandow, 1965). Experimentally denervated mammalian skeletal muscle has
been extensively studied, and defined alterations characterise the chronic
case (Vrbová et al., 1978). Mononeural innervation (i.e. one MEP per
muscle cell) is a feature of normal mammalian skeletal muscle but
denervation alters this distribution and multiple extra-junctional
cholinergic receptors explain the supersensitivity displayed by such
muscle to cholinergic drugs (Fambrough, 1970). Systemic and regional
administration of succinylcholine to humans with traumatic nerve injury, resulted in contractures in the denervated musculature and regional administration of fractional systemic doses has been proposed to be of diagnostic use in MH (Baraka, 1978). In vitro skeletal muscle fibres from MH susceptible swine contracted spontaneously upon the addition of succinylcholine (Okumura et al., 1979), but acetylcholine at similar concentrations failed to contract MH muscle (Okumura et al., 1980). This differential response to apparently related cholinergic drugs indicates that succinylcholine-induced contractures in MH muscle are not mediated by a cholinergic mechanism and that the extra junctional spread of cholinergic receptors does not occur in MH muscle.

Similar conclusions have been reached by Moulds (1977, 1978), who found that denervated mouse muscle behaved quite differently to MH human muscle when assessed by a variety of parameters. Denervated muscle displayed a prolongation of the "active state" in response to electrical stimulation, a failure to contract when treated with 3% halothane and a contracture induced by succinylcholine blocked any subsequent response to either potassium chloride or to electrical stimulation (Moulds, 1977, 1978). MH muscle by contrast displayed no change in "active state", a large contracture in response to 3% halothane, and a non-depolarising succinylcholine contracture, as evidenced by the maintenance of twitch and the ability to subsequently contract to potassium chloride (Moulds, 1977, 1978). The collective results of Moulds (1977, 1978) and Okumura et al. (1980) exclude denervation as the primary skeletal muscle abnormality predisposing individuals to MH, although certain similarities do exist.

2.3.2 Sarcolemmal lesions in malignant hyperpyrexia

Excluding the specialised region at the end plate, the primary
function of the sarcolemma is to amplify the radial spread of the activation signal initially generated at the neuromuscular junction. The sarcolemma and T-tubular network are collectively responsible for the maintenance of the steep ionic gradients which are functionally expressed as the differential electrical potentials at either side of the surface membrane system. Alterations in resting membrane potential (RMP) in porcine MH skeletal muscle on exposure to halothane have been noted in forelimb digital extensor (Gallant et al., 1979) and intercostal (G. Galloway, J. Bornstein, J. Sullivan and M.A. Denborough, unpublished results) muscles. An earlier study (Bradley et al., 1973), also utilising intercostal muscle failed to observe any change in RMP on exposure to halothane, but these experiments may have been carried out at room temperature, where halothane contractures (Nelson et al., 1975) and the halothane-induced partial depolarisation do not occur (G. Galloway, J. Bornstein, J. Sullivan and M.A. Denborough, unpublished results). Indeed the failure by Bradley et al. (1973) to obtain an \textit{in vitro} halothane contracture would tend to support this possibility. Halothane-induced depolarisation did not occur in normal muscle preparations thus indicating that the partial depolarisation (average 5.1 mV, G. Galloway, J. Bornstein, J. Sullivan and M.A. Denborough, unpublished results) is a real phenomenon and is indicative of an underlying surface membrane lesion.

Kendig and Bunker (1972) have shown that under halothane anaesthesia the \textit{in vivo} RMP of rat gastrocnemius muscle was depressed by an average of 6 mV compared to RMP recorded 3 hours after the cessation of anaesthesia in the equivalent spinalised animal. This may reflect a normal \textit{in vivo} response to prolonged halothane anaesthesia (i.e. > 3 hours (Kendig and Bunker, 1972)) as normal pig, frog and mouse muscle RMP was not altered by thirty minutes \textit{in vitro} exposure to halothane (Gallant et al.,
1979). Thus it is possible that the partial depolarisation of porcine MH muscle obtained on in vitro exposure to halothane may be indicative of a much greater in vivo depolarisation. In isolation this depolarisation does not explain the enhanced contractility displayed by porcine MH muscle on in vitro exposure to halothane (Okumura et al., 1979), but the lower mechanical threshold of porcine MH muscle (Bryant and Anderson, 1977) provides the necessary link correlating depolarisation to contraction. This shift in mechanical threshold activation effectively brings the electrical threshold quite close to the RMP (Bryant and Anderson, 1977) and hence the small halothane induced depolarisation may be sufficient to induce a contracture. The electrophysiological evidence favouring a surface membrane lesion in MH is good (reviewed above) but comparable studies on the structural composition of MH surface membranes have yet to be attempted and hence no attempt can be made to correlate such functional changes with defined structural abnormalities. The demonstration that this surface membrane lesion occurs in a white (Gallant, 1979; Gallant et al., 1979) and a red (G. Galloway, J. Bornstein, J. Sullivan and M.A. Denborough, unpublished results) muscle is important as it indicates that this abnormality occurs independently of muscle fibre type.

2.3.3 Triad abnormalities in malignant hyperpyrexia

Glycerol-induced destruction of the T-tubule or replacement of the normal Ringer buffer with deuterium oxide-Ringer (D$_2$O-Ringer) abolished twitch and blocked the hypersensitive contractures of porcine MH skeletal muscle in response to a number of drugs (Okumura et al., 1980). E-C coupling has been shown to be inhibited in the presence of D$_2$O (Sandow et al., 1976) or by pretreatment with glycerol (Dulhunty and Gage, 1973) and the inhibitory effects of such treatments on drug-induced contractures in MH muscle indicate that E-C coupling is abnormal (Okumura et al., 1980).
The extent of T-tubular destruction induced by glycerination is generally incomplete (Fatkin et al., 1979) and the actual site of uncoupling is unknown. In MH muscle the return of hypersensitive drug contractures on returning to normal Ringer indicates that the uncoupling of the E-C process in the presence of D$_2$O is completely reversible (Okumura et al., 1980). The triad is the functional site at which E-C coupling occurs and electron microscopy has shown (Franzini-Armstrong, 1975) that the portions of the T-tubular and terminal cisternae which constitute the triad are distinct from the bulk of these membrane systems. Structural asymmetry is a characteristic feature of the triad (Franzini-Armstrong, 1975) with junctional "feet" being localised specifically on the terminal cisternae. These "feet" constitute a direct physical link between the junctional membranes, and an increase in their number appears to accompany muscle activation (Eisenberg et al., 1979). Such structures have been proposed to be involved in specific step(s) coupling excitation to eventual calcium release in frog skeletal muscle (Eisenberg et al., 1979) and the rate of formation and/or destruction of these "feet" may be abnormal in MH skeletal muscle. Stereological analysis of the dystrophic chicken pectoralis muscle has shown an increase in surface and volume densities of the T-tubular system compared to the normal chicken muscle and this increase has been proposed (Crowe and Baskin, 1979) to partially account for the altered contractile properties characteristic of this form of dystrophy. Although no published evidence exists supporting either abnormal triad "feet" formation or alterations in the surface area of the T-system, both are consistent with the hyperexcitability that is an in vitro feature of MH skeletal muscle.

2.3.4 Sarcoplasmic reticular abnormalities in malignant hyperpyrexia

Specialisation of the SR has resulted in specific regions fulfilling
quite distinct functions (Winegrad, 1970). Thus the terminal cisternae modulate calcium release while the longitudinal SR is responsible for calcium uptake. In the previous section the role of the terminal cisternae has been briefly considered as an integral functional unit of the triad and in this section the biochemical evidence favouring abnormalities in the ability of SR from porcine and human MH muscle to metabolise calcium is discussed. Britt and co-workers have found that calcium accumulation by SR from MH human muscle was less than normal (Britt et al., 1973a) while the equivalent process in MH swine SR was greater than normal (Britt et al., 1975), and the extent of these differences increased in the presence of halothane. The greater than normal basal uptake of calcium by MH swine SR (Britt et al., 1975) contrasts with the decreased calcium uptake and reduced calcium binding found in more recent studies (Nelson, 1978; Gronert et al., 1979). The in vivo assessment of the isometric twitch displayed by the tibialis anterior muscle of stress susceptible swine showed that the contracture time and the twitch tension were greater and the half relaxation longer than in the equivalent muscle of stress resistant swine (Campion et al., 1974, 1979). This indicates that in PSS skeletal muscle there is a lesion in the processes modulating calcium release and/or re-uptake. Campion and Topel (1975), Campion et al. (1976), Britt (1977, 1979) and Gronert et al. (1979) have extensively reviewed the experimental evidence favouring an SR abnormality in porcine MH, and it is generally believed that such an abnormality does exist but that its pathological contribution is unable to fully explain MH susceptibility in either the human or porcine forms of the syndrome.

2.3.5 Mitochondrial abnormalities in malignant hyperpyrexia

Mitochondrial metabolism is primarily concerned with the aerobic dependent generation of ATP. As a consequence, the metabolic competence
of this organelle is under tight regulatory control. At very low adenosine diphosphate (ADP) levels a resting or state 4 respiratory rate prevails but upon the addition of ADP, active or state 3 respiration results, with an associated increase in oxygen uptake. The respiratory rate in the presence (state 3) or absence (state 4) of ADP, gives the respiratory control index (RCI) or more correctly the acceptor control index (ACI) which in intact, freshly prepared mitochondria may be 10 or more (Gronert and Heffron, 1979). Molar ADP utilisation per mole of oxygen, the ADP/O ratio, together with the ACI gives two simple techniques with which to assess mitochondrial respiratory competence in vitro.

Early results obtained using swine (Denborough et al., 1973b; Mitchelson, 1974) and human (Britt et al., 1973b) muscle mitochondria showed that mitochondrial functioning was similar in normal and MH preparations both in the presence and absence of halothane. Mitochondria isolated from PSS muscle displayed similar state 3, state 4, ACI and ADP/O ratios to normal muscle mitochondria (Campion et al., 1975, 1976) and it is unlikely that a mitochondrial abnormality is a primary determinant of PSS susceptibility. Gronert and Heffron (1979) have shown that state 3 respiration and the rate of calcium binding in porcine MH mitochondria are approximately half those of normal porcine mitochondria and the authors have proposed that in MH, mitochondria are less "tightly coupled". This depression in the basal mitochondrial respiratory rate is indicative of an underlying myopathy (Gronert and Heffron, 1979) but in isolation this lesion could not account for the metabolic disturbance that characterises an episode of MH. Decreased mitochondrial calcium binding in porcine MH (Gronert and Heffron, 1979) is consistent with the increased mitochondrial calcium efflux rates reported by Cheah and Cheah (1978, 1979).
If the *in vitro* preference for calcium uptake at the expense of oxidative phosphorylation (Carafoli, 1975) is also a mitochondrial feature *in vivo* then the raised myoplasmic calcium levels that are maintained during MH would result in mitochondrial calcium uptake with eventual uncoupling of oxidative phosphorylation. The energy dependent uptake of calcium together with the decreased calcium binding capacity, increased calcium efflux rates and uncoupling of oxidative phosphorylation would quickly deplete ATP levels and eventually lead to cell necrosis. This "vicious cycle" of mitochondrial calcium overload and ATP depletion has been proposed to be a common terminal event in a wide variety of muscle disorders (Wrogemann and Pena, 1976).

2.3.6 Contractile protein abnormalities in malignant hyperpyrexia

The regulation of contractile activity distal to calcium release is complex with primary control being exerted by the interaction of calcium with the thin filament localised troponin complex (Murray and Weber, 1974; Perry, 1979). Alterations in intracellular pH which accompany MH (Bennet *et al.*, 1978) complicate any attempts to correlate altered contractile protein functioning with an underlying abnormality.

Tension development in glycerinated porcine fibres is pH dependent (Izumi *et al.*, 1977) and the lower pH values which characterise PSE muscle ensure that rigor occurs more readily as a consequence of irreversible actomyosin cross bridge formation. Specific differences in the thick filament protein myosin have been reported (Park *et al.*, 1975), but these probably reflect altered activities rather than a specific difference in myofibrillar protein composition (Kany *et al.*, 1978), and this altered functioning is most likely a result of the lower pH in PSE muscle.
2.3.7 Energy metabolism and malignant hyperpyrexia

Muscle energy consumption is unevenly divided in the contraction-relaxation cycle with 2/3 utilised during the active contractile phase (Lehninger, 1975). Thus energetic insufficiency would have quite dramatic effects on the skeletal muscle contraction and relaxation.

The role of CPK in muscle energetics is crucial, as its presence and normal functioning are essential in maintaining energetic sufficiency within the cell. Elevations in serum CPK levels may characterise a number of muscle disorders, but such an elevation should not be regarded as indicative of any single muscle disorder. Such diverse factors as age, sex, race, level of fitness, neurological condition, drug ingestion and trauma have been shown to effect serum CPK levels (Zsigmond, 1978). The elevated serum CPK levels often encountered in MH individuals are most likely indicative of a "fragile" sarcolemma (Britt, 1977), and it is possible that a substantial loss of CPK from muscle may affect the energetic competence of the cell (Pennington, 1978). In the absence of halothane both MH human (Isaacs et al., 1975) and swine (Van den Hende et al., 1976) muscle possess lower amounts of creatine phosphate (CP) than normal muscle, and when challenged with halothane MH muscle is rapidly depleted of CP and ATP (Harrison et al., 1969; Nelson et al., 1972; Bremen and Kench, 1973; Britt et al., 1977; Van den Hende et al., 1976) while such decreases are small in normal muscle. The rapid depletion in the energetic "currency" of the cell would have dramatic effects on contraction, where irreversible actomyosin cross-linking occurs (Britt, 1977, 1979), and on relaxation where myoplasmic calcium levels remain above the contractile threshold as a direct consequence of the critically low intracellular CP and ATP concentrations. This decrease in ATP levels may be due to a primary lesion in the functioning of one or more of the enzymes associated with ATP
regeneration or as a secondary response to the altered intracellular pH and raised calcium levels. The former possibility has been proposed by Schmidt et al. (1974) and deficiencies in the activity of adenylate kinase (AK) in human MH muscle have been documented (Schmidt et al., 1974; Schmidt and Heller, 1977). The latter enzyme catalyses the reaction,

\[ 2 \text{ADP} \rightarrow \text{AMP} + \text{ATP} \]

and in energetically active tissues (e.g. muscle) it plays an important role in maintaining the energy charge (Atkinson, 1968) of the cell. Partial (<70% activity, K. Schmidt, personal communication) and almost complete (<20% activity, Schmidt et al., 1974) AK deficiencies have been described. Two individuals in the latter category failed to survive halothane anaesthesia while an individual with a partial AK deficiency survived an episode of MH. Halothane binds specifically in the adenine niche of AK (Sachsenheimer et al., 1977) and as such may be considered an unusual, reversible inhibitor of AK. The decreased presence and/or activity of AK (Schmidt et al., 1974) together with the specific AK-halothane interaction (Sachsenheimer et al., 1977) may result in a breakdown in the cellular processes mediating ATP regeneration during MH and death may or may not occur depending on the extent of the AK deficiency (K. Schmidt, personal communication).

Futile cycling (Williams, 1976) and an enhanced glycolytic rate (Bremen and Kench, 1973) are time dependent features observed in MH swine under halothane anaesthesia and represent additional breakdown points in cellular energy metabolism.

2.3.8 Cyclic nucleotides and malignant hyperpyrexia

Since an enhanced glycolytic rate is an apparently common feature of PSS, PSE, MH skeletal muscle, a number of studies (Ono et al., 1976a, b,
1977a, b) have attempted to correlate the known stimulatory effects of cyclic AMP (cAMP) on glycogenolysis (Britt et al., 1977, 1979) with stress susceptibility. Although cAMP levels were significantly higher at three minutes post mortem in stress susceptible swine (Ono et al., 1976a, 1977a) no differences were detected in the activities of adenylate cyclase or phosphodiesterase (Ono et al., 1977b) in normal and stress susceptible porcine skeletal muscle. Abnormalities in the metabolism of cAMP have been reported in human dystrophic muscle (Mawatari et al., 1974; Canal et al., 1977) but the consequences of such abnormalities are far from understood. The recently demonstrated association between cAMP and calcium release (Raible et al., 1978) and cyclic GMP (cGMP) and decreased glycogenolysis (Beitner et al., 1978) in skeletal muscle provide important sites of action for the cyclic nucleotides but the relevance of such associations to MH muscle is unknown.

2.3.9 Histochemical and morphological abnormalities in malignant hyperpyrexia

The extent and type of innervation determines many of the functional properties of mammalian skeletal muscle (Vrbová et al., 1978) and many such different properties have been exploited in the development of histochemical techniques to identify different fibre types. Classificatory schemes are numerous (Addis, 1978) and most are based on the relative presence of a number of key metabolic enzymes. The most widely used categorises fibres as one of three types:

Type 1; low ATPase, high succinate dehydrogenase, oxidative, slow twitch, red fibres.

Type 2a; intermediate ATPase and succinate dehydrogenase, oxidative-glycolytic, fast twitch, pale fibres.
Type 2b; high ATPase, low succinate dehydrogenase, glycolytic, fast twitch, white fibres.

Thus histochemical designation is indicative of a more subtle biochemical association, with myoglobin content, glycolytic rate, mitochondrial content, the extent of development of the SR, SR calcium uptake capacities and the muscle relaxation rate (Beecher et al., 1965; Fiehn and Peter, 1971; Eisenberg and Kuda, 1975) all being related to muscle fibre type. Therefore, the accurate classification of muscle fibre types is essential in the detailed assessment of muscle function in any muscle disorder as simple alterations in muscle fibre type may result in dramatic changes in associated contractile properties (Close, 1972; Vrbová et al., 1978).

Morphological and histochemical examination provide simple and rapid means with which to assess muscle damage and their application in studies related to MH has been widely exploited (Isaacs, 1978; Palmer et al., 1978; K. Hopkinson and M.A. Denborough, unpublished results). In MH human muscle pathological findings have been variable, but their occurrence supports the contention that an underlying myopathy exists in at least some susceptible individuals. These changes have included an increase in type 1 fibres, fibre type grouping, peripheral fibre atrophy, variable fibre size, the presence of central cores, altered localisation of nuclei, ruptured mitochondria, and distended SR (Denborough et al., 1973a; Isaacs et al., 1973; Gullotta and Helpap, 1975; Isaacs and Heffron, 1975; Harriman et al., 1978; Hull et al., 1978; Isaacs, 1978; Riske-Nielson, 1978; K. Hopkinson and M.A. Denborough, unpublished results).

In porcine muscle the most common pathological finding relates to the increased presence of giant myofibres in the musculature of stress susceptible swine (Dutson et al., 1978). Supercontracted fibres appear more frequently in MH swine muscle than in comparable normal muscle (Venable,
1973; Palmer et al., 1978) and it is possible that there is a correlation between the occurrence of giant myofibres and supercontraction (Dutson et al., 1978). The pathological features that are common in human MH skeletal muscle are less frequent in MH porcine muscle (Palmer et al., 1978).

2.3.10 Dantrolene sodium, skeletal muscle and malignant hyperpyrexia

The ability of dantrolene to preferentially act on skeletal rather than on smooth or cardiac muscle or other non-muscle tissues, prompted its initial use in the treatment of MH (Harrison, 1975) and its successful use in vivo (Harrison, 1975; Gronert et al., 1976c; Jardon et al., 1976; Harrison, 1977; Kerr et al., 1978) together with its known tissue site of action are consistent with the primary MH lesion being skeletal muscle localised. Its in vitro effectiveness in preventing or reversing abnormal drug contractures (Anderson et al., 1976, 1978; Austin and Denborough, 1977; Okumura et al., 1980) provides definitive support for the extent and importance of skeletal muscle lesions in MH. Experiments on skeletal muscles from a number of vertebrate and invertebrate sources has shown that dantrolene, at low doses, has little effect on the electrical properties of the surface membrane (Ellis and Bryant, 1972), the neuromuscular junction (Ellis and Carpenter, 1972), SR calcium uptake (Yamamoto et al., 1977) or SR ATPase activities (Green et al., 1976; Yamamoto et al., 1977) and its primary effect is exerted via antagonism of intracellular calcium release (Ellis and Carpenter, 1972; Van Winkle, 1976; Francis, 1978). Intact fibre experiments utilising the calcium sensitive photoprotein aequorin (Desmedt and Hainaut, 1979; Lopez et al., 1979) have confirmed the specific antagonism by dantrolene of calcium release. Although the detailed molecular site of action of dantrolene is as yet unclear a number of processes have been considered, including the antagonism

2.4 SUMMARY

In MH, a wide body of evidence favours skeletal muscle as the crucially affected site, and the dysfunction induced in this tissue on exposure to an appropriate trigger is directly related to the high mortality rate characteristic of the syndrome.

Specific abnormalities have been described in a number of muscle processes including sarcolemmal depolarisation, electrical threshold activation, excitation-contraction coupling, sarcoplasmic reticular and mitochondrial calcium metabolism, ATP regeneration and drug-induced contraction. An attempt has been made to correlate the descriptive approach of normal muscle structure and function with the experimental observations on specific abnormalities reportedly found in MH skeletal muscle.

A more precise identification of the skeletal muscle lesion(s) and of their relative importance in the expression of the syndrome has been afforded with successful therapeutic and experimental utilisation of the thiohydantoin drug dantrolene sodium. This latter drug completely abolishes the drug-induced hypercontractility that is an in vitro feature of isolated MH muscle and in addition offers in vivo protection against the initiation of MH in susceptible swine.
CHAPTER 3

ISOLATION AND CHEMICAL CHARACTERISATION OF NORMAL AND
MALIGNANT HYPERPYREXIC PORCINE SKELETAL MUSCLE MICROSONES

3.1 INTRODUCTION

3.1.1 Muscle membrane abnormalities in malignant hyperpyrexia

The evidence favouring skeletal muscle as the primary affected tissue in MH has been presented in Chapters 1 and 2. Despite the widely held view that the skeletal muscle abnormality in MH is membrane localised (Britt, 1977, 1979; Denborough, 1980), no systematic studies have been undertaken to characterise the chemical composition of muscle membranes in either porcine or human MH skeletal muscle. Such an approach has documented alterations in the lipid (Takagi et al., 1973; Rubsmen et al., 1976; De Kretser and Livett, 1977; Swift et al., 1979) and protein (Scales et al., 1977) composition of muscle microsomes in a number of forms of dystrophy.

There is limited evidence favouring the notion that in MH, muscle membranes are characterised by a structural abnormality (McIntosh and Berman, 1974; Cheah and Cheah, 1978). Whole muscle homogenates and subcellular fractions from PSE susceptible porcine skeletal muscles are characterised by a higher degree of fatty acid unsaturation in membrane phospholipids, and such changes have been proposed to explain the altered permeability characteristics that are a feature of this myopathic condition (McIntosh and Berman, 1974). Arrhenius plots of calcium-stimulated mitochondrial respiration show that MH mitochondria display higher transition temperatures than those of normal preparations and this difference has been proposed to reflect a more saturated fatty acid profile.
in phospholipids of MH muscle mitochondria (Cheah and Cheah, 1978). Therefore the reports which have relevance to this topic have produced conflicting evidence as to the nature and extent of this abnormality, as both an increase (Cheah and Cheah, 1978) and a decrease (McIntosh and Berman, 1974) in the saturated/unsaturated fatty acid ratio of muscle membrane phospholipids have been proposed to be the underlying structural abnormality predisposing to MH.

The procedures utilised in isolating membrane fractions are of importance in any study designed to chemically characterise the isolated product and the fractionation scheme used and the rationale behind its use is presented in the following section.

3.1.2 Fractionation of skeletal muscle membranes

The isolation of pure subcellular muscle fractions was not a prerequisite in this study. The major requirements were for a mild and reproducible fractionation procedure based on established methods which would permit a comparative study of microsomal membrane composition between normal and MH skeletal muscles. A survey of the literature related to skeletal muscle membrane fractionation procedures revealed that a wide variety of methods were available which isolated membrane material of varying degrees of purity. A common feature in most of these isolation schemes has been the use of sucrose density gradient centrifugation, which utilises buoyant density as the deciding parameter in the isolation of membrane material.

The application of density gradient centrifugation to skeletal muscle membrane separation was first attempted in the mid 1960s (Hasselbach and Makinose, 1963; Konoshita et al., 1964; Searydarian and Mommaerts, 1965) and its early success in isolating discrete muscle subfractions has
ensured its continued use. Sucrose density gradient centrifugation in combination with high ionic strength buffers (to minimise contamination with contractile proteins) has been widely used to isolate surface (Boegman et al., 1970; Festoff and Engel, 1974; Schapira et al., 1974; Agapito and Cabezas, 1977; Smith and Appel, 1977; Bermego et al., 1978; Reddy et al., 1978; Biarchi et al., 1979) and intracellular (Martonosi and Halpin, 1971; Meissner, 1975; McIntosh et al., 1977) membranes from skeletal muscle. Recent evidence suggests that morphological alterations and a loss of peripherally associated membrane proteins occur when high ionic strength buffers are included in isolation procedures (Lau et al., 1977; Campbell et al., 1979). A number of other reports have appeared where the use of high ionic strength buffers has been avoided and the isolation of a variety of subcellular fractions has been achieved with minimal contractile protein contamination (Kidawi et al., 1973; Kidawi, 1974; Caswell et al., 1976; Lau et al., 1977; Biarchi et al., 1977, 1979). These latter isolation methods were compatible with the requirements initially set out (i.e. mild and reproducible) and the isolation scheme utilised here represents a modification of these methods.

This chapter outlines the isolation procedure employed in the fractionation of microsomal membranes from normal and MH porcine skeletal muscle. Low ionic strength solutions in combination with discontinuous sucrose density gradient centrifugation have been used to fractionate muscle microsomes on the basis of buoyant density. The chemical composition of these microsomal fractions has been characterised with respect to protein and lipid content in an attempt to detect compositional differences which may reflect susceptibility to MH.
3.2 MATERIALS AND METHODS

3.2.1 Animals and methods of anaesthesia

The animals in these studies were bred at the John Curtin School of Medical Research experimental farm at Braidwood, New South Wales. These animals were produced from crosses of MH susceptible Landrace and Large White breeds. Normal animals were obtained from the same source. In all cases MH susceptibility was assessed by recognised pharmacological criteria (Okumura et al., 1979).

Similar procedures were utilised for the collection of muscle from normal and MH swine. Animals were premedicated with the neuroleptic Azaperone (4'-fluoro-4-(4-(2-pyridyl)-1-piperazinyl)butyrophenone) 4 mg/ml intramuscularly and thiopentone was administered by an ear vein cannula. Tracheal intubation was carried out after anaesthesia was induced with 3-5 mg/kg of thiopentone. Anaesthesia was maintained with nitrous oxide in oxygen and supplemented with thiopentone when required. Segments (50-100 grams) of the trapezius and longissimus dorsi (l. dorsi) muscles were removed from the animal and immediately frozen in liquid nitrogen. The section of l. dorsi muscle used in this study was excised ventral from the first lumbar vertebra. Animals were then dispatched with a bolt gun and rapidly exsanguinated. In all experiments muscle was removed under anaesthesia and post mortem collection of muscle was not employed.

3.2.2 Halothane anaesthesia

In a number of normal (2) and MH (6) swine, additional muscle was removed after halothane (1-2%) anaesthesia. After collecting muscle from one side under nitrous oxide-barbiturate anaesthesia, animals were maintained for an additional hour under halothane and segments of trapezius and l. dorsi muscle were then removed from the opposite side. These animals
were then shot and exsanguinated.

Nitrous oxide-barbiturate anaesthesia was uneventful in all the normal and MH swine. Mild hypothermia was the only noticeable feature of halothane anaesthesia in the two normal swine. The clinical features of MH (Chapter 1) although slow to develop were present in four of the six MH swine after 1 hour of halothane anaesthesia. In the two remaining MH swine halothane anaesthesia was uneventful and susceptibility was confirmed by the development of the syndrome in response to i.v. succinyl choline (2 mg/kg).

3.2.3 Muscle fractionation

Muscle was maintained at -60°C and fractionation was initiated within 24 hours of removal. Frozen muscle was partially thawed, fat and connective tissue removed and then finely diced. The diced muscle was then homogenised in 3 volumes of 8% (w/v) sucrose in a Waring blender for 1 minute (40 seconds low speed, 20 seconds high speed). The blades of the blender were reversed to minimise organelle damage (Roesenthal et al., 1965) and to decrease extraction of contractile proteins. The fractionation scheme is shown in Figure 3.1. The homogenate was filtered through 20 layers of cheese cloth and the residue discarded. The filtrate was centrifuged at 59,000 g for 45 minutes utilising a Beckman 30 rotor and a Beckman L-35 ultracentrifuge. The pellets were resuspended in 8% (w/v) sucrose and layered (10 ml/tube) on a discontinuous sucrose gradient consisting of 15 ml of 45% (w/v) sucrose and 12 ml of 30% (w/v) sucrose. Cellulose nitrate tubes (3½ by 1 inch) were used and centrifugation was carried out in a Beckman SW 27 swing out rotor at 96,000 g for two hours. The suspensions recovered from the 8-30% and 30-45% interfaces were pelleted at 59,000 g for 45 minutes in the 30 rotor. The membrane samples
MUSCLE 30 - 60 grams wet weight

HOMOGENISE IN 3 vols 8%(w/v) SUCROSE
FILTER

CENTRIFUGE FILTRATE

DISCARD SUPERNATANT

PELLET

RESUSPEND

CENTRIFUGE DISCONTINUOUS SUCROSE GRADIENT

96000 g 120 mins, 4°C

COLLECT INTERFACE BANDS

CENTRIFUGE

59000 g 45 mins, 4°C

RESUSPEND PELLETS

RETAIN FRACTIONS FOR:
1). LIPID ANALYSIS
2). AMINO ACID ANALYSIS
3). PROTEIN ELECTROPHORESIS
4). N M R

Figure 3.1: Schematic outline of skeletal muscle fractionation procedure
were resuspended in 10 ml of distilled water and aliquots assayed for protein content. Samples of known protein concentration were removed for chemical and physical analysis and stored at -60°C until required.

3.2.4 Binding of tritiated ouabain

A total of four (2 normal, 2 MH) swine were used in these experiments and only the l. dorsi muscle was used. Tritiated ouabain (20 µCi (approximately 20 pmoles), The Radiochemical Centre) was dissolved in 10 ml of a modified Ringer buffer (121 mM NaCl, 5.4 mM KCl, 1.2 mM MgSO₄, 1.2 mM NaH₂PO₄, 15 mM NaHCO₃, 11.5 mM d-glucose and 2.5 mM CaCl₂). Under nitrous oxide-barbiturate anaesthesia the l. dorsi muscle was exposed and the ouabain solution was injected directly into the muscle at several points. Anaesthesia was continued for a further 20 minutes and the injected segment of the muscle was removed prior to dispatching the animal with a bolt gun. Microsomal fractionation was initiated within one hour of excision and the fate of the label was followed by liquid scintillation spectroscopy. Aliquots (200 µl) were removed throughout the fractionation procedure, added to 10 ml of an aqueous based liquid scintillant mixture (66% xylene (v/v), 33% triton x-100 and 0.5% (w/v) 2,5-diphenyloxazole) and counted in a Packard Tri-Carb Liquid Scintillation Spectrometer.

The ouabain concentration was calculated by counting an aliquot of the tritiated ouabain solution and using a value of 49 Ci/mmol for the specific activity (Radiochemical batch analysis, The Radiochemical Centre). Ouabain binding was expressed as pmoles bound per individual fraction.
3.2.5 **Electron microscopy**

Pelleted fractions were fixed in 5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4 and post fixed in 1% osmium tetroxide. Graded dehydration was achieved through a series of acetone washes (30% up to 100% (v/v)) and the samples were then embedded in Spurs resin. Sections were cut with a diamond knife (Dupont) on a Reichert OME2 ultramicrotome and post stained in saturated solutions of uranyl acetate (45 minutes) and "Reynolds" lead citrate (20 minutes).

The sections were observed in a Phillips 300 Electron Microscope.

3.2.6 **Protein estimation and amino acid analysis**

Protein content in fractions was estimated as described by Lowry *et al.* (1951) with a two point double log plot used to construct a standard curve (Peterson, 1977). Amino acid analysis was performed by Mr L.B. James, Department of Biochemistry, John Curtin School of Medical Research, Canberra, by the method of Moore *et al.* (1958).

3.2.7 **Protein electrophoresis**

Slab gels containing 4.5% (stacking gel) and 10% (separating or running gel) acrylamide were prepared from a stock solution of 29.2% (w/v) acrylamide and 0.8% (w/v) N,N'-methylenebisacrylamide. Final concentrations in the separating gel were as follows; 10% (w/v) acrylamide, 0.375 M Tris-HCl (pH 8.8), 0.1% (w/v) sodium dodecyl sulphate (SDS), 0.05% (v/v) N,N,N',N'-tetraethylmethylenediamine (TEMED) and 0.33% (w/v) ammonium persulphate. The gels were poured to within 2 cm of the top of the plate, overlayed with water and allowed to polymerise for 1-2 hours. The stacking gel consisted of; 4.5% (w/v) acrylamide, 0.125 M Tris-HCl (pH 6.8) 0.1% (w/v) SDS, 0.06% (v/v) TEMED and 0.029% (w/v) ammonium persulphate.
Sample channels were created in the stacking gel by the presence of a teflon "comb" containing eight 1 cm wide "teeth". Polymerisation of the stacking gel generally took 2-3 hours. The electrode buffer contained 0.25 M Tris-HCl, 0.192 M glycine, and 0.1% (w/v) SDS (pH 8.3).

Solubilising buffer contained 0.01 M phosphate (pH 7.1), 5% (w/v) SDS, 10% (v/v) 2-mercaptoethanol and 10% (w/v) sucrose (Glossman and Lutz, 1970). Lyophilised membrane samples were dissolved in the solubilising buffer by heating at 100°C for 3 minutes. Aliquots containing between 30 and 80 μg protein were applied to individual channels and electrophoresis was carried out with a current of 35 mA. Running time varied from 3 to 4 hours and was followed by the frontal movement of the tracking dye, bromophenol blue.

The gels were fixed in 10% (v/v) isopropyl alcohol and 10% (w/v) trichloroacetic acid for 1-2 hours. These were then stained overnight in a 50% (v/v) methanol and 10% (v/v) acetic acid containing 0.1% (w/v) Coomassie brilliant blue. The gels were diffusion destained by exhaustive washing in 10% (v/v) acetic acid and 5% (v/v) methanol solutions and stored in 10% (v/v) acetic acid. Electrophoresis was carried out in a unit constructed to published specifications (Madjar et al., 1977) with the exception that no cooling facilities were built into the apparatus. The preparation of the acrylamide gels represents a modified version of the method published by Laemmli (1970).

The protein stained gels were photographed and densitometric scans obtained of the prints. Densitometry was performed on a model SD 3000 Spectrodensitometer (Schoeffel Instrument Corp.) equipped with a model 300 density computer. Scans were performed at 550 nm using a single beam mode of reference.
3.2.8 Lipid analysis

Membrane suspensions were lyophilised and extracted overnight in 10 ml of chloroform/methanol (2/1; v/v). These samples were filtered through Whatman 41 filter paper into preweighed graduated glass tubes and the filtrate was aqueous washed (Folch et al., 1957). Aliquots were removed for cholesterol (Owens and Hughes, 1970) and total lipid phosphorus (Bartlett, 1959) determinations and the remaining lipid was dried under nitrogen. Total lipid content was determined gravimetrically. Neutral and polar lipid separation was achieved by thin layer chromatography (TLC) on 0.25 mm thick silica gel G plates in a hexane:diethyl ether:acetic acid (90:10:1; v/v/v) solvent system (De Moreno et al., 1976). The phospholipid bands were visualised by spraying with 0.1% (w/v) 2,7-dichlorofluorescein in 95% methanol and were methylated in 14% (w/v) boron trifluoride-methanol at 100°C for 10 minutes (Morrison and Smith, 1964). The methylated fatty acids were extracted into hexane and analysed by gas liquid chromatography (GLC). Analysis of the methylated fatty acids was performed on a Packard 7300 gas chromatograph equipped with a model 805 oven.

The glass column (180 cm by 4 mm i.d.) was packed with 10% DEGS-PS on 80/100 mesh Supelcoport (Supelco Inc.) and operated at 190°C. Injection and detection ports were maintained at 225°C and gas flow rates were as follows; N₂, 20 ml/minute; H₂, 20 ml/minute and air, 200 ml/minute. An inlet column pressure of 20 psi was maintained at the injection port. Peak identification was referenced against known standards (PUFA 1 and 2; Supelco Inc.) and log plots of retention time versus carbon number were used to confirm identification. A Mini Lab digital integrator (Columbia Scientific Instruments) was used for peak quantitation.

All lipid values are reported as means ± S.D.
3.2.9 Materials

Sucrose, SDS, ammonium persulphate, glycine, bromophenol blue, and boron trifluoride were obtained from British Drug Houses. Silica gel-G was from Merck, azaperone from Ethnor and TEMED from Koch-Light Laboratories. Coomassie brilliant blue, dichlorofluorescein, and trizma base were obtained from Sigma Chemical Company and acrylamide, N,N'-methylenebisacrylamide and 2-mercaptoethanol were from Eastman. Additional reagents (i.e. buffer salts, scintillation reagents etc.) were obtained from British Drug Houses.

3.3 RESULTS

3.3.1 Buoyant density patterns

Figure 3.2 illustrates the density gradient pattern of normal skeletal muscle microsomes from trapezius and 1. dorsi muscles. The fractions designated as light, heavy and sediment correspond to material banding at 8-30%, 30-45% interfaces and at the bottom of the tube, respectively. Similar patterns were obtained for both MH muscles. The variable occurrence of light material in 1. dorsi muscles was a feature of both normal and MH muscles. Halothane anaesthesia failed to alter this gradient fractionation pattern in either normal or MH muscles.

3.3.2 Electron microscopy

Representative electron micrographs of the various microsomal fractions are shown in Figures 3.3 and 3.4. Micrographs of the crude microsomal material and of the sediment fractions are not shown but in both cases contractile protein contamination was evident. Vesicular material accounted for at least 70% of the former material.

Light vesicles isolated from normal (Figure 3.3a) and MH (Figure 3.3b)
Figure 3.2: Buoyant density pattern of normal porcine skeletal muscle microsomes in discontinuous density gradients

The gradient consisted of 8%, 30% and 45% (w/v) sucrose.

Centrifugation was for 2 hours at 96,000 g. Buoyant density patterns of normal trapezius (a) and l. dorsi (b) microsomes are shown. MH muscles displayed similar buoyant density patterns.
Figure 3.3: Electron micrographs of microsomal membranes from normal and MH trapezius muscle

Micrographs of light (A) and MH (B) microsomal vesicles and of heavy normal (C) and MH (D) microsomal vesicles are shown. T-tubular material is indicated by single arrows and mitochondria by double arrows. The bar line represents 500 nm.
Figure 3.4: Electron micrographs of microsomal membranes from normal and MH l. dorsi muscles

Micrographs of light normal (A) and MH (B) microsomal vesicles, and of heavy normal (C) and MH (D) microsomal vesicles are shown. T-tubular material is indicated by single arrows and mitochondria by double arrows. The bar line represents 500 nm.
trapezius muscle displayed many common features including closed vesicles of variable size, an absence of mitochondria and an absence of electron dense material within the vesicles.

The heavy vesicles isolated from normal (Figure 3.3c) and MH (Figure 3.3d) trapezius muscle were characterised by the predominance of variable sized vesicles, some mitochondria (double arrows in Figure 3.3), the presence of T-tubular material (single arrows in Figure 3.3) and the presence of electron dense material within many of the vesicles.

The light banding material from both normal (Figure 3.4a) and MH (Figure 3.4b) 1. dorsi muscle, displayed characteristics similar to those described for light microsomes from trapezius muscle (Figure 3.3a and b). Mitochondrial contamination was more extensive and T-tubular material less evident in heavy 1. dorsi microsomes (Figure 3.4c and d) than in trapezius heavy microsomes (Figure 3.3c and d) but as with the other microsomal fractions vesicular material predominated.

Halothane treated normal and MH muscle fractions were not analysed by electron microscopy.

3.3.3 Ouabain binding

An average of 1% (0.33-1.25%) of the total ouabain injected was recovered in the crude microsomal fraction, with MH microsomes binding twice (3.8 pmoles, n = 2) as much as normal (1.8 pmoles, n = 2) microsomes. Arbitrarily setting the amount of ouabain bound by the light microsomal fractions equal to 1, then ratios of 1, 3.4 and 6.7 and 1, 3.5 and 2.4 were obtained for the light, heavy and sediment fractions of normal and MH 1. dorsi muscles, respectively. In normal muscle, of the 1.8 pmoles of bound ouabain applied to the gradient, 6%, 18% and 38% was recovered in the light, heavy and sediment fractions, respectively. Of the 3.8 pmoles of
ouabain bound by the MH crude microsomal fraction, 9%, 30% and 20% were bound by the light, heavy and sediment fractions, respectively. Thus, in these limited experiments both qualitative and quantitative differences characterised ouabain binding in normal and MH l. dorsi muscles.

3.3.4 Protein analysis

Quantitative recovery of muscle membranes was not attempted in this study, hence no attempt was made to correlate membrane protein recovery per amount of muscle homogenised. The generally low yield (in terms of protein) of light microsomal material from normal and MH l. dorsi muscles resulted in only limited analysis being carried out on these fractions. Therefore only electron microscopic and protein electrophoretic analysis were consistently carried out on the l. dorsi light microsomal material.

3.3.5 Amino acid composition

Total hydrolysis of the individual fractions yielded the amino acid compositional profiles shown in Table 3.1. The high occurrence of acidic amino acids (i.e. glutamic and aspartic acids) was a feature in all samples and the % contribution of individual amino acids was similar in all fractions.

The low occurrence of glycine (<9%) is indicative of minimal contamination by collagen and other connective tissue-like proteins. The sulphur containing amino acid cysteine is destroyed in the hydrolytic step employed for this analysis and separate attempts were not made to assess its relative contribution. Amino acid analysis was not performed on either halothane treated normal or MH fractions.
Table 3.1: Amino acid composition of sub-cellular fractions of normal and MH porcine skeletal muscles

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Residues per 100 Total Amino Acids</th>
<th>Trapezius Normal</th>
<th>Trapezius MH</th>
<th>L. Dorsi Normal</th>
<th>L. Dorsi MH</th>
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<tr>
<td></td>
<td></td>
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<td>Heavy</td>
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1 Values are reported as means (n = 3)
3.3.6 Protein SDS acrylamide gel analysis

Molecular weight standardisation of the 10% acrylamide gels used in this study was achieved with the use of the known molecular weight standards shown in Figure 3.5. Molecular weight assignments for 10% acrylamide gels are reliable within the 95,000 to 20,000 molecular weight range, as it is only within this range that the straight line relationship between the log of the molecular weight and protein mobility is linear (Figure 3.5).

The large number of proteins was a feature of the light and heavy fractions from both muscle types in normal and MH microsomes. Figure 3.6 (a and b) shows sample gels and their respective densitometric scans of trapezius light microsomal proteins isolated from normal and MH muscles, respectively. The comparable light 1. dorsi fractions are shown in Figure 3.6a and b for normal and MH muscle, respectively. Quantitative differences were apparent between the normal and MH fractions of both muscle types but such differences were of a variable nature. The protein profiles of the heavy microsomal membranes are shown in Figure 3.7 and as for the light microsomal proteins quantitative rather than qualitative differences were apparent. Sediment fractions (Figure 3.8) displayed the characteristic myosin (molecular weight 200,000) and actin (molecular weight 43,000) proteins, indicating that in these samples contractile protein contamination was extensive. Apparent differences in densitometric scans are primarily due to variable sample application with samples b and d (Figure 3.8) being overloaded.

The sample gels shown represent muscle fractions which were scanned under identical conditions. Variations in the width and length of the scanning beam affect resolution in densitometry but in these sample gels this factor has been minimised. As many of the samples (not shown)
Figure 3.5: Electrophoretic mobility as a function of molecular weight

Molecular weight on a log scale is plotted against relative mobility for a standard mix of proteins. These included:
β-galactosidase (120,000), phosphorylase A (93,000), bovine serum albumin (67,000), catalase (60,000), glutamate dehydrogenase (53,000), ovalbumin (43,000), alcohol dehydrogenase (37,000), triosephosphate isomerase (26,500), adenylate kinase (23,000), ribonuclease A (13,500) and cytochrome c (12,000). Electrophoresis and staining were carried out as described in Section 3.2.7.
Figure 3.6: Polyacrylamide gel patterns of light microsomal membranes from normal and MH skeletal muscles

Representative patterns of light microsomal proteins from normal (A) and MH (B) trapezius and normal (C) and MH (D) l. dorsi muscles are shown. Molecular weight standards are indicated by arrows and from left to right comprise: phosphorylase A (93,000), bovine serum albumin (67,000), ovalbumin (43,000) and adenylate kinase (23,000). Sample solubilisation, electrophoresis, staining, and densitometry were carried out as described in Section 3.2.7.
Figure 3.7: Polyacrylamide gel patterns of heavy microsomal membranes from normal and MH skeletal muscles

Representative patterns of heavy microsomal proteins from normal (A) and MH (B) trapezius and normal (C) and MH (D) 1. dorsi muscles are shown. Molecular weight standards are indicated by arrows and from left to right comprise: phosphorylase A (93,000), bovine serum albumin (67,000), ovalbumin (43,000) and adenylate kinase (23,000). Sample solubilisation, electrophoresis, staining, and densitometry were carried out as described in Section 3.2.7.
Representative patterns of sediment proteins from normal (A) and MH (B) trapezius and normal (C) MH (D) 1. dorsi muscles are shown. Molecular weight standards are indicated by arrows and from left to right comprise: phosphorylase A (93,000), bovine serum albumin (67,000), ovalbumin (43,000) and adenylate kinase (23,000). Sample solubilisation, electrophoresis, staining, and densitometry were carried out as described in Section 3.2.7.
were scanned over a six month period the variation in protein profiles may be partially caused by alterations in scanning conditions.

In a number of MH microsomal fractions the diminished occurrence of a low molecular weight protein distinguished susceptible from normal muscles and this deficiency was found in both trapezius and l. dorsi muscles. This feature is illustrated in Figure 3.7 where the absence of this protein (molecular weight 20-25,000) in the heavy MH fraction from l. dorsi muscle contrasts with its presence in the equivalent fraction from normal muscle.

Halothane anaesthesia failed to modify the protein profiles of either normal and MH muscle microsomes in a consistent manner but a quantitative decrease in a number of densitometric peaks in some halothane treated MH muscle fractions might reflect a generalised loss of peripherally attached membrane proteins (gels and scans not shown).

3.3.7 Lipid composition

a) Light microsomes

Light microsomes isolated from normal and MH trapezius muscle yielded total lipid to protein (w/w) ratios of $1.15 \pm 0.29$ (n = 10) and $0.94 \pm 0.25$ (n = 8), respectively. Cholesterol content was similar in both, but there was a significant decrease ($p < 0.05$) in the lipid phosphorus content in the light microsomal fraction isolated from MH trapezius muscle (Table 3.2). In the light MH fraction the cholesterol to lipid phosphorus ratio was approximately double that found in normal muscle (Table 3.2). The low occurrence of light microsomal material from normal and MH l. dorsi muscle was a consistent feature and was probably indicative of a heavier banding surface membrane which, because of the limited number of density "steps" in the gradient, banded with the bulk of the microsomal material at
Two comparisons have been made: normal and MH muscle pre-halothane and MH muscle pre- and post-halothane.

*p > 0.05

Ratio of averages

I. deep muscle yielded insufficient material for analysis

<table>
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<tr>
<th></th>
<th>Normal (pre-halothane)</th>
<th>MH (pre-halothane)</th>
<th>MH (post-halothane)</th>
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<tr>
<td></td>
<td>(5 = u) 8.0 ± 3.65</td>
<td>(8 = u) 197.36 ± 76.06</td>
<td>(8 = u) 1.27 ± 0.36</td>
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<td>(5 = u) 9.0 ± 2.0</td>
<td>(8 = u) 161.88 ± 47.16</td>
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<td>9.9</td>
<td>(5 = u) 12.66 ± 3.16</td>
<td>(8 = u) 124.48 ± 31.56</td>
<td>(10 = u) 1.15 ± 0.20</td>
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Table 3.2: Lipid composition of normal and MH light microsomes

TRAPEZIUS
Two comparisons have been made: normal and MH muscle pre-halothane and MH muscle pre- and post-halothane.

<table>
<thead>
<tr>
<th></th>
<th>Normal (pre-halothane)</th>
<th>MH (pre-halothane)</th>
<th>MH (post-halothane)</th>
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<td>7.7</td>
<td>(4 = n) 10.22 ± 0.20</td>
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<td>7.8</td>
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<td>6.6</td>
<td>(7 = n) 10.35 ± 0.43</td>
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<td>68.54 ± 0.26</td>
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** p < 0.01

B. L. DORISI

<table>
<thead>
<tr>
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<th>MH (post-halothane)</th>
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<td>8.5</td>
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<td>13.2</td>
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<td>9.6</td>
<td>(4 = u) 10.35 ± 1.08</td>
<td>0.57 ± 0.00</td>
<td>79.28 ± 2.14</td>
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A. TRAPEZIUS

Table 3.3: Lipid composition of normal and MH heavy microsomes

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<th>(mg/mg)</th>
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<th>Cholesterol</th>
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Table 3.4: Lipid composition of normal and MH sediment fractions

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<td>9.6</td>
<td>2.96 ± 1.16</td>
<td>28.41 ± 18.33</td>
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<td>7.7</td>
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<td>0.24 ± 0.06</td>
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<td>17.0</td>
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<td>MH (pre-halothane)</td>
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<td>5.5</td>
<td>4.14 ± 2.10</td>
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<td>0.25 ± 0.11</td>
<td>Normal (pre-halothane)</td>
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</table>
Figure 3.9: Polar fatty acid composition of the light microsomal fraction from normal and MH trapezius muscles

The mean percentage (±S.D.) of individual fatty acids are shown. Fatty acids contributing less than 5% are excluded. Two comparisons have been made (a) MH muscle pre-halothane with normal muscle pre-halothane (significance values are represented by *); and (b) MH muscle post-halothane with MH muscle pre-halothane (significance values are indicated by *).

* p < 0.05  
*** p < 0.001
Figure 3.10: Polar fatty acid composition of the heavy microsomal fraction from normal and MH trapezius muscles

The mean percentage (±S.D.) of individual fatty acids are shown. Fatty acids contributing less than 5% are excluded. Two comparisons have been made (a) MH muscle pre-halothane with normal muscle pre-halothane (significance values are represented by *); and (b) MH muscle post-halothane with MH muscle pre-halothane (significance values are indicated by **).

*** p < 0.01

★ p < 0.05

★ p < 0.001
The mean percentage (±S.D.) of individual fatty acids are shown. Fatty acids contributing less than 5% are excluded. Two comparisons have been made: (a) MH muscle pre-halothane with normal muscle pre-halothane (significance values are represented by *); and (b) MH muscle post-halothane with MH muscle pre-halothane (no significant differences were found in this group).

* p < 0.05

Figure 3.11: Polar fatty acid composition of the heavy microsomal fraction from normal and MH 1. dorsi muscles
the 30-45% interface. Phospholipid fatty acid composition of the trapezius light fraction is shown in Figure 3.9. A significant increase in the palmitic acid (C16:0) content (p < 0.05) and a decrease in the linoleic acid (C18:2) content (p < 0.001) were a feature of light microsomal phospholipids from MH trapezius muscle.

There were no significant differences in light microsomal lipid, cholesterol or lipid phosphorus contents after halothane anaesthesia (Table 3.2) indicating that the lipid compositional profiles of normal and MH muscle are not grossly altered by a one hour halothane challenge. The decrease in arachidonic acid (C20:4) levels was the only significant change that occurred in MH light microsomal membrane composition in response to halothane anaesthesia (Figure 3.9).

b) **Heavy microsomes**

Total lipid and lipid phosphorus content of heavy microsomal fractions from trapezius muscle was significantly lower (p < 0.01) than in normal heavy microsomal fractions (Table 3.3A). Cholesterol levels were similar (Table 3.3A) in both normal and MH heavy microsomal fractions and with the exception of linoleic acid (C18:2) phospholipid fatty acid composition was essentially similar (Figure 3.10).

Lipid composition of normal and MH 1. dorsi heavy microsomal fractions was similar (Table 3.3B). The levels of oleic acid (C18:1) were lower in MH heavy microsomes (Figure 3.11) and this represented the only statistically significant difference in lipid composition between normal and MH 1. dorsi heavy microsomal fractions.

Table 3.3A and Figure 3.10 summarise the effects of halothane anaesthesia on MH trapezius heavy microsomal lipid composition. Total lipid content was increased (before halothane = 0.40 ±0.07; after halothane
= 0.64 ± 0.16; p < 0.01, Table 3.3A) and there was a significant increase (p < 0.01) and decrease (p < 0.05) in the levels of palmitic (C16:0) and arachidonic (C20:4) acids, respectively, after halothane treatment (Figure 3.10).

No changes were recorded in the lipid composition of MH 1. dorsi heavy microsomes isolated before and after halothane anaesthesia (Table 3.3B and Figure 3.11). Halothane anaesthesia also had little effect on membrane lipid composition for both normal trapezius and 1. dorsi muscles (results not shown).

The lipid data from the sediment fractions are shown in Table 3.4. Due to extensive contamination by non-membranous material no attempt has been made to statistically analyse the data and it has been included merely for comparative purposes. In a number of sediment fractions phospholipid fatty acid profiles were also determined but as the compositional distribution reflected that found in the light and heavy microsomal fractions it has not been included here.

3.4 DISCUSSION

A major aim of this study was the development of a mild and reproducible fractionation procedure for porcine skeletal muscle microsomes which avoided the use of high salt solutions, hypotonic solutions or incubations at elevated temperatures. These latter procedures are used in most skeletal muscle membrane fractionation schemes. This seemed a prerequisite for experiments designed to structurally and functionally characterise muscle membranes from a normal and myopathic source. The methods described here fulfil this aim and their application to both red and white muscles from normal and MH swine has provided data on the nature
of the structural abnormality in MH muscle membranes.

The identification of muscle microsomal fractions was carried out on the basis of electron microscopic and chemical compositional characteristics. In the case of l. dorsi muscle the binding of tritiated ouabain also permitted the tracking of T-tubular material (Caswell et al., 1976) during the fractionation process. The high cholesterol/protein and total lipid/protein ratios in the light fractions are characteristic features of the muscle sarcolemma (Fiehn et al., 1971; Schapira et al., 1976; Agapito and Cabezas, 1977; Headon et al., 1977; Somer et al., 1977). The diminished occurrence of light banding material from l. dorsi muscle indicates that in this muscle sarcolemmal membranes band at heavier densities. Similar observations have been made on the rat extensor digitorum longus muscle which, like the porcine l. dorsi muscle, is a white muscle (Smith and Appel, 1977). Light banding material from rabbit muscle also bands at this density (i.e. 30% sucrose) in continuous gradients and this material has been proposed to constitute SR membrane vesicles which are "inside-out" (Sarzala and Michalak, 1978). Thus the light vesicle fraction characterised in the present study probably constitutes membrane material which originates from the sarcolemma (Kidawi, 1974) and the SR (Meissner, 1975; Sarzala and Michalak, 1978).

The lower total lipid/protein and cholesterol/protein ratios observed in the heavy vesicle fractions were consistent with their heavier banding position on the gradient (McIntosh et al., 1977) and indicate that the bulk of this membrane material was intracellular in origin (Headon et al., 1977).

The differences in buoyant density profiles obtained were characteristic of the type of muscle used (i.e. red or white) and failed to
distinguish normal from MH muscles. T-tubular entrapment of tritiated ouabain (Caswell et al., 1976) was qualitatively and quantitatively different in normal and MH l. dorsi muscles and a proliferation of the junctional tubular system, as has recently been demonstrated in dystrophic muscle (Scales et al., 1977; Crowe and Baskin, 1979) might explain the increased capacity of MH l. dorsi muscle to bind ouabain. It is possible, therefore, that hypersensitive E-C coupling which is an in vitro pharmacological feature of MH muscle (Okumura et al., 1980) may simply reflect such a proliferation of the tubular membrane system. This possibility remains to be fully explored.

Electron microscopy provided additional evidence favouring the notion that all light and heavy fractions constituted predominantly vesicular membrane material. A heterogeneous size and shape distribution were characteristic features of normal and MH light and heavy fractions from both muscle types, indicating that these physical parameters were of limited importance in determining vesicle buoyancy in sucrose density gradients. Tentative identification of the skeletal muscle site of origin of the vesicle-like material was attempted only for mitochondria and T-tubular material and identification was based on published micrographs (Kidawi et al., 1973; Kidawi, 1974; Caswell et al., 1976; Lau et al., 1977). The preferential localisation of porcine mitochondrial and T-tubular material in the heavier banding fraction accords with the banding position of this material from rat and rabbit skeletal muscle (Kidawi et al., 1973; Caswell et al., 1976). The remaining vesicular material, constituting the bulk of both the light and heavy fractions, probably originated from the terminal cisternae and from the longitudinal sections of the SR (Meissner, 1975). Furthermore Meissner (1975) has proposed that the light and heavy banding vesicles might be preferentially derived from the longitudinal
sections and terminal cisternae of SR, respectively, but it is unknown if such a distribution characterises the vesicle fractions isolated in the present study.

Amino acid compositional data have been used in a number of studies of muscle membranes (Martonosi and Halpin, 1971; Vandenburgh et al., 1974) and these data provide a useful indicator of connective tissue contamination. The high occurrence of glycine and proline indicates that the membrane preparation isolated by Vandenburgh et al. (1974) contained extensive amounts of collagen-like proteins. The results obtained in the present study closely reflect the amino acid compositional profile of rabbit SR (Martonosi and Halpin, 1971) and in both of these studies the low occurrence of glycine and proline indicates that contamination with connective tissue proteins was minimal.

The SDS gel electrophoresis study showed that quantitative rather than qualitative differences characterise the various microsomal fractions but the variable nature of these differences restricted any detailed comparisons.

The complexity of the membrane protein acrylamide gel patterns contrasts with most published reports on muscle membranes (McIntosh et al., 1977; Lau et al., 1977; Smith and Appel, 1977) and may reflect differences in isolation and electrophoretic conditions or cross contamination with different membrane fractions.

The similarity in membrane protein compositions between the red and white fractions characterised here agrees with published data on rat red and white muscle microsomes (Smith and Appel, 1977) but contrasts with the differences reported to exist between red and white microsomal proteins in porcine l. dorsi muscle (McIntosh et al., 1977). Normal and MH
microsomes were essentially similar in qualitative protein content and it would seem that the abnormal functioning that characterises MH muscle is not related to gross changes in membrane protein constituents. The quantitative differences may in themselves reflect MH susceptibility but their variable occurrence tends to downplay such a possibility.

Significant differences were found in the lipid composition of MH trapezius fractions with increased cholesterol and decreased lipid phosphorus values being a consistent feature in all the trapezius fractions analysed. The decrease of linoleic acid content in MH trapezius muscle membranes is in line with the above observations on cholesterol and lipid phosphorus contents and indicates that in MH swine the red trapezius muscle is in a more solid or rigid state. Thus on exposure to halothane the membranes may become abnormally fluid (Austin, 1976). The inability to detect similar differences in the white 1. dorsi muscle questions the generality of this change in membrane lipid composition in MH, and of its importance in explaining its aetiology. Abnormal drug-induced contractures are an in vitro feature of the white gracilis (Okumura et al., 1979) and the red trapezius (B. Crocker, J. Sullivan and M.A. Denborough, unpublished results) muscles, indicating that the abnormality is expressed irrespective of muscle fibre type. It is possible that the lipid changes described in MH trapezius muscle may be a secondary event and may reflect changes in fibre type distribution. The differences described in phospholipid fatty acid profiles of the normal red trapezius and the white 1. dorsi muscles, contrast with evidence presented on phospholipid fatty acid composition in red and white segments of procine 1. dorsi muscle (McIntosh et al., 1977). Possible contamination with adipose material (Takagi et al., 1973) may have contributed to the differences observed in lipid composition of normal and MH trapezius muscle microsomes.
The inability to detect consistent lipid and/or protein compositional differences in the crude microsomal fractions characterised in the present study suggests that (1) the lipid and protein content of MH muscle microsomes is not grossly abnormal and (2) a further purification of the microsomal fractions is required to properly assess whether a consistent compositional abnormality is present in specific MH skeletal muscle membranes (e.g. T-tubule membrane).

3.5 SUMMARY

Discontinuous sucrose density gradient centrifugation was employed to isolate discrete light and heavy banding microsomal fractions from normal and MH porcine skeletal muscles. These fractions were characterised with respect to lipid and protein content. Differences in lipid composition were apparent only in the red trapezius muscle, where an increase in cholesterol and a decrease in phospholipid unsaturated fatty acid content characterised the MH trapezius muscle. The similar lipid compositional profiles for normal and MH white 1. dorsi muscles suggests that the lipid changes observed in MH trapezius may reflect a secondary pathological event. Consistent differences in membrane protein content were not apparent in any of the muscle fractions analysed although variable protein differences were observed in the low molecular weight range. It is suggested that gross changes in lipid and protein content do not characterise MH muscle membranes.
CHAPTER 4

NUCLEAR MAGNETIC RESONANCE STUDIES OF
ANAESTHETIC-MEMBRANE INTERACTIONS

4.1 INTRODUCTION

4.1.1 Theories of anaesthesia

"General anaesthesia is a pharmacologically induced reversible
disruption resulting in a coordinated sequence of changes in neural
activity" (Kaufmann, 1977). The clinical value of anaesthetics derives
from their ability (at low concentrations) to reversibly impair higher
neural functions without seriously disrupting the functions of the
cardiocirculatory and respiratory systems. The molecular mechanisms under­
lying the complex processes of anaesthesia largely remain a mystery.
Nonetheless, four key observations suggest that anaesthesia is the result
of a physical interaction between the anaesthetic and certain constituents
of the cell rather than a specific chemical reaction. First, the in vivo
and in vitro (Mastrangelo et al., 1978) reversibility of anaesthetic
action; second, the potency of an anaesthetic is related to its lipid
solubility (Meyer, 1937); third, anaesthetic properties are shown by a
wide variety of simple but chemically unrelated substances (Miller, 1977)
and fourth the effects of anaesthetics can be reversed by high pressures
(Seeman, 1977). The site of this "physical interaction" is generally
considered to be membrane localised (Miller, 1977; Trudell, 1977).

Hydrophobic (apolar) and hydrophilic (polar) environments
characterise specific regions in biological membranes (Singer, 1972;
Singer and Nicolson, 1972) and the predominance of chemically neutral
anaesthetics (e.g. nitrous oxide, cyclopropane, halothane etc.) has led
to the recognition that predominantly hydrophobic forces are involved in anaesthetic action (Miller, 1977). Candidates for this anaesthetic "site" are the phospholipid moieties of the membrane bilayer which constitutes an integral part of all biological membranes. This does not rule out hydrophilic interactions between the anaesthetic and specific membrane constituents (Sandorfy, 1978); such mechanisms of anaesthesia have not been as extensively studied. Theories of anaesthesia which address themselves to the lipid bilayer as the primary site of action of anaesthetics, represent elaborations on the "critical volume" hypothesis (Mullins, 1954; Seeman, 1972; Kaufmann, 1977; Miller, 1977). The "critical volume" hypothesis proposes that it is not the concentration of anaesthetic within the membrane, but the anaesthetic-induced expansion in membrane volume that actually causes anaesthesia (Miller, 1977).

The most simple of these, the "fluidised lipid" hypothesis, proposes that the interaction of the anaesthetic with membrane results in a generalised increase in lipid fluidity or mobility (Trudell et al., 1973). The "lateral phase separation" hypothesis (Trudell, 1977) represents a refinement of the "fluidised lipid" hypothesis in that it postulates that anaesthetics "melt" or fluidise a region of co-existing liquid-like and solid-like lipid surrounding a membrane protein, thus reducing the protein's ability to undergo conformational changes associated with normal functioning. Major determinants of membrane fluidity include the degree of unsaturation, the chain length and the head group specificity of component phospholipids, the cholesterol content and the temperature (Emmelot and Van Hoeven, 1975; Marsh, 1975) and these factors may in turn modulate the effects anaesthetics have on biological membranes (Pang and Miller, 1978).
The "phase transition" hypothesis (Lee, 1976) represents an additional variation on this common theme and, although initially developed to explain the mode of action of local anaesthetics, it is also applicable to general anaesthetics. The annular lipids surrounding specific membrane proteins are proposed to be in a solid state and the addition of the anaesthetic "melts" this lipid with a resultant decrease in the activity of these proteins (Lee, 1976, 1979).

The "plate" model of membrane structure proposes that the biomembrane is composed of a number of relatively rigid plates or patches (composed of phospholipids in a crystalline or solid state) that are in relative motion with respect to each other (Jain and White, 1977). These rigid plates constitute discrete cooperative units which are separated from each other by relatively fluid, disorganised phospholipid regions. Anaesthetics reduce the size of the cooperative unit in membranes, thus altering the physical environment of its constituent functional proteins (Koehler et al., 1978).

The assumption that the primary site of action of anaesthetics is the phospholipid of the membrane, is a common feature in the "fluidised lipid", the "lateral phase separation", the "phase transition" and the "cooperative plate" theories of anaesthesia (discussed above). The interaction of the anaesthetic with the phospholipid portions of the bilayer is somehow "felt" by the functional proteins in the membrane and therefore the effects of anaesthetics are mediated by this indirect effect on membrane proteins (Miller, 1977; Lenaz et al., 1978a, b). The mechanism by which this indirect effect of anaesthetics is mediated differs in each of the lipid-based theories outlined above but all propose that labilisation of lipid-protein interactions is the end result of this "indirect" effect.
The presence of discrete hydrophobic and hydrophilic zones in membrane proteins is a central tenet of the fluid mosaic model of membrane structure (Singer and Nicolson, 1972) and therefore a direct interaction between the anaesthetic and the hydrophobic regions of the protein cannot be ruled out as a primary site of anaesthetic action. Evidence in favour of such a direct anaesthetic-protein interaction stems from studies on the effects of anaesthetics on isolated protein structure (Balasubramanian and Wetlaufer, 1966; Barker et al., 1975; Sachsenheimer et al., 1977) and function (Ueda and Kamaya, 1973). The failure of anaesthetics to inhibit solubilised red blood cell acetylcholinesterase at concentrations which readily inhibit the membrane bound enzyme argues against such a direct interaction (Lenaz et al., 1978a).

The resume of the proposed mechanisms by which anaesthetics exert their effects is by no means complete. It is biased towards those theories which have the lipid bilayer as the primary site of action of anaesthetics. This bias reflects the majority held view that the anaesthetic site is lipid localised. The next section considers the contribution biophysical techniques, especially nuclear magnetic resonance (NMR) spectroscopy, have made to our understanding of the mechanisms of anaesthesia.

4.1.2 Biophysical studies of anaesthetic-membrane interactions

The molecular events which follow the binding of anaesthetics to membrane components lie at the heart of anaesthetic action. A number of biophysical techniques have been used to study the mechanisms of action of anaesthetics including fluorescence spectroscopy (Okuda and Ogli, 1979), differential scanning calorimetry (Jain et al., 1975; Koehler et al., 1978), X-ray and neutron diffraction (Franks and Lieb, 1978, 1979), electron spin resonance spectroscopy (Trudell et al., 1973; Boggs et al., 1976; Miller
and Pang, 1976; Rosenberg et al., 1977; Lenaz et al., 1978a, b; Mastrangelo et al., 1978; Pang and Miller, 1978) and NMR spectroscopy (Shieh et al., 1975, 1976; Trudell and Hubbell, 1976; Koehler et al., 1977a, b; Vanderkooi et al., 1977; Koehler et al., 1978, 1979). With few exceptions (see Boggs et al., 1976; Franks and Lieb, 1978, 1979) these studies implicate the lipid bilayer as the primary site of anaesthetic action. In principle, NMR spectroscopy represents the most useful of these biophysical approaches in that it is non-perturbing and can supply detailed molecular information on a number of naturally occurring, biologically important nuclei (e.g. $^1$H, $^{13}$C, $^{19}$F and $^{31}$P). Details regarding the theory of NMR are outside the scope of this study but suffice it to say that certain nuclei are characterised by a magnetic moment which in the presence of an externally applied magnetic field can be manipulated to obtain information regarding the molecular environment of the nucleus.

In this study the functional characterisation of anaesthetic-membrane interactions has been carried out using $^1$H, $^{19}$F and $^{31}$P NMR spectroscopy. The effects of halothane on muscle membranes has been examined using $^1$H NMR and some aspects of halothane membrane interactions have been studied in artificial lipid membranes using $^{31}$P and $^{19}$F NMR. This latter approach has permitted monitoring of lipid and anaesthetic environments in the same sample.

4.2 MATERIAL AND METHODS

4.2.1 Methods

a) $^1$H NMR

Muscle membrane fractionation and the preparation of total lipid extracts were carried out as described in Sections 3.2.3 and 3.2.8, respectively. Fractions were lyophilised and reconstituted in a glucose
free, modified D$_2$O-Ringer buffer (121 mM NaCl, 5.4 mM KCl, 1.2 mM MgSO$_4$, 1.2 mM Na$_2$HPO$_4$, 15 mM NaHCO$_3$, 2.5 mM CaCl$_2$ in D$_2$O; pH 7.1-7.2). Sonication of muscle membrane fractions was not employed but total lipid extracts were sonicated as described for egg lecithin preparation (see below). Egg lecithin (L-α-phosphatidylcholine; henceforth referred to as EPC) and EPC-cholesterol vesicles were formed by sonication at maximum output for 15 minutes in an MSE 100 watt ultrasonic disintegrator (MSE Ltd, London, England). During sonication the lipid suspensions were kept on ice. Standard concentrations of EPC (50 mg/ml) and cholesterol (12.5 mg/ml) were always used. Vortexed lipid samples were prepared by intermittent mixing (5-10 minutes total duration) on a model K-500J vortex mixer (Scientific Industries Inc. Mass. U.S.A.). $^1$H NMR spectra were obtained on a JOEL NMH-100, continuous wave spectrometer operating at 100 MHz. Ambient probe temperature was 26°C and all spectra were obtained by a single sweep of 250 seconds over 1080 Hz. A capillary of tetramethylsilane (TMS) in carbon tetrachloride was used as an external reference and the position of all peaks was reported relative to this compound.

b) $^{19}$F and $^{31}$P NMR

50 mg/ml of EPC or dipalmitoyl lecithin (L-α-dipalmitoylphosphatidylcholine; henceforth referred to as DPL) was sonicated in a glucose free, modified Ringer buffer (121 mM NaCl, 5.4 mM KCl, 1.2 mM MgSO$_4$, 1.2 mM Na$_2$HPO$_4$, 15 mM NaHCO$_3$, 2.5 mM CaCl$_2$; pH 7.1-7.2). Sonication conditions were as described for $^1$H NMR samples with the exception that cooling was not employed during sonication of the DPL samples. Spectra were obtained on a JOEL FX-90Q, fourier transform spectrometer operating at 36.2 MHz for $^{31}$P and 84.26 MHz for $^{19}$F. The magnet was tuned to optimise field homogeneity using a deuterated solvent and the instrument was then switched
to external lock, thus avoiding the requirement for deuterated solvents in the test sample. Chemical shift data were reported relative to the usual standards of 85% orthophosphoric acid and 100% trifluoroacetic acid (TFA) for $^{31}$P and $^{19}$F nuclei, respectively; however the primary external references used were $1M$ methylene diphosphonic acid (MDP) in $D_2O$, $pH = 9.0$ ($^{31}$P) and 5% TFA in $H_2O$ ($^{19}$F). All $^{31}$P and $^{19}$F spectra were recorded at 30°C.

4.2.2 Materials

TFA and TMS were obtained from Aldrich Chemical Company. MDP was from Sigma Chemical Company and orthophosphoric acid from British Drug Houses. $D_2O$ (99.7%) was obtained from the Australian Atomic Energy Commission.

4.3 RESULTS

4.3.1 $^1H$ NMR spectra of lecithin and lecithin-cholesterol membranes

The type of information available from $^1H$ NMR spectroscopy is simply shown in Figure 4.1, where the resonance signals arising from the choline, methylene and terminal methyl protons give an almost complete picture of the lipid environments within a model phospholipid membrane. The method of preparation and the presence of cholesterol had dramatic effects on the resonance signals from these 3 groups (Figure 4.2). The co-sonication of cholesterol (12.5 mg/ml) with EPC (50 mg/ml) almost abolished the methylene and methyl proton signals and reduced the choline signal by a factor of 3 (Figure 4.2A compared to Figure 4.3C). There was no discernible resonance peaks in the vortexed EPC samples (Figure 4.2B). The sonicated EPC spectrum shown in Figure 4.2C is similar to that used in Figure 4.1 and is included for comparative purposes.
Figure 4.1: $^1$H NMR spectrum of sonicated EPC vesicles

This schematically illustrates the regions of the EPC molecule which contribute to the spectrum. Chemical shifts were referenced against TMS.
Figure 4.2: Effect of cholesterol and method of preparation on the $^1$H NMR spectrum of EPC

(A) Sonicated EPC-cholesterol vesicles (50 and 12.5 mg/ml) respectively.
(B) Vortexed EPC multi bilayers (50 mg/ml).
(C) Sonicated EPC vesicles (50 mg/ml).

Chemical shifts were referenced against TMS.
The addition of halothane (up to 94.7 mM) had little effect on $^1$H resonance signals in vortexed EPC samples or in sonicated EPC and EPC-cholesterol lipid samples (results not shown). Greater volumes of halothane (up to 0.47 M) increased the choline, methylene and methyl resonances in vortexed EPC and EPC-cholesterol samples, but actually decreased choline and methylene resonances in sonicated EPC lipid samples (results not shown).

4.3.2 $^1$H NMR spectra of normal and MH microsomal membranes and lipid extracts

$^1$H spectra were obtained of the light and heavy microsomal trapezius fractions and of the heavy microsomal l.dorsi fraction and of their lipid extracts, from both normal and MH muscles. Figure 4.3 shows spectra obtained of a MH trapezius light microsomal fraction (A) and its equivalent lipid extract (B). The addition of halothane (up to 94.7 mM) resulted in an increase in the intensity of the methylene resonances in both the membrane and lipid extract samples. Similar spectra were obtained for the other normal and MH muscle fractions and in all cases the effects of halothane failed to distinguish normal from MH fractions.

The effects of temperature were monitored in the light and heavy microsomal membrane fractions from normal and MH trapezius muscle. Temperature effects, in the 10°C to 70°C range, are shown in Figure 4.4 for a normal trapezius light microsomal fraction. The small increase in width of the methylene resonance is indicative of an increase in the number of nuclei contributing to the signal. Temperatures in the 10°C to 70°C range had only small effects on $^1$H resonances and such behaviour was characteristic of both normal and MH muscle fractions.
Figure 4.3: Effect of halothane on the $^1$H NMR spectra of MH trapezius light microsomal membrane and sonicated lipid extract. (A) Light microsomal membrane. (B) Light microsomal membrane lipid extract. Bars indicate methylene peak height. Similar spectra were obtained for other normal and MH membrane and lipid extract preparations. Chemical shifts were referenced against TMS.
Figure 4.4: Effect of temperature on the $^1$H NMR spectrum of normal trapezius light microsomal membranes

Samples were equilibrated at the appropriate temperature for 10 minutes prior to obtaining a spectrum. Similar spectra were obtained for other normal and MH membrane and lipid extract preparations. Chemical shifts were referenced against TMS.
Figure 4.5: $^{31}P$ NMR spectrum of sonicated EPC vesicles

Typical $^{31}P$ spectrum of sonicated EPC (50 mg/ml) vesicle preparation. Chemical shifts were reported relative to 85% orthophosphoric acid. $^{31}P$ assignments were according to Berdon et al, 1975.
Figure 4.6: Effect of halothane on the $^{31}$P NMR spectrum of sonicated EPC and DPL vesicle preparations

(A) EPC (no additions); (B) EPC (47.4 mM halothane);
(C) DPL (no additions); (D) DPL (47.4 mM halothane).

Samples contained 50 mg/ml lipid. Chemical shifts (ppm) were referenced against 85% orthophosphoric acid.
Figure 4.7: Effect of environment on chemical shift positions of the $^{19}$F nucleus of halothane

The external references (A) and (G) were 5 and 100% TFA, respectively. Halothane (47.4 mM) was added to the various samples (B, C, D and F) and the $^{19}$F spectra recorded 10 minutes later. Chemical shifts were referenced against 100% TFA (G). The structure of halothane is shown at the top of the figure.
4.3.3 $^{31}$P NMR studies of halothane-phospholipid interactions

The $^{31}$P NMR spectrum of a sonicated EPC preparation is shown in Figure 4.5. The orthophosphate resonance (not shown) appears quite close to the phospholipid resonances and for this reason MDP was used as the primary reference (Figure 4.5). The environments in the outer (EPC out) and inner (EPC in) regions of EPC vesicles differ as evidenced by the different shift positions for the EPC phosphorus at either side of the vesicle (Figure 4.5). The separation between the outside and inside resonances was 5.2 Hz and the EPC out/EPC in ratio was 1.6 (Figure 4.5).

Figure 4.6 shows the $^{31}$P spectra of sonicated EPC and DPL vesicles in the absence and presence of 47.4 mM halothane. The spectrum of the sonicated EPC vesicles (Figure 4.6A) is similar to that shown in Figure 4.5. The addition of halothane had no effect on the splitting between the resonances but the EPC out/EPC in ratio increased from 1.60 (Figure 4.6A) to 1.80 (Figure 4.6B). In the absence of halothane outside and inside DPL resonances were broad with a splitting of 6.4 Hz and a DPL out/DPL in ratio of 1.45 (Figure 4.6C). Halothane (47.4 mM) sharpened both the outside and inside resonances of DPL vesicles, decreased the splitting between resonances to 5.1 Hz and decreased the DPL out/DPL in ratio to 1.3 (Figure 4.6D).

4.3.4 $^{19}$F NMR studies of halothane-phospholipid interactions

The $^{19}$F spectrum of the fluorine on halothane is a doublet peak (Figure 4.7) caused by the interaction of the fluorine nuclear spin with that of the proton on the adjacent carbon. The sensitivity of halothane to its environments is simply shown in Figure 4.7 where the shift positions of the halothane doublet are shown in a number of different solvents. The halothane resonances shown in Figure 4.7 (i.e. traces B, C, D, E and F)
represent results of individual experiments but were included on the same figure to illustrate how solvent environment affects fluorine chemical shifts. Thus using 100% TFA (G) as reference, the doublet centre was upfield of the reference, at 0.95 ppm in hexane (F), at 1.29 ppm in concentrated halothane (E), at 2.73 ppm in a DPL vesicle suspension (D), at 2.72 ppm in an EPC vesicle suspension (C) and at 2.87 ppm in Ringer buffer (B) (Figure 4.7).

The $^{19}$F spectra of halothane in EPC (Figure 4.7C) and DPL (Figure 4.7D) vesicles were recorded on the same samples used for the $^{31}$P spectra shown in Figure 4.6B and D, respectively. The $^{19}$F spectra show (Figure 4.7) that the partitioning of halothane into increasingly hydrophilic or polar solvents results in a progressive upfield shift of the halothane from the 100% TFA reference (Figure 4.7). The appearance of a single doublet in the $^{19}$F spectra of halothane in EPC and DPL vesicles, intermediate between Ringer and hexane, implies that the halothane in the lipid vesicles rapidly exchanges between the lipid and aqueous phases and that the amount of halothane permanently bound within the vesicle is small.

4.4 DISCUSSION

The $^1$H spectra of EPC membranes demonstrate how structural composition and organisation affect individual proton resonances. Sonication of EPC resulted in the formation of vesicles with well resolved choline, methylene and methyl resonances, while vortexed samples were characterised by the absence of resolved resonances. The co-sonication of cholesterol and EPC resulted in the formation of vesicles which were characterised by a marked decrease in the choline resonance while the methylene and methyl resonances were almost abolished. These
observations are consistent with EPC forming small, highly curved vesicles when sonicated and large multi-bilayer structures when vortexed (Sheetz and Chan, 1972; Chan et al., 1973). The absence of methylene and methyl resonances in EPC-cholesterol vesicles indicates that the molecular mobility (on the NMR time scale) of the fatty acyl side chains of the phospholipid is restricted in the presence of cholesterol. Such an interpretation is consistent with currently held views on the function of cholesterol in model and biological membranes (Drake et al., 1972; Demel and De Kruyff, 1976; Papaladjopoulos, 1976). These simple experiments using $^1$H NMR spectroscopy show how structure and composition affect the $^1$H spectrum. This provided a useful basis upon which subsequent $^1$H NMR studies of halothane-muscle membrane interactions were carried out.

High resolution $^1$H NMR spectra have been obtained for a number of biological membrane preparations, including SR membranes (Davis and Inesi, 1971; Eletr and Inesi, 1972; Davis and Inesi, 1972; Robinson et al., 1972; Davis et al., 1976; Vanderkool et al., 1977), erythrocyte membranes (Chapman et al., 1968), retinal rod outer segment disc membranes (Brown et al., 1977) and yeast and rat liver mitochondrial membranes (Brown et al., 1975). These $^1$H spectra were remarkably similar, with the predominant signals arising from the choline, methylene and methyl protons of the phospholipids (Davis et al., 1976; Brown et al., 1975; Brown et al., 1977). This indicates that the molecular environments of these phospholipid functional groups are similar and that structural organisation in biological membranes displays features common to all membrane systems. Lipid mobility at the molecular level comprises the rotational motion about the bonds of the fatty acid side chains and the translational motion of the lipid molecule within the plane of the membrane (Marsh, 1975). Thus the $^1$H spectrum provides qualitative information on the physical state of the
membrane with the resonance intensity (i.e. the percentage of the total resonances contributing to the signal) reflecting the "fluid" state of the membrane. Elevations in temperature (Davis et al., 1976) or the addition of halothane (Vanderkooi et al., 1977) intensified methylene, methyl and, to a lesser extent, choline resonances in SR membrane preparations, indicating that such treatments have a common mode of action. The fluidising effects of halothane on SR membranes reported by Vanderkooi et al. (1977) agree with the effects of halothane on normal and MH microsomal preparations observed in the present study.

The absence of a choline resonance in the membrane and sonicated lipid preparations studied here, contrasts with its predominance in $^1$H spectra of SR membranes (Davis and Inesi, 1971; Eletr and Inesi, 1972; Davis and Inesi, 1972; Davis et al., 1976) and of sonicated SR lipid extracts (Robinson et al., 1972). Possible explanations for this include a specific interaction between the choline head group and membrane proteins, resulting in significant line broadening, or that the $^1$H spectrum reflects not only phospholipid resonances but also mono-, di- and triglyceride and free fatty acid resonances. The absence of a choline resonance has been proposed to reflect membrane integrity (McLaughlin et al., 1977) and as sonication was not employed in the preparation of the microsomal membranes, head group immobilisation by membrane proteins cannot be ruled out. In the sonicated microsomal lipid extracts the absence of a choline resonance (over all the conditions studied) indicates that in these preparations other possibilities must be considered. Non-steroidal neutral lipids (i.e. mono-, di-, and triglycerides and free fatty acids) constitute a major percentage of the total lipid (up to 40%; J. Sullivan and M.A. Denborough, unpublished results) in muscle microsomal membrane preparations. Therefore, their contribution might explain the predominance
of methylene and methyl resonances and a complete absence of choline resonances in the $^1$H spectra of microsomal membrane preparations. Neutral lipids occur in most biological membranes and although many studies have documented structural and functional roles for cholesterol, similar studies have not been carried out on non-steroidal neutral lipids. The numerous centrifugation steps employed during the fractionation procedure, rule out the possibility that such lipids were contaminants but in the absence of a defined role for non-steroidal lipids in biological membranes the significance of their contribution to the $^1$H NMR spectrum remains to be clarified.

Temperature-induced enhancement of methyl, methylene and choline resonances characterise $^1$H spectra of model (Sheetz and Chan, 1972) and biological membranes (Brown et al., 1975; Davis et al., 1976; Brown et al., 1977). Elevations in temperature enhanced methyl and methylene resonances in both normal and MH microsomal membrane and lipid preparations and responses failed to differentiate the two muscle types. The resonance enhancement was small but was indicative of an increase in the pool size of "melted" lipids. Such small effects of temperature may in part reflect the immobilising presence of cholesterol or that the resonances arise from non-phospholipid molecules (i.e. the non-steroidal neutral lipids discussed earlier). These possibilities require further study.

The inability to differentiate normal from MH muscle preparations on the basis of $^1$H NMR spectroscopy, indicates that the molecular environments surrounding the contributing resonances are similar. This indicates that the abnormality underlying MH is subtle and is probably localised at the direct level of protein structure and/or function, although an indirect effect on the stability of lipid-protein interactions cannot be ruled out. The $^1$H spectra indicate that a gross alteration in the structure
of MH muscle microsomal membranes is unlikely but this may reflect the heterogeneous nature of the membranes included in the microsomal preparations. Further work using purer membrane fractions would be required to assess such a possibility.

$^{31}P$ NMR of sonicated EPC vesicles permitted the simultaneous monitoring of PC moieties on each side of the membrane bilayer. The EPC outside/inside ratio of 1.6 to 1.8 agreed with previously published values for the outside/inside distribution of EPC and a variety of other unsaturated PCs in vesicle preparations (Berdon et al., 1975; De Kruijff et al., 1976). The predominance of unsaturated fatty acids in the EPC used in the current study (unsaturated/saturated ratio 1.4) ensured that at 30°C the EPC was in a fluid state. The phase transition or the melting temperature of DPL is 41°C and therefore at 30°C this lipid is in a solid state. The $^{31}P$ spectra reflected this physical state of the membrane with the fluid EPC vesicles displaying well resolved outside/inside resonances while the solid DPL vesicles displayed a broader, less resolved splitting of outside and inside resonances. The addition of 47.4 mM halothane to sonicated EPC vesicles had little effect on the $^{31}P$ spectrum. This was not unexpected as halothane was added to an already fluid membrane. Sonicated DPL vesicles by contrast displayed a sharpening of the outside/inside resonances and greater resolution of the splitting between these resonances when 47.4 mM halothane was added. This effect of halothane on the $^{31}P$ spectrum of sonicated DPL vesicles was similar to that observed when the spectrum was obtained at 50°C in the absence of halothane (Berdon et al., 1974).

$^{19}F$ NMR was employed to monitor halothane environments in the same EPC and DPL vesicle preparations from which $^{31}P$ spectra were obtained. In
agreement with previous reports (Trudell and Hubbell, 1976; Koehler et al., 1977a, b) the halothane chemical shift positions reflect the environment surrounding the halothane molecule. Direct comparisons between the chemical shift positions of the halothane doublet and the environmental factors contributing to these shifts were possible only with Trudell and Hubbell's (1976) study where shift positions were referenced against 100% TFA. The use of hexafluoroacetone by Koehler et al. (1977a, b) as an external fluorine reference meant that chemical shift comparisons between their results and the results obtained in the present study could not be carried out. Trudell and Hubbell (1976) obtained centered chemical shifts of 2.79, 2.57 and 0.99 ppm upfield of 100% TFA when halothane was dissolved in water, sonicated EPC vesicles or hexane respectively. The comparable values obtained in the present study of 2.87, 2.72 and 0.95 ppm for halothane in Ringer, sonicated EPC vesicles and hexane, respectively are in good agreement with Trudell and Hubbell's (1976) results. The similar shift positions for halothane in sonicated EPC (2.72 ppm) and DPL (2.73 ppm) vesicles, indicates that in these preparations, halothane environments are similar. The difference in peak height might reflect the lower partitioning of halothane into DPL vesicles at 30°C (i.e. below its phase transition temperature) compared to its partitioning into EPC vesicles at the same temperature. Indeed it has been shown that at 25°C halothane partitioning into EPC vesicles is four-fold higher than that into DPL vesicles (Simon et al., 1979).

The use of $^{19}$F and $^{31}$P NMR to study halothane-phospholipid interactions provides a simple means with which to monitor halothane and phospholipid environments in the same sample. The application of such an approach to study MH muscle membranes could provide important information as to the nature of the interaction between halothane and MH muscle
membranes. Such an approach remains for future investigations.

4.5 SUMMARY

NMR techniques are well suited to provide structural and dynamic information in a non-perturbing manner on model and biological membranes. In this study a number of aspects of membrane structure and function have been investigated using $^1$H, $^{19}$F and $^{31}$P NMR spectroscopy. In normal and MH microsomal muscle membranes and their lipid extracts elevations in temperatures or the addition of halothane enhanced methylene and methyl resonances in the $^1$H spectrum, but such effects were similar in normal and MH preparations. Choline resonances were absent in all muscle preparations studied. The molecular environments of sonicated EPC and DPL vesicles differed as evidenced by the $^{31}$P NMR spectrum, the EPC spectrum being well resolved into signals from each side of the bilayer while the DPL spectrum was broad and outside/inside resonances were less well resolved. Halothane interactions with these vesicle systems were studied using $^{19}$F NMR and it was shown that halothane environments were similar in sonicated EPC and DPL vesicles, although the amount bound by the former preparation was greater.
CHAPTER 5

TEMPERATURE DEPENDENCE OF SKELETAL MUSCLE FUNCTION

IN PORCINE MALIGNANT HYPERPYREXIA

5.1 INTRODUCTION

Contractile hypersensitivity in human MH skeletal muscle to the calcium releasing drug caffeine was first demonstrated in 1970 by Kalow and co-workers in Toronto. On the basis of this finding, it was concluded that in MH there is a disturbance in intracellular calcium metabolism in skeletal muscle (Kalow et al., 1970). These experiments were carried out at 25°C, and at this temperature halothane failed to induce a contracture in MH muscle. Subsequent work has shown that halothane-induced contractures are an in vitro pharmacological feature of MH muscle (Ellis et al., 1972; Moulds and Denborough, 1972), but that their occurrence is temperature dependent (Nelson et al., 1975). Caffeine contractures are temperature dependent in mammalian skeletal muscle with contractile sensitivity being greater at 37°C than at room temperature in human (Nelson et al., 1977) and in rat (Frank and Buss, 1967) skeletal muscle. The temperature dependence of caffeine-induced contractures has not previously been investigated in MH skeletal muscle. In rat skeletal muscle contractures in response to the isotonic replacement of NaCl with KCl are greater at 37°C than at 22°C (Frank and Buss, 1967). Succinylcholine, KCl and changes in temperature are some of the additional diverse stimuli which induce an abnormal contracture in MH skeletal muscle (Moulds and Denborough, 1974), but a systematic investigation of the temperature dependence of these processes has not previously been attempted.
In the 20° to 40°C temperature range isometric twitch behaviour in rat (Close and Hoh, 1968; Hanson, 1974; Ranatuanga, 1977a), and cat (Ranatuanga, 1977b) skeletal muscle is dependent on muscle fibre type. Over this temperature range the pattern of isometric twitch changes little in red muscles, while in white muscle cooling significantly increases the size of the twitch response (Close and Hoh, 1968; Ranatuanga, 1977a, b). Thus isometric twitch of mammalian muscles as a function of temperature can be used as an indicator of the predominant fibre type which characterises any individual muscle.

The objectives of the experiments described in this chapter were;

(1) to examine the effects of temperature on isometric twitch and contracture in response to halothane, caffeine, succinylcholine, and KCl in normal and MH porcine skeletal muscle, and

(2) to determine the optimum temperature at which the \textit{in vitro} pharmacological assessment of MH susceptibility should be carried out.

5.2 MATERIALS AND METHODS

5.2.1 Methods

Premedication and nitrous oxide-barbiturate anaesthesia were carried out as described in section 3.2.1. A total of 14 MH and 3 normal swine were biopsied. In all cases susceptibility was assessed by standardised pharmacological methods (Okumura \textit{et al.}, 1979). Clamped biopsy samples of approximate dimensions 4 cm by 1 cm by 0.3 cm, length, width and depth respectively were removed from the gracilis muscle and placed in a modified Ringer buffer (121 mM NaCl, 5.4 mM KCl, 1.2 mM MgSO$_4$,}
1.2 mM NaH2PO4, 15 mM NaHCO3, 11.5 mM d-glucose, 2.5 mM CaCl2; pH 7.4) at 37°C, which was continuously bubbled with carbogen (95% O2/5% CO2). From 3 to 5 clamps were taken from each animal. Prior to use a fibre bundle (approximately 100 mg wet weight) was cut from the bulk muscle and tied off at either end to small (0.3 cm diameter) metal rings using Ethicon size '00' silk thread. One end of the fibre bundle was fixed to a glass rod, and the other to a force transducer (Watson Victor), and the muscle preparation was lowered into a jacketed organ bath containing 25 ml of modified Ringer buffer. Isometric tension was recorded after amplification (San-Ei 6M52 strain amplifier); the resting tension was 1 gram. Twitch responses were obtained with DC square waves generated by a Grass Model S4E stimulator, via platinum electrodes, to the following specifications; 3 msec duration, 30 V and a frequency of 0.1 Hz. This level of stimulation constituted supra maximal twitch at 25°C and 37°C.

Temperature control was maintained with a Braun thermomix, equipped with a mercury contact thermometer. For fixed temperature experiments in the 20° to 45°C range the circulating water bath temperature was set at the appropriate level and the muscle buffer temperature was permitted to equilibrate to the pre-set level prior to the transfer of the fibre bundles to the muscle bath. Utilising this procedure, buffer temperature was maintained to within ±0.5°C of the pre-set level. Lower temperatures were maintained by the addition of ice to the circulating water bath. This procedure maintained temperatures to within ±1.0°C of the pre-set level. In experiments designed to follow twitch and tension responses to a gradual rise in temperature both the circulating water bath and muscle buffer temperatures were separately monitored. Fibre bundles were first equilibrated at 5°C and the final temperature set at 37°C. This resulted in a temperature rise in the buffer of approximately 2.5°C/min.
Throughout the experiments the bulk muscle was maintained at 37°C in carbogen treated Ringer and the order in which the experiments were carried out was varied from day to day.

The stock solutions of the drugs used were: caffeine, 100 mM in Ringer; potassium chloride, 4 M in Ringer; acetylcholine, 125 mM in distilled water; dantrolene sodium, 3 mM in dimethyl sulphoxide and tubocurarine 1.47 mM in distilled water. Halothane was administered by passing carbogen through a temperature calibrated Dragewick vaporizer. Physostigmine (0.5 µM) was added directly to the buffer. Calcium was omitted when preparing calcium free buffers and when required, 2 mM ethylene glycol-bis-(β-aminoethylether)N,N'-tetraacetic acid (EGTA) was added to this calcium free Ringer. Deuterium oxide (D₂O) Ringer was prepared by dissolving the normal buffer salts in D₂O. No correction was made for pH in such solutions (note: pD = pH + 0.4 [Huxtable and Bressler, 1974]). D₂O purity was in excess of 99.7% and re-use after redistillation did not modify its action. Glycerinated fibres were prepared by pretreatment of separated, tied, fibre bundles in Ringer supplemented with 400 mM glycerol. This treatment was carried out at 37°C in carbogen treated buffer for one hour. pH corrections for the modified Ringer buffer at 5°C were calculated from the formula relating pH to temperature as used by Creese et al. (1958).

One fibre bundle per animal was used for each drug at any one temperature while up to 3 fibre preparations per animal were used in the twitch experiments. Significance values were obtained using the Student's t-test. In drug contracture experiments significance values, for MH muscle only, were expressed relative to the contracture induced at 37°C. In twitch recovery experiments significance values for MH muscles were
expressed relative to normal twitch at a similar temperature. Values expressed in Figures 5.2 to 5.6 are reported as means ± S.E.M.

5.2.2 Materials

The buffer salts, potassium chloride, glycerol, and acetylcholine chloride were obtained from British Drug Houses. Caffeine, physostigmine, and EGTA were from Sigma Chemical Company. Succinylcholine chloride (Scoline) and tubocurarine chloride (Tubarine) were obtained from Wellcome. Deuterium oxide was obtained from the Australian Atomic Energy Commission. Procaine hydrochloride was from David Bull Laboratories, and halothane from Imperial Chemical Industries. Dantrolene sodium was the kind gift of Dr K.O. Ellis, Norwich Eaton Pharmaceuticals.

5.3 RESULTS

5.3.1 Temperature effects on tension and twitch

Twitch recovery as a function of temperature was similar in normal (Figure 5.1) and MH muscle but in most cases recovery proceeded faster in the latter muscle (Figure 5.2). The temperature at which maximal twitch occurred (Figure 5.2) was significantly different (p < 0.05) in MH (21.2 ± 0.8°C, n = 12) and normal (24.1 ± 0.7°C, n = 7) muscle preparations. Additional differences in twitch recovery were noted at temperatures below 20°C (Figure 5.2) where recovery of fibre twitch occurred earlier in MH muscle preparations. A slight increase in muscle tension occurred on heating the fibre bundles from 5°C to 37°C but this was always less than 0.1 gram in both normal (Figure 5.1) and MH muscle preparations. A further increase in temperature from 37°C to 45°C resulted in a small increase in tension in normal (0.21 ± 0.1 grams, n = 5) and MH (0.20 ± 0.05 grams, n = 9) fibre bundles which was not significantly
Figure 5.1: Effect of temperature on isometric twitch in normal porcine gracilis muscle

Fibre preparations were incubated at 5°C for 6 minutes and then the temperature was raised (2.5°C/minute) to 37°C. Similar profiles characterised MH fibres with the exception that twitch recovery and twitch maximum occurred earlier than in normal muscle.
Figure 5.2: **Effect of temperature on isometric twitch in normal and MH porcine gracilis muscle**

Conditions were as described in Figure 5.1. Values are expressed as % of the maximum twitch recorded. Each point represents the mean ± S.E.M. of 7 (open circles) and 12 (closed circles) individual fibre preparations from 3 normal and 5 MH swine, respectively.

* p < 0.05
different. Twitch responses were not studied in the 37° to 40°C temperature range. The addition of d-tubocurarine (final concentration 0.06 mM) to the muscle bath prior to the initiation of a temperature run failed to alter the twitch recovery pattern in normal (n = 3) or MH (n = 3) muscle preparations.

5.3.2 Temperature dependence of halothane contractures

The temperature dependence of 3% halothane-induced contractures is shown in Figure 5.3 for normal and MH muscle preparations. Alterations in temperature from 20°C to 45°C had little effect on the ability of halothane to cause contractures in normal muscle and in this temperature range contractures were always less than 0.05 grams. In MH muscle halothane-induced contractures were minimal at 25°C (0.14 ± 0.06 grams, n = 8) and plateaued at 37°C (1.09 ± 0.17 grams, n = 14). Large contractures were induced at 5°C both in normal (1.45 ± 0.17 grams, n = 3) and MH (1.31 ± 0.27 grams, n = 8) muscle preparations (Figure 5.3).

5.3.3 Temperature dependence of caffeine contractures

Temperature effects on 2 mM caffeine contractures in normal and MH muscles (Figure 5.4) were qualitatively similar to those obtained with halothane (Figure 5.3). Normal muscle failed to contract to 2 mM caffeine in the 20°C to 45°C temperature range, while in MH muscle 2 mM caffeine contractures increased as the temperature was raised from 20°C (0.12 ± 0.04 grams, n = 11) to 37°C (0.64 ± 0.11 grams, n = 14). Both normal and MH muscle displayed enhanced caffeine sensitivity at 5°C with contractures of 1.52 ± 0.19 grams (n = 3) and 2.26 ± 0.60 grams (n = 7) for normal and MH muscles, respectively (Figure 5.4).
5.3.4 Temperature dependence of succinylcholine contractures

Contractures induced by 1 mM succinylcholine displayed a similar qualitative contractile dependence on temperature (Figure 5.5) as halothane (Figure 5.3) and caffeine (Figure 5.4). In MH muscle succinylcholine induced small contractures in the 20°C to 30°C temperature range and these increased to a maximum at 45°C (0.56 ± 0.16 grams, n = 7). Normal muscle failed to contract in this temperature range. Large contractures were induced at 5°C in normal (1.45 ± 0.17 grams, n = 3) and MH (1.3 ± 0.27 grams, n = 8) muscle (Figure 5.5).

5.3.5 Temperature dependence of KCl contractures

80 mM KCl-induced contractures displayed a qualitatively and quantitatively distinct temperature dependence pattern (Figure 5.6) compared with halothane (Figure 5.3), caffeine (Figure 5.4) and succinylcholine (Figure 5.5). Between 5°C and 45°C KCl had little effect on muscle tension (<0.1 grams) in normal muscle preparations. In MH muscle KCl had little effect on tension at 5°C, 20°C or 25°C (<0.1 grams). The maximum contracture recorded in MH muscle in response to KCl occurred at 37°C (1.77 ± 0.33 grams, n = 14) (Figure 5.6).

5.3.6 Drug induced contractures at 5°C

As mentioned above 3% halothane (Figure 5.3), 2 mM caffeine (Figure 5.4) and 1 mM succinylcholine (Figure 5.5) induced large contractures in both normal and MH muscle fibres at 5°C. 80 mM KCl had no effect on muscle tension in either normal or MH muscle at 5°C (Figure 5.6).

Repeated drug treatments in a calcium free buffer at 5°C, resulted in a progressive decrease in the extent of the contracture in normal and MH muscle preparations. This was true for halothane, caffeine and
Figure 5.3: Temperature dependence of 3% halothane-induced contractures in normal and MH porcine gracilis muscle

Fibre bundles were equilibrated at the appropriate temperature for a minimum of 5 minutes prior to the addition of halothane. Each point represents the mean ± S.E.M. of 3 normal (open circles) and 7-14 MH (closed circles) separate fibre preparations. Significance values are relative to the contracture induced at 37°C and apply to MH preparations only.

** p < 0.01

*** p < 0.001
Figure 5.4: Temperature dependence of 2 mM caffeine-induced contractures in normal and MH porcine gracilis muscle

Fibre bundles were treated as described in Figure 5.3. Each point represents the mean ± S.E.M. of 3 normal (open circles) and 7-14 MH (closed circles) separate fibre preparations. Significance values are referenced against the contracture induced at 37°C and apply to MH preparations only.

** p < 0.01
*** p < 0.001
Figure 5.5: Temperature dependence of 1 mM succinylcholine-induced contractures in normal and MH porcine gracilis muscle

Fibre bundles were treated as described in Figure 5.3. Each point represents the mean ± S.E.M. of 3 normal (open circles) and 7-14 (closed circles) separate fibre preparations. Significance values are referenced against the contracture induced at 37°C and apply to MH preparations only.

** p < 0.01

*** p < 0.001
Figure 5.6: Temperature dependence of 80 mM KCl-induced contractures in normal and MH porcine gracilis muscle

Fibre bundles were treated as described in Figure 5.3. Each point represents the mean ± S.E.M. of 3 normal (open circles) and 7-14 MH (closed circles) separate fibre preparations. Significance values are referenced against the contracture induced at 37°C and apply to MH preparations only.

* p < 0.05
** p < 0.01
*** p < 0.001
succinylcholine (n = 2, for each drug in normal and MH muscle preparations). Prior treatment with 2 mM EGTA at 37°C for one hour abolished 2 mM caffeine and 3% halothane contractures in both normal and MH muscle in a calcium free buffer at 5°C (single experiments only).

Acetylcholine (1 mM) in the presence of the choline-esterase inhibitor physostigmine (0.5 μM), did not induce a contracture in normal (n = 2) or MH (n = 2) fibre bundles at 5°C. Glycerol-induced T-tubular disruption failed to alter the time course or the extent of contracture to halothane, caffeine or succinylcholine in MH muscle (n = 2 for each drug) at 5°C. Replacing the normal modified Ringer buffer with D2O Ringer buffer did not affect halothane- or caffeine-induced contractures in MH muscle (n = 2, for each drug). Dantrolene sodium (12 μM) did not block halothane- or caffeine-induced contractures at 5°C in normal or MH muscle preparations (single experiments only).

The modified Ringer buffer used in these studies gave a pH of 6.9 at 5°C, but correcting this (by the addition of NaHCO3) to give a pH of 7.4 at 5°C did not affect the halothane- or caffeine-induced contractures in MH muscle preparations (n = 2 for each drug).

In MH muscle at 5°C 10 mM procaine blocked halothane contractures by over 50% (n = 2), and 5 mM procaine blocked caffeine contractures by up to 70% (n = 2).

5.4 DISCUSSION

The isometric twitch behaviour of gracilis muscle from normal and MH swine in the 20°C to 40°C temperature range is qualitatively characteristic of mammalian fast twitch white fibres (Turang et al., 1964; Close
and Hoh, 1968; Hoh, 1974). Histochemical studies (K. Hopkinson and M.A. Denborough, unpublished results) show that porcine gracilis muscle is a fast twitch white muscle.

The inability of the acetylcholine antagonist d-tubocurarine to modify either the recovery or the pattern of twitch as a function of temperature, argues against the involvement of acetylcholine release and the MEP as possible contributory agents responsible for the temperature-twitch profile displayed by porcine gracilis muscle.

The increase in twitch displayed by swine gracilis muscle (Figures 5.2 and 5.2) on decreasing temperature from 37°C to 20°C, contrasts with the decreased contractile responses to halothane, caffeine, succinylcholine and KCl over the same temperature range. A similar situation has been described in rat extensor digitorum longus muscle where twitch (Close and Hoh, 1968) and caffeine contractures (Frank and Buss, 1967) increase and decrease, respectively as temperatures are lowered. As the final events in contraction are common to both twitch and drug-induced contraction (i.e. calcium release and myofibrillar interdigitation), an explanation for their different temperature sensitivities may reside in the sequence of events occurring prior to calcium release. Thus it is proposed that in MH muscle the initial events of E-C coupling, as induced by electrical stimulation and by the drugs tested here, differ.

It is possible that the hypersensitive drug-induced contractures, which are observed in MH muscle at 37°C may reflect the ability of such drugs such as halothane, caffeine and succinylcholine to initiate a calcium-induced calcium release process (Endo, 1977; Ebashi, 1979) which does not occur in normal muscle. Such a drug-induced release of trigger calcium does not presumably occur at 20°C or 25°C where this calcium pool
is effectively immobilised. The occurrence and relative importance of calcium-induced calcium release in pathological muscle needs further investigation.

Biochemical studies in isolated rat (Becker and Willis, 1975) and porcine (McIntosh et al., 1977) SR preparations have shown that Ca ATPase activity decreases as temperatures are lowered from 37°C to 20°C. Thus a decrease in calcium uptake may underlie the greater twitch recorded in normal and MH muscles in the 20°C to 25°C temperature range, but this effect cannot explain the loss of drug induced contractility that occurs over the same temperature range in MH muscle. It is thus possible that the primary abnormality in MH resides in the processes modulating the release of activator calcium although our limited knowledge about such processes even in normal muscle (Adrian, 1979; Caputo, 1979) restricts detailing such an abnormality.

The decreased drug-induced contractility displayed by MH muscle on reducing temperature from 37°C to 25°C suggests that the hypersensitive step(s) coupling excitation to subsequent calcium release has been dissociated and it is proposed that this site is localised at the level of the functional interaction between the T-tubule and the sarcoplasmic reticulum.

Calcium uptake by rabbit SR fragments is abolished at 2°C (Martonosi and Feretox, 1964) thus suggesting that the large drug contractures induced at 5°C may reflect a decrease in the ability of porcine SR to accumulate calcium. The calcium dependence of the 5°C drug contractures was illustrated by their abolition after treatment at 37°C with calcium-free EGTA buffer, and by their diminution with repeated drug treatments in calcium-free Ringer. The disappearance of
twitch and the loss of KCl-induced contractures at 5°C imply a loss of surface excitability, and it is possible that halothane, caffeine and succinylcholine induce muscle contraction at 5°C by a direct action on sarcoplasmic reticular stores of calcium. The similarity between the extent of contracture induced by these drugs at 5°C in normal and MH muscle indicates that under these conditions the amount of calcium released does not differ between the two muscle preparations.

The local anaesthetic procaine was the only drug tested which had any inhibitory effect on these low temperature contractures. Membrane stabilisation or a more direct antagonism of intracellular calcium release are two proposed mechanisms by which procaine antagonises drug-induced contractures in skeletal muscle (Moulds, 1974) and either mechanism is compatible with the results reported here.

Contractures in response to 3% halothane, 2 mM caffeine or 1 mM succinylcholine at 5°C were not modified by:

(1) pretreatment with dantrolene sodium (12 μM)
(2) replacement of the normal modified Ringer buffer with a D₂O Ringer buffer
(3) glycerol-induced T-tubular disruption.

The latter three treatments have been used to study the role of abnormal E-C coupling in MH muscle at 37°C (Okumura et al., 1980). The inability of these E-C uncoupling treatments to modify halothane-, caffeine- or succinylcholine-induced contractures at 5°C suggests that surface membrane excitability is not a requirement for such low temperature contractures and is further evidence for a direct action of these drugs on the SR to induce calcium release.
Alterations in buffer pH as a function of temperature have been noted for Hepes (Dahl and Henquin, 1978) and Ringer (Creese et al., 1958) buffers. Bicarbonate based Ringer buffers display a complicated pH temperature relationship (Creese et al., 1958) but in general pH decreases with decreasing temperature. Correcting the buffer pH to 7.4 at 5°C had little effect on halothane or caffeine contractures, indicating that the decrease in pH which occurred in the modified Ringer at 5°C had no effect on drug-induced contractures.

The rapid cooling contracture (RCC) which occurs in frog muscle (Conway and Sakai, 1960) in response to caffeine, at concentrations which constitute sub-threshold contractile doses at room temperature, bears some resemblance to the low temperature contractures studied in this investigation. An important difference resides in the temperature procedures used, as the caffeine RCC technique involves the rapid transfer of muscle from a 20°C buffer to one maintained at 0°C (Conway and Sakai, 1960) while the fixed temperature method employed in the present study allows equilibration at 5°C prior to the addition of any drug. An additional difference resides in the reported ability of dantrolene to inhibit caffeine RCC in frog muscle (Homma et al., 1976) compared with the inability of dantrolene to block halothane or caffeine-induced contractures in porcine muscle at 5°C.

The optimum temperature at which to assess MH susceptibility by in vitro pharmacological methods has been suggested to be 37°C (Denborough, 1979). A similar conclusion had been reached by Nelson and co-workers on the basis of their observations on the temperature dependence of halothane-induced contractures in porcine MH muscle (Nelson et al., 1975) and of caffeine-induced contractures in normal human muscle (Nelson et al., 1977).
In this study a detailed assessment of in vitro drug-induced contractures in porcine MH muscle, and to a lesser extent normal porcine muscle, over a range of temperatures indicates that the in vitro pharmacological assessment of MH susceptibility should be carried out at 37°C.

5.5 SUMMARY

The temperature dependence of twitch in normal and in MH swine gracilis muscle is characteristic of mammalian fast twitch, white fibres. Drug induced contractures are temperature dependent with normal and MH muscle preparations displaying unique temperature response profiles. In MH muscle, halothane, caffeine and succinylcholine induced maximum contractures at 5°C which were not inhibited by dantrolene, D$_2$O or glycerination. Increasing the temperature from 20° to 45° enhanced the contractile effectiveness of halothane, caffeine, succinylcholine and KCl in MH muscle. The hypersensitive E-C coupling that occurs in MH muscle at 37°C is abolished at 25°C and it is proposed that the site of this abnormal coupling is localised at the level of the triad. It is recommended that the in vitro assessment of MH susceptibility should be carried out at 37°C.
CHAPTER 6

EFFECTS OF TMB-8 ON NORMAL AND MALIGNANT HYPERPYREXIC PORCINE SKELETAL MUSCLE

6.1 INTRODUCTION

The role of calcium ions in the physiological activation of skeletal muscle contractile proteins is now firmly established (Sandow, 1965; Ebashi and Endo, 1968). Skeletal muscle is characterised by a low resting level of calcium (i.e. \( <10^{-7} \)M) in the myoplasm which increases rapidly prior to and during the development of a contracture (Sandow, 1970; Endo, 1977). Drugs which antagonise calcium release have been widely used in studies designed to assess the role of calcium in skeletal muscle function (Andersson, 1978). Such antagonists may act at a number of levels, but the end experimental result (i.e. antagonism of contractile responses) is a consequence of the maintenance of low intracellular calcium levels. In skeletal muscle calcium fluxes are primarily intracellular (Ebashi and Endo, 1968), although the qualitative and quantitative contribution of an influx of extracellular calcium remains to be clarified (Moulds and Denborough, 1974b; Barrett and Barrett, 1978; Spiecker et al., 1979). Calcium antagonists may be classified according to their site of action with extracellular influx and intracellular mobilisation being the most commonly antagonised processes (Rahwan et al., 1979). The mode of action of the muscle relaxant dantrolene sodium (Section 2.3.2) displays characteristics consistent with both extracellular (Putney and Bianchi, 1974; Takauiji et al., 1975; Takauiji et al., 1977; Takauiji and Nagai, 1977, Yamamoto et al., 1977) and intracellular (Ellis and Bryant, 1972; Ellis and Carpenter, 1972; Hainaut...
and Desmedt, 1974; Van Winkle, 1976; Morgan and Bryant, 1977; Francis, 1978; Desmedt and Hainaut, 1979) antagonism of calcium fluxes in skeletal muscle. This bimodal inhibition of calcium mobilisation by dantrolene has been proposed to be concentration dependent with low concentrations inhibiting extracellular influx and higher concentrations directly antagonising SR calcium release (Putney and Bianchi, 1974; Nelson and Denborough, 1977). The \textit{in vivo} effectiveness of dantrolene in blocking the development of the syndrome in susceptible swine (Harrison, 1975, 1977) indicates that in MH the primary abnormality resides in the skeletal musculature. \textit{In vitro} drug-induced hypercontractility in MH muscle preparations is abolished by pretreatment with dantrolene (Anderson \textit{et al.}, 1976, 1978; Okumura \textit{et al.}, 1980), and the effectiveness of low concentrations of dantrolene in attenuating hypercontractility has been proposed to reflect antagonism of the E-C coupling step(s) proximal to activator calcium release from the terminal cisternae (Nelson and Denborough, 1977).

The \(\omega\)-(N,N-diethylamino)-alkyl-3,4,5-trimethoxybenzoates) (TMB) constitute a group of compounds which have been widely used as calcium ion antagonists in studies of smooth muscle (Malagodi and Chiou, 1974a; Creese, 1979), skeletal muscle (Malagodi and Chiou, 1974b), platelets (Charo \textit{et al.}, 1976a, b; Le Breton and Dinerstein, 1977; Rittenhouse-Simmons and Deykin, 1978; Gorman \textit{et al.}, 1979) and leukocytes (Matsumoto \textit{et al.}, 1979). Robinson (1971) has shown that as the length of the intermediate alkyl chain is increased, not only does the potency of these drugs increase, but the dose range for blockage of a wide variety of contractile agents in smooth and skeletal muscles narrows as well. The length of the alkyl chain in the TMB compound is therefore a major determinant of potency and tissue specificity (Robinson, 1971). In smooth
muscle TMB-8 (8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride) non-competitively inhibits responses to a number of contractile stimuli, but competitive antagonism occurs only in response to barium chloride (Malagodi and Chiou, 1974a). Collectively this data provides evidence of an intracellular site of action for TMB-8 in smooth (Malagodi and Chiou, 1974a) and skeletal (Malagodi and Chiou, 1974b) muscles. In addition TMB-8 markedly inhibits caffeine-induced calcium release from a skeletal muscle SR preparation, while having no effect on calcium uptake rates (Chiou and Malagodi, 1975). Short chain TMB compounds possess properties characteristic of cholinesterase inhibitors (Robinson, 1971), but the inclusion of physostigmine (a cholinesterase inhibitor) in the buffer does not modify the inhibitory effects of TMB-8 on the contractile responses induced by a number of agents in smooth and skeletal muscle preparations (Malagodi and Chiou, 1974a, b).

In platelets TMB-6 and TMB-8 have been shown to markedly depress aggregation and secretion induced by a variety of agents (Charo et al., 1976a; Le Breton and Dinerstein, 1977; Gorman et al., 1979) and it seems that in these cells TMB compounds 'anchor' intracellular calcium in situ (Rittenhouse-Simmons and Deykin, 1978).

In a number of tissues TMB compounds attenuate calcium mobilisation in response to diverse stimuli and, although the exact mechanism(s) of action are unknown, calcium immobilisation in situ is the most likely mechanism of action. The effects of TMB compounds have not previously been assessed on MH skeletal muscle.

This chapter examines the effects of the calcium ion antagonist TMB-8 on halothane-, caffeine-, succinylcholine- and potassium chloride-induced contractures in normal and MH porcine skeletal muscle.
6.2 MATERIALS AND METHODS

6.2.1 Methods

Anaesthesia (Section 3.2.1), the removal of clamped biopsy material, the preparation of fibre bundles and the assessment of contractile responses (Section 5.2) were carried out as described earlier. Experiments were carried out at 37°C in a jacketed organ bath under an initial resting tension of 1 gram. A total of 8 normal and 13 MH swine were biopsied.

Results were expressed as the mean ± S.E.M., and compared using the Student's t-test. A maximum of two fibre preparations per individual animal was used for any single or combination drug treatment.

6.2.2 Materials

TMB-8 was synthesised by Dr D.J. Brown, Medical Chemistry Group, John Curtin School of Medical Research, Canberra, by the method of Malagodi and Chiou (1974a). Stock solutions of TMB-8, 5 and 50 mM, were prepared in the modified Ringer buffer. Physostigmine was obtained from Sigma Chemical Company. Additional materials were as described in Section 5.2.2.

6.3 RESULTS

6.3.1 Drug contractures in porcine skeletal muscle

Contractile responses to 3% halothane, 2 mM caffeine, 1 mM succinylcholine and 80 mM KCl differentiated normal from MH muscle (Figure 6.1). 1 mM TMB-8 induced contractures in MH muscle (1.28 ± 0.22 grams, n = 5) which were significantly different from contractures in normal muscle (0.10 ± 0.10 grams, n = 4) (Figure 6.1).
Tension changes in response to 100 µM TMB-8 were always less than 0.1 grams in both muscle types.

6.3.2 Effects of TMB-8 on normal muscle

Contractile responses to 3% halothane, 2 mM caffeine, 1 mM succinylcholine and 80 mM KCl were minimal in normal muscle preparations (Figure 6.1) and pre-treatment with 100 µM TMB-8 had no effect on these responses. A minimum of 4 muscle preparations was used for any TMB-8-drug combination, and for all such combinations contractile responses were less than 0.1 grams (results not shown). Isometric twitch was abolished by treatment with 1 mM TMB-8 (Figure 6.2D).

6.3.3 Effects of TMB-8 on MH muscle

Figure 6.2 shows the dose dependency of TMB-8-induced contractures in MH muscle. Low concentrations of TMB-8 (i.e. 100 µM) enhanced the contractile sensitivity of MH muscle to halothane, caffeine and succinylcholine. Typical tracings are shown in Figure 6.3. Prior treatment with 100 µM TMB-8 significantly increased the magnitude of drug contractures induced by 3% halothane (229%), 2 mM caffeine (156%) and 1 mM succinylcholine (131%) (Figure 6.4). However this concentration of TMB-8 reduced KCl-induced contractures by a factor of 3 (Figure 6.4).

The effectiveness of TMB-8 in blocking KCl-induced contractures was concentration dependent with half maximal inhibition occurring at approximately 50 µM TMB-8 (Figure 6.5). The inhibitory effect of 100 µM TMB-8 on KCl-induced contractures was not modified in a calcium supplemented (10 mM) buffer (n = 2).

In addition to potentiating halothane-, caffeine- and succinylcholine-induced contractures, 100 µM TMB-8 induced an additional contracture when added to halothane or succinylcholine contracted muscle.
Contractile responses to 3% halothane (HAL), 2 mM caffeine (CAFF), 1mM succinylcholine (SCC), 80 mM KCl, and 1 mM TMB-8 were recorded under an initial resting tension of 1 gram. Contractile responses are shown for normal (diagonal bars) and MH (closed bars) fibre preparations. Standard errors are not included for normal muscle responses as S.E.M.s were always ≤ 0.01 grams.

** p < 0.01

*** p < 0.001
Figure 6.2: Contractile responses of MH porcine skeletal muscle to TMB-8

These tracings show the contractile responses of individual MH fibre preparations to increasing concentrations of TMB-8 (traces, A, B, and C). The effect of 1 mM TMB-8 on normal muscle is shown for comparison (trace D).
Figure 6.3: Modification of halothane-, caffeine-, succinylcholine- and KCl-induced contractures by TMB-8 (100 μM) in MH porcine skeletal muscle

Typical responses are shown for 3% halothane (HAL), 2 mM caffeine (CAFF), 1 mM succinylcholine (SCC) and 80 mM KCl (traces A1, B1, C1 and D1, respectively). TMB-8 (100 μM) was added where indicated and drugs added 10 minutes after this (A2, B2, C2 and D2). Trace A3 shows the potentiating effect of 3% halothane on 100 μM TMB-8-induced contractures. Each trace represents a separate fibre preparation.
Figure 6.4: Effect of TMB-8 (100 µM) on the contractile responses of MH porcine skeletal muscle to halothane, caffeine, succinylcholine and KCl

Contractile responses to 3% halothane, 2 mM caffeine, 1 mM succinylcholine, and 80 mM KCl (closed bars) are shown in Figure 6.1. TMB-8 (100 µM) was added 10 minutes prior to each drug and the maximum tension generated recorded (horizontal bars). Abbreviations for drugs are as shown in Figure 6.1. Each observation represents a separate fibre preparation.

** p < 0.01

*** p < 0.001
Figure 6.5: Inhibition of KCl-induced contractions in MH porcine skeletal muscle by TMB-8

The traces show contractile responses to KCl (80 mM, arrows indicate when added) in the absence or presence of increasing concentrations of TMB-8. TMB-8 (at the concentrations indicated) was added 10 minutes prior to KCl. Each trace represents a separate fibre preparation.
The addition of 100 μM TMB-8 to halothane treated muscle (Figure 6.3A3) resulted in a contracture (1.42 ± 0.20 grams, n = 17), and a similar result was recorded in succinylcholine treated muscle (0.40 ± 0.16 grams, n = 6). By comparison 100 μM TMB-8 alone had no effect on MH muscle (< 0.1 grams, n = 37). The addition of 100 μM TMB-8 after a KCl-induced contracture had no effect on muscle tension (n = 6). Potentiation of TMB-8-induced contractures by caffeine was not investigated.

Physostigmine (0.5 μM) was added to the buffer on a number of occasions but the hyperreactive responses in MH muscle preparations were not altered in its presence (results not shown).

6.3.4 Attenuation of TMB-8 induced hyperreactivity in MH porcine skeletal muscle

Glycerol-induced detubulation abolished both the contractile effect of 1 mM TMB-8 and the drug contractile potentiating effect of 100 μM TMB-8 in MH muscle preparations. In MH muscle the E-C coupling antagonist dantrolene sodium (6 μM) completely abolished;

(1) 100 μM TMB-8-induced potentiation of halothane-induced contractures (Figure 6.6A) (n = 3);
(2) halothane-induced potentiation of 100 μM TMB-8 induced contractures (Figure 6.6B) (n = 2);
(3) the contracture induced by 1 mM TMB-8 (Figure 6.6C) (n = 2).

Contractility in response to 1 mM TMB-8 was abolished by a 30 minute incubation in a 2 mM EGTA, calcium free buffer (n = 2).

6.4 DISCUSSION

The hyperreactivity that characterises MH skeletal muscle on exposure to TMB-8 contrasts with the inhibitory effect on drug-induced
Figure 6.6: Inhibition of TMB-8-induced hyperreactivity in MH porcine skeletal muscle by dantrolene sodium

Potentiation of 3% halothane-induced contractures by 100 μM TMB-8 (trace A1) and of 100 μM TMB-8-induced contractures by 3% halothane (trace B1) was completely abolished by 6 μM dantrolene (traces A2 and B2, respectively). 1 mM TMB-8-induced contractures (trace C1) were also abolished by 6 μM dantrolene (trace C2). Abbreviations for drugs are as shown in Figure 6.1. Each trace represents a separate fibre preparation.
contractures in a variety of muscle tissues. In smooth (Malagodi and Chiou, 1974a) and skeletal (Malagodi and Chiou, 1974b) muscles TMB-8 non-competitively inhibits contractile responses to agents that act on the surface membrane (e.g. acetylcholine, KCl, direct and indirect electrical stimulation, and nicotine), while competitive antagonism characterises the effects of TMB-8 on contractures induced by agents which have an intracellular site of action (e.g. caffeine and barium chloride). Non-competitive inhibition occurs at low doses, while 5- to 10-fold higher concentrations of TMB-8 are required to competitively antagonise the direct action of barium chloride and caffeine on intracellular calcium release in smooth and skeletal muscles, respectively (Malagodi and Chiou, 1974a, b).

In frog skeletal muscle the slow diffusion of TMB-8 into intracellular membrane sites has been proposed to underly the requirement for high doses of TMB-8 to inhibit caffeine contractures (Malagodi and Chiou, 1974b) and this proposal is supported by the effectiveness of low doses of TMB-8 (i.e. 5 μM) in inhibiting the release of $^{45}$Ca$^{2+}$ by caffeine from an isolated skeletal muscle SR preparation (Chiou and Malagodi, 1975).

In normal porcine skeletal muscle contractures were not induced by 3% halothane, 2 mM caffeine, 1 mM succinylcholine or 80 mM KCl in the absence or presence of 100 μM TMB-8. In MH muscle the inhibitory effects of low doses of TMB-8 (< 100 μM) on KCl-induced contractures accords with the concentrations required to inhibit KCl-induced contractures in a variety of skeletal muscle preparations (Malagodi and Chiou, 1974b).

The inability of low doses of TMB-8 to antagonise halothane-, caffeine- or succinylcholine-induced contractures suggests that in MH muscle these drugs do not act on the sarcolemma. The similarity between
MH muscle responses in the presence or absence of physostigmine rules out the possible inhibition of acetylcholinesterase as an explanation of TMB-8-induced hyperreactivity in MH muscle preparations. The rapidity with which contractures develop in response to 1 mM TMB-8 argues against an intracellular site of action since, in intact muscle, the effect of TMB-8 on SR membranes has been proposed to be time dependent with up to 20 minutes required for full expression (Malagodi and Chiou, 1974b). Thus in MH muscle TMB-8 appears to exert its abnormal effects distal to the sarcolemma and proximal to SR calcium release (i.e. at the level of the triad). The effectiveness of E-C uncoupling treatments (i.e. prior glycerination or pretreatment with dantrolene sodium) in abolishing TMB-8-induced hyperreactivity is also consistent with a triadic site of action.

The mechanism by which TMB-8 immobilises calcium remains obscure but the lack of highly charged functional groups on the TMB-8 molecule (Figure 6.7) would suggest that a direct interaction with calcium is unlikely. A direct interaction of TMB-8 with biological membranes is an alternative mechanism of action, and is consistent with the structure and length of the molecule. The length of the TMB-8 molecule approximates to the half width of a synthetic phospholipid bilayer and the hydrocarbon diethylamino-octyl side chain could conceivably become inserted parallel to the phospholipid fatty acids with the bulky trimethoxybenzoyl group stabilised by hydrogen bonding between the carboxyl group and the polar head groups of the phospholipids. This interaction is schematically illustrated by Figure 6.7. Stabilisation of the membrane might be the likely result of this TMB-8-phospholipid interaction and the slow rate of transverse diffusion of TMB-8 across the membrane (Malagodi and Chiou, 1974b) might reflect its rigid deposition in the lipid bilayer. A similar
Figure 6.7: Hypothetical localisation of TMB-8 within a phospholipid membrane

The structural similarities between TMB-8 and Coenzyme Q-3 are shown in the upper section of the figure. Below is shown a possible site of action of TMB-8 in a phospholipid membrane.
site of action has been proposed for the synthetic ubinquinone, Co-enzyme Q-3 (Lenaz et al., 1978a). The site of action of TMB-8 as shown in Figure 6.7 is relatively non-specific; other interactions might occur which would confer a greater degree of specificity. These might include;

(1) a direct interaction between TMB-8 and specific membrane proteins (e.g. calcium binding proteins);

(2) an indirect effect of TMB-8 on membrane proteins, mediated by an interaction between the drug and the 'layer' or 'annulus' of phospholipids which surround specific membrane proteins. This represents a more specific interaction than that described in Figure 6.7.

Although such a model would be consistent with the effects of TMB-8 in a number of normal tissues it is difficult to explain the initial hyperreactivity that occurs in MH muscle on exposure to TMB-8 in terms of such a model. It is possible that the hyperreactive effect of TMB-8 on MH muscle has little to do with its function as a calcium antagonist and that the interaction of the drug with the T-tubular membrane in some way facilitates or itself directly initiates the release of trigger calcium (Endo, 1977; Ebashi, 1979). Thus the mechanism by which TMB-8 contracts and potentiates drug-induced contractures remains obscure but this drug provides a useful tool to further study the nature and site of the skeletal muscle abnormality in MH.

6.5 SUMMARY

TMB-8 contracted porcine MH skeletal muscle and potentiated the contractile actions of 3% halothane, 2 mM caffeine, and 1 mM succinylcholine. 80 mM KCl-induced contractures were antagonised by TMB-8. E-C uncoupling
treatments abolished TMB-8-induced hyperreactivity. Immobilisation of
calcium in situ appears to underlie the mode of action of TMB-8 in
normal muscle but the mechanism underlying TMB-8-induced hyperreactivity
in MH muscle remains obscure.
CHAPTER 7

PLATELET FUNCTION IN MALIGNANT HYPERPYREXIA

7.1 INTRODUCTION

7.1.1 Platelet structure and function

The platelet possesses a number of structural and functional characteristics similar to those of skeletal muscle, including a muscle-type contractile system and a complete dependence on calcium for normal functioning (White and Gerrard, 1978; White, 1979). Stimulus-secretion coupling, the process whereby stimulation of the surface membrane is coupled to eventual secretion of intracellular contents, is similar to E-C coupling in skeletal muscle (Day and Holmsen, 1971; Barnhart, 1978). Structurally the platelet is complex, and consists of three discrete regions or zones; the peripheral zone, the sol-gel zone and the organelle zone (Henry, 1977; White, 1979). The peripheral zone consists of the plasma membrane and surface connected canalicular membrane, and its chemical constituents modulate specificity for stimuli triggering platelet activation (White, 1979). The sol-gel zone constitutes the matrix of the platelet cytoplasm and contains the contractile protein systems which maintain the discoid shape of the resting or unchallenged platelet and modulate shape change, internal contraction and secretion in the stimulated platelet (Henry, 1979; White, 1979). The organelle zone contains a specialised group of secretory vesicles which include dense bodies, α-granules and lysosomal vesicles (Holmsen and Weiss, 1979).

Upon stimulation the signal initiating the changes in the platelet surface membrane may or may not be translated and amplified through the membrane, depending on the nature and the level of stimulation employed.
When platelets are exposed to aggregating agents in vitro the change in surface membrane properties results in the eventual formation of aggregates. This process of aggregate formation reflects an increase in platelet "stickiness" (White, 1979). Above a certain threshold level of stimulation the aggregating signal is propagated into the cell and activation of the platelet contractile machinery results in the mobilisation, fusion and active extrusion of secretary vesicle contents through the surface-connected canalicular system. The release reaction represents the final stage in the activation process and the released products (particularly ADP) in turn transmit the signal to surrounding, unstimulated platelets, causing further aggregation.

Platelets contain both metabolic and storage pools (dense granule localised) of adenine nucleotides and the distribution of the nucleotides is characteristic for each pool (Holmsen and Weiss, 1979). In normal human platelets approximately 65% of the total content of adenine nucleotides are stored in the dense granules where the ratio of ATP to ADP is 2:3 (Holmsen and Weiss, 1979). In the metabolic pool ATP predominates and the ATP/ADP ratio may be greater than 8 (Holmsen and Weiss, 1979). Storage pool diseases constitute a broad group of disorders which are all associated with the abnormal storage and/or release of constituents from the dense granules. In such individuals the failure to release intracellular platelet constituents (i.e. serotonin and non-metabolic adenine nucleotides) is due to a deficiency in the number of dense granules (Lusher and Barnhart, 1977).

An additional group of disorders has been described where the release mechanism is defective, despite a normal storage pool (Deykin, 1974; Walsh and Gagnatelli, 1974). Defective release characterises aspirin treated platelets and this supports the conclusion that the release
defect has some characteristics in common with the effect of aspirin on normal platelets (Paratti et al., 1974). Acetylation of the cyclooxygenase enzyme represents the primary site of action of aspirin (Roth and Majerus, 1977) and this results in a block in the arachidonic acid cascade at a step prior to the formation of the potent aggregatory endoperoxides and thromboxanes (Harris et al., 1979).

Platelet aggregation is usually studied in vitro using the turbidimetric method first developed by Born (1962). Despite the in vitro nature of the test its application has provided substantial and useful data on platelet function in both the normal and diseased states (Lusher and Barnhart, 1977; Barnhart, 1978). Platelet aggregation may be reversible or irreversible and monophasic or biphasic depending on the nature and strength of the stimulus employed. The second phase of biphasic aggregation is associated with the occurrence of the release reaction (Charó et al., 1977, Barnhart, 1978) and its inhibition by the cyclooxygenase inhibitor aspirin, suggests that secondary aggregation, the release reaction and the generation of prostaglandin and endoperoxide metabolites are closely linked phenomena.

7.1.2 Platelet function in malignant hyperpyrexia

Platelet aggregatory responses have been characterised in a number of studies in an attempt to correlate MH susceptibility with a clinically demonstrable platelet abnormality (Isaacs, 1978; Zsigmond et al., 1978). A number of susceptible individuals have shown decreased responsiveness to aggregatory agents, especially adrenaline and collagen (Isaacs, 1978; Zsigmond et al., 1978). Secondary aggregation, and the associated release reaction, in response to adrenaline was absent in 2 out of 13 (Isaacs, 1978) and 5 out of 8 (Zsigmond et al., 1978) patients screened by these
investigators. Ristocetin- and ADP-induced aggregation was always normal (Zsigmond et al., 1978). Aggregatory responses to arachidonic acid, thrombin, or to the calcium ionophore A-23187 have not been assessed in M:\H platelets. It would appear therefore that abnormal aggregation is a feature found in some susceptible individuals but the presence of borderline to normal responses in others questions the usefulness of this method in assessing M:\H susceptibility (Isaacs, 1978).

Platelet synthesis of purine nucleotides is modified in the presence of halothane and this effect is most dramatic in platelets from M:\H susceptible individuals (Solomons et al., 1978). Utilising radioactive $^{14}$C-labelled adenine, Solomons has shown that halothane alters the nucleotide synthesising capabilities of M:\H platelets and results in an abnormally low ratio of $^{14}$C-ATP $+$ $^{14}$C-ADP/$^{14}$C-AMP (i.e. $\leq 3$) in M:\H susceptible individuals (Solomons et al., 1978). Comparable experiments with normal platelets gave a ratio greater than 8 (Solomons et al., 1978). This decreased capacity for synthesis of adenine nucleotides in the presence of halothane represents a useful link to the M:\H skeletal muscle response where halothane rapidly depletes ATP levels in vitro (Section 2.3.7).

This chapter examines the effects of a variety of aggregating agents on M:\H platelets in an attempt to develop a simple, nontraumatic procedure with which to assess M:\H susceptibility. The aggregatory effects of ADP, collagen, adrenaline, thrombin, arachidonic acid, and A-23187 on M:\H platelets have been examined in some detail, and the results correlated with the in vitro pharmacological assessment of susceptibility on the basis of drug-induced contractures in isolated skeletal muscle preparations.
7.2 MATERIALS AND METHODS

7.2.1 Methods

Blood was collected atraumatically via a 21 gauge butterfly cannula with a 12 inch extension tube. 9 vol of blood were collected into 1 vol of citrate (0.1 M trisodium citrate, pH 7.4). Platelet-rich plasma (PRP) was prepared by centrifugation at 100 g for 15 minutes at room temperature. PRP was stored in stoppered syringes at 37°C and aggregation experiments were completed within three hours of blood collection. Platelet-poor plasma (PPP) was prepared from PRP by centrifugation at 8000 g for two minutes at room temperature. Patients were requested to abstain from ingesting medications known to influence platelet behaviour for at least ten days prior to blood collection (Barrer and Ellison, 1977; Verstraete, 1978).

Platelet counts in PRP were performed on a Model ZF Coulter Counter (Coulter Electronics, England). PRP samples (5 μl) were added to 15 ml of isoton (Coulter Electronics) and counted within 30 minutes of dilution. Values are given as means ± S.D.

Platelet aggregation was monitored turbidometrically (Born, 1962) using a Payton dual channel Aggregation Module (Payton Association Ltd, Canada). The following stock solutions were maintained; ADP, 10 mM in reagent diluent (RD); adrenaline bitartarate, 10 mM in RD; collagen, 1 mg/ml in acetic acid; bovine thrombin, 1000 units/ml in RD; potassium arachidonate, 14.5 mM in RD; A-23187, 2 mg/ml in DMSO. Stock solutions of collagen and A-23187 were stored at 4°C; all others were stored at -20°C. RD contained 147 mM NaCl, 2.7 mM KCl, 9.4 mM NaHCO₃ and 0.42 mM NaH₂PO₄; pH 7.4 and was prepared fresh each day. Prior to use, working dilutions were prepared from the stock solutions and maintained on ice during use.
The release reaction was assessed by measuring the release of radioactivity from platelets previously labelled with $^{3}$H-serotonin (5-hydroxytryptamine binoxalate-$^{3}$H; 25 Ci/mmol. PRP was incubated with $^{3}$H-serotonin (0.3 $\mu$Ci/ml of PRP) at 37°C for 30 minutes. The release reaction was stopped by the addition of paraformaldehyde (Costa and Murphy, 1975) and radioactivity was determined in PPP and PRP samples. The samples (100 $\mu$l) were added to 15 ml of scintillation mixture (Bray, 1960) and counted in a Packard Tri-carb Liquid Scintillation Spectrometer. The released $^{3}$H-serotonin was expressed as a percentage of the total radioactivity in the PRP.

Electron microscopy was carried out on pelleted platelet fractions essentially as described in Section 3.2.5.

7.2.2 Materials

Patients were referred to Dr M.A. Denborough for assessment of MH susceptibility by recognised pharmacological criteria (Moulds and Denborough, 1974a). This involved the in vitro exposure of isolated skeletal muscle preparations to 3% halothane, 2 mM caffeine, 1 mM succinylcholine and 80 mM KCl. Abnormal contractures to the aforementioned drugs differentiated MH susceptible from normal individuals.

ADP, collagen (from bovine achilles tendon) and arachidonic acid (99% pure) were obtained from Sigma Chemical Company. Adrenaline bitartarate was from Calbiochem, and bovine thrombin from Parke Davis. A-23187 was kindly supplied by Eli Lilly Research Laboratories and $^{3}$H-serotonin was obtained from New England Nuclear.
7.3 RESULTS

7.3.1 Platelet counts

Platelet counts varied from 165,000 to 602,500 /µl in normal PRP (Table 7.1) and from 238,600 to 623,400 /µl in MH PRP (Table 7.2). Average counts were significantly higher in MH PRP (normal = 341,100 ± 98,600 /µl, n = 13, and MH = 435,100 ± 96,000 / µl, n = 19; p < 0.05).

7.3.2 Platelet aggregation

Normal aggregation responses to a variety of agents are shown in Figure 7.1. The range of threshold concentrations of arachidonic acid (0.29 to 0.73 mM), thrombin (0.05 to 0.2 units/ml) and A-23187 (5 to 20 µg/ml) required to induce irreversible aggregation were similar in normal and MH PRP. Excluding patient B.R. (Table 7.2) both normal and MH platelets responded normally to ADP (range, 1 to 10 µM). However responses to adrenaline and collagen varied considerably and 3 response groups could be identified with some overlap between the normal and MH subjects (Tables 7.1 and 7.2).

(a) Group 1; irreversible aggregation in response to 1 µM adrenaline and 0.25 µg/ml collagen (Figure 7.1) characterised platelet responses in this group. Platelets from six normal (Table 7.1) and four MH (Table 7.2) individuals gave Group 1 responses.

(b) Group 2; an absence of first phase and a considerable delay in the onset of aggregation characterised adrenaline responses in this group (Figure 7.2A) and/or responses to collagen were also considerably reduced (Figure 7.2A). Platelets from seven normal (Table 7.1) and fourteen MH (Table 7.2) individuals gave Group 2 responses.

(c) Group 3; in one MH patient (B.R.; Table 7.2) ADP, adrenaline and collagen failed to induce irreversible aggregation (Figure 7.3) even
Figure 7.1: Group 1 platelet aggregation and release responses

The final concentrations of the aggregatory agents used were as follows: ADP, 2.5 μM; adrenaline (ADR), 1 μM; collagen (COLL), 0.25 μg/ml; arachidonic acid (AA), 0.6 mM; A-23187, 20 μg/ml; thrombin (THR), 0.1 units/ml. Release of $^3$H-serotonin is indicated at the end of each tracing. Aggregation responses were typical for all Group 1 patients but $^3$H-serotonin release was only monitored in patient Y.M.
Figure 7.2: Group 2 platelet aggregation and release responses

The final concentrations of aggregatory agents used were as described in Figure 7.1. Aggregation in response to arachidonic acid, A-23187 and thrombin was similar to Group 1 platelets. The % release of $^3$H-serotonin refers to patient J.R. only.
Figure 7.3: Group 3 platelet aggregation and release responses

The final concentrations of aggregatory agents used were as described in Figure 7.1. Aggregation in response to arachidonic acid, A-23187 and thrombin was similar to Group 1 platelets. Group 3 responses were found only in one patient, B.R., an MH susceptible individual.
"Normal" humans are deemed as those giving a negative biopsy result.

<table>
<thead>
<tr>
<th>Group</th>
<th>Adrenaline collagen</th>
<th>Platelet aggregation</th>
<th>Platelet aggregation and platelet counts in &quot;normal&quot; humans</th>
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Mean ± S.D.: 341,100 ± 98,600

Table 7.1: Platelet aggregation and platelet counts in "normal" humans
Table 7.2: Platelet aggregation and platelet counts in MH susceptible humans

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Mean ± S.D.: 435,100 ± 96,000

Abn = abnormal aggregation
N = normal aggregation

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at very high concentrations (e.g. ADP, 10 μM; adrenaline, 10 μM; collagen, 20 μg/ml). The failure of these agents to aggregate B.R.'s platelets contrasts with the normal aggregatory behaviour displayed in response to thrombin, arachidonic acid, and A-23187 and indicates that these platelets are characterised by a release defect.

7.3.3 Platelet release reaction

$^3$H-serotonin release was monitored in B.R. (Group 3, Figure 7.3) on three separate occasions, and on all occasions, ADP (2.5 μM), adrenaline (1 μM) and collagen (0.25 μg/ml) failed to induce any release of radioactivity.

Percentage release of $^3$H-serotonin from Y.M.'s platelets (Group 1) and from J.R.'s platelets (Group 2) was essentially similar in response to ADP, (percentages in Figures 7.1 and 7.2, respectively), and failed to differentiate these two groups. Release of $^3$H in response to thrombin, arachidonic acid, and A-23187 was similar in each of the three groups. In all of the patients tested, $^3$H-serotonin uptake reached a maximum within 15 minutes and was maintained over a ninety minute period.

7.3.4 Electron microscopy

The high concentration of dense granules in the platelets of B.R. are similar to the numbers found in normal platelets (Figure 7.4). The addition of 10 μM adrenaline (approximately 5-10 times the concentration required to aggregate Group 1 platelets) had little effect on B.R.'s platelets while the normal platelets displayed extensive aggregation and release of intracellular contents (Figure 7.5).

7.4 DISCUSSION

In the present investigation abnormal platelet aggregation was
Figure 7.4: Platelet morphology in an MH susceptible and a normal individual

The upper micrograph shows platelets isolated from an MH susceptible patient (B.R., Table 7.2 and Figure 7.3). The lower micrograph shows platelets isolated from a normal individual. Morphological details are similar. The designated structural features comprise: the surface connected canalicular system (CS), dense bodies (DB), the dense tubular system (DTS), specific storage granules (G), glycogen granules (Gly) and mitochondria (M). Magnification: upper, x 10,000; lower, x 13,000.
Figure 7.5: **Effect of adrenaline on platelet morphology in an MH susceptible and a normal individual**

Platelets were pelleted and fixed 5 minutes after the addition of 10 μM adrenaline. The upper micrograph shows adrenaline treated platelets from an MH susceptible patient (B.R., Table 7.2 and Figure 7.3) and the lower micrograph adrenaline treated platelets from a normal individual. Aggregation and release did not occur in B.R.'s platelets while in normal platelets adrenaline induced irreversible aggregation and release (as evidenced by pseudopod formation and a decrease in granules). Magnification: upper, x 8,000; lower, x 13,000.
demonstrated in a large number of individuals shown to be susceptible to MH, but there was considerable overlap between platelet responses in normal and MH subjects. In a random study in South Africa, involving several members of families known to be MH susceptible, abnormal platelet aggregation was shown in many carriers but, as in this study, normal responses were also recorded in some susceptible individuals (Isaacs, 1978). Details regarding the nature of these abnormal responses have not been reported, but it would appear that irreversible aggregation in response to collagen and adrenaline did occur but that higher concentrations of the agents than normal were required to bring about this effect (Isaacs, 1978). The Group 2 responses described here best approximate to the abnormal platelet responses reported by Isaacs (1978). Separate studies on two MH susceptible families in Michigan (Zsigmond et al., 1978) identified additional variations on these abnormal aggregation patterns. In one family, secondary aggregation was not observed in response to adrenaline or collagen while in the second family, only adrenaline failed to induce secondary aggregation. The variety of aggregating responses reported for MH individuals in this and other studies (Isaacs, 1978) may reflect methodological differences, but such a possibility seems unlikely. A more likely explanation is that this variability in platelet responses reflects heterogeneity in the types of cell affected, and/or the degree of expression of the abnormality in any single cell type.

Abnormalities in platelet function are associated with either hypo- or hyperreactivity (White and Gerrard, 1978) and the abnormality (when expressed) in MH platelets falls within the former category. Decreased platelet activity is a characteristic feature of a number of congenital disorders including Bernard-Soulier syndrome, von Willebrand's disease, thrombasthenia, and the heterogeneous storage pool diseases
The nature of the abnormality underlying the Group 2 platelet response (i.e. no first phase to adrenaline and decreased collagen-induced aggregation) remains obscure, but the delay and decreased responsiveness to specific agents may reflect a direct surface membrane abnormality and/or an indirect functional decrease in the transmission of the activation stimulus from the surface membrane to the rest of the platelet. The normal ADP, thrombin, arachidonate and A-23187 mediated aggregation responses further support such a selective abnormality.

Group 3 responses display many features indicative of a release or aspirin-like defect. The failure to aggregate and undergo the release reaction (despite the normal complement of secretory products) in response to ADP, adrenaline and collagen is common to a number of congenital connective tissue disorders (Estes, 1972; Hathaway et al., 1972), and in one of these, namely osteogenesis imperfecta (OI) the abnormality is thought to be diffuse affecting many cell types, including leukocytes and platelets (Hathaway et al., 1972). Platelet aggregation and release in response to thrombin, A-23187, and in an individual (K.C.) susceptible to OI displayed a similar response profile to that shown by MH patient B.R. (J. Sullivan, N.G. Ardlie and M.A. Denborough, unpublished results).

The abnormal metabolism of adenine nucleotides in both MH (Solomons et al., 1978) and OI platelets (Hathaway et al., 1972) further underlines the similarities and the generalised nature of each of these syndromes. Electron micrographs show that B.R.'s platelets contain normal levels of dense bodies (Figure 7.4) and together with the demonstrated ability of thrombin, arachidonate, and A-23187 to induce serotonin release, indicate that these platelets display characteristics consistent with a release defect (Luscher and Barnhart, 1977). Group 3 responses have also been
described in two additional MH susceptible individuals (N.G. Ardlie and M.A. Denborough, unpublished results) indicating that the release defect characteristic of B.R.'s platelets may be more widespread than was suggested by this current study. The overlap in platelet aggregation responses between normal and MH individuals limits the application of platelet function tests in assessing MH susceptibility. Additionally, the sensitivities of the platelet to a wide variety of drugs (Barrer and Ellison, 1977; Verstraete, 1978) necessitates the carrying out of such tests under conditions where the ingestion of drugs by patients is strictly controlled. The demonstrated abnormalities in platelet aggregation responses in a large number of MH susceptible individuals further underlines the generality of the defect predisposing to MH. However the variable presence and extent of the platelet abnormality limits the use of platelet function tests in assessing MH susceptibility. Furthermore the occurrence of normal platelet aggregation in individuals unambiguously identified as being MH susceptible, together with the ability of a wide variety of drugs to alter aggregation responses in normal individuals (Barrer and Ellison, 1977; Verstraete, 1978) indicates that platelet function tests alone should not be used to identify individuals at risk for MH.

7.5 SUMMARY

In this investigation abnormal aggregation responses occurred in fifteen of the nineteen MH susceptible individuals screened, although in only one of these fifteen was the ability to aggregate in response to ADP, abolished. In the remaining fourteen a diminished response to adrenaline and collagen characterised susceptible individuals. Seven of the thirteen normal individuals screened displayed decreased aggregatory responses to adrenaline and to collagen. Under conditions where the ingestion of drugs
is strictly controlled, platelet function tests serve as an additional method to complement currently available techniques in assessing MH susceptibility. In isolation such tests should not be used to identify individuals at risk for MH.
CHAPTER 8

GENERAL DISCUSSION

8.1 INTRODUCTION

The studies presented in this thesis were undertaken to further characterise the nature of the abnormality predisposing to MH. A multidisciplinary approach was adopted in which biochemical, pharmacological and biophysical techniques were used to achieve this objective.

8.2 GENERAL DISCUSSION

Earlier investigations on MH clearly implicate skeletal muscle as the primary affected tissue. Within this tissue it appears that the underlying abnormality is directly associated with the processes modulating intracellular calcium concentration and/or distribution, although there is still no direct evidence to support this view. The most important level of control of calcium ion movements in cells is carried out by cell membranes, and an abnormality in the processes modulating intracellular calcium concentrations could reflect a structural and/or functional abnormality at the membrane level. In the case of skeletal muscle a variety of membrane linked processes have been implicated (Chapter 2). Despite the evidence favouring a variety of functional abnormalities in discrete subcellular muscle fractions, few studies have attempted to correlate abnormal function to an underlying structural abnormality. Such an approach was adopted in this study and the chemical composition of normal and MH muscle membranes was presented in Chapter 3. These findings suggest that there are no consistent changes in membrane composition in MH skeletal muscle. A decrease in the unsaturated fatty acid content of
membrane phospholipids and an increase in the levels of cholesterol were found in MH trapezius muscle, but the similar composition of normal and MH l. dorsi muscle suggests that the changes in the lipid composition of the trapezius muscle probably reflect a secondary phenomenon.

The protein profiles of the muscle membrane fractions characterised in the present study were complex (Section 3.3.6) and contrast with the simpler profiles for porcine skeletal muscle membranes obtained by McIntosh et al. (1977). Differences in acrylamide gel composition and the use of a cylindrical disc gel electrophoretic system might account for the simpler muscle membrane protein profiles obtained by McIntosh et al. (1977). Indeed the running of comparable muscle membrane fractions on disc and slab gels demonstrated the greater resolving power of the latter electrophoretic system (J. Sullivan and M.A. Denborough, unpublished results). Although cross contamination between various membrane systems cannot be ruled out as an explanation for the complex electrophoretic patterns, the results from the present investigation suggest that the simpler protein profiles reported for skeletal muscle membranes using disc gel electrophoresis might reflect the limitations of this electrophoretic method. Further work is required to clarify this point.

Consistent quantitative or qualitative abnormalities in membrane protein composition were not found in either the red trapezius or the white l. dorsi muscles from MH swine. This appears to rule out a gross alteration in muscle membrane protein composition as the primary cause of MH, but a more subtle protein abnormality in a discrete membrane system cannot be ruled out. Further purification of the membrane fractions isolated in the present study would be required to assess such a possibility.

Elevations in body temperature represent an important clinical
feature during the development of MH (Denborough, 1979) the skeletal muscle plays a major role in this process (Lucke et al., 1979). As body temperature rises the development of the syndrome, within skeletal muscle, becomes self sustaining since the increased temperature reduces the calcium requirement for actinomyosin cross-bridge formation (Fuchs, 1975), SR calcium uptake (Britt et al., 1975) and ATP synthesis (Britt, 1979). Thus the elevation in muscle temperature represents a key event in MH and in Chapters 4 and 5 controlled variations in temperature were exploited to study two different aspects of muscle function. In Chapter 4 the functional aspects of isolated muscle membranes were characterised using $^1$H NMR spectroscopy.

In model phospholipid membranes $^1$H NMR provides simultaneous information on the surface, hydrocarbon and middle regions of the membrane bilayer. Peak height and width are related to the molecular mobility of individual proton groups and this property in turn reflects the physical state (i.e. solid or fluid) of the membrane bilayer. The $^1$H spectra of both the microsomal membranes and the sonicated lipid extracts gave similar well resolved methylene and methyl resonances, indicating that proteins do not appreciably alter lipid mobility in the muscle membrane. As there are many non-phospholipid containing fatty acid molecules (i.e. mono-, di- and triglycerides) in the membrane and lipid extracts the contribution of glycerides to the methylene and methyl resonances must also be considered. Elevations in temperature generally sharpen phospholipid proton resonances in model and biological membranes (reviewed in Chapter 4) and the small effects of temperature on the $^1$H spectra obtained in the present study are consistent with the view that non-phospholipids are contributing to the $^1$H spectrum. In the absence of specific structural and/or functional roles for non-steroidal neutral
In isolated cut-fibre preparations the controlled variation of temperature produced dramatic changes in the muscle response to a variety of agents and such effects readily differentiated normal from MH muscles (Chapter 5). There was a significant decrease in the contractile responses of MH muscle preparations to halothane, caffeine, succinylcholine and KCl in the 20 to 25°C temperature range compared to the responses obtained at 37°C. The mechanism by which lower temperatures reduce MH skeletal muscle hyperreactivity is unknown. Alterations in the physical state of the muscle membrane might explain this decrease in hyperreactivity but ¹H NMR spectroscopy failed to detect significant differences in the organisation of MH muscle membranes over a wide temperature range (Section 4.3.2) indicating either

(i) fluidity differences do not differentiate MH from normal muscle membranes or,

(ii) the ¹H NMR techniques that were employed were not sufficiently sensitive to detect organisational differences in the membrane preparations.

Recent work has shown that MH porcine skeletal muscle is partially depolarised on exposure to halothane (Gallant et al., 1979; G. Galloway, J. Bornstein, J. Sullivan and M.A. Denborough, unpublished observations) and together with a lower mechanical threshold (Anderson and Bryant, 1977) this provides a rational explanation for the hyperreactivity that characterises MH skeletal muscle (Section 3.2.2). Neither the partial depolarisation (G. Galloway, J. Bornstein, J. Sullivan and M.A. Denborough, unpublished results) nor the halothane-induced contractures (Section 5.3.2) were obtained at room temperature suggesting that the two phenomena may be
related. The effects of lower temperatures on the mechanical threshold of MH muscle have yet to be investigated.

Antagonists of calcium ion movements have been used in a variety of clinical and experimental studies on MH (Section 6.1). In particular, the muscle relaxant dantrolene sodium has been used successfully to attenuate the syndrome in vivo and to block hypercontractility in vitro (Section 6.1). The ability of dantrolene to abolish drug-induced hypercontractility in MH muscle contrasts with the hyperreactive effects of TMB-8 (Chapter 6). The calcium ion antagonist TMB-8 has been shown to block drug-induced contractures in a variety of smooth and skeletal muscle preparations (Malagodi and Chiou, 1974a, b; Chiou and Malagodi, 1975) but TMB-8 contracted at high concentrations and potentiated drug-induced contractures at low concentrations in MH porcine skeletal muscle (Chapter 6). These effects of TMB-8 are clearly inconsistent with the mode of action of a calcium ion antagonist and it seems that TMB-8-induced hyperreactivity occurs by a mechanism independent of calcium immobilisation.

Despite its potentiating effects on halothane-, caffeine- and succinylcholine-induced contractures, TMB-8 inhibited KCl-induced depolarising contractures in MH skeletal muscle. The differential effects of TMB-8 on KCl- and succinylcholine-induced contractures supports the contention that in MH skeletal muscle the succinylcholine contracture occurs by a mechanism independent of membrane depolarisation (Moulds, 1978). TMB-8 therefore represents a useful tool to differentially probe the mechanisms underlying abnormal drug-induced contractures in MH skeletal muscle.

The final chapter examines platelet function in MH and the results presented confirm that platelet abnormalities occur in some MH susceptible
individuals. Previous work had shown that abnormal platelet aggregation is found in a number of MH susceptible individuals (Isaacs, 1978; Zsigmond et al., 1978). In these studies when abnormal platelet function occurred it was always associated with decreased platelet reactivity. This contrasts with the hyperreactivity that is found in MH skeletal muscle. Furthermore, it indicates that although there are many similarities between excitation-secretion coupling in the platelet and excitation-contraction coupling in skeletal muscle (Day and Holmsen, 1971; Barnhart, 1978) there are important differences. The inability of the excitation-contraction coupling inhibitor dantrolene sodium to modify platelet aggregatory responses to collagen, adrenaline or ADP in normal and MH individuals emphasises such differences (J. Sullivan, N.G. Ardlie and M.A. Denborough, unpublished results).

In all the individuals screened in this study MH was suspected either because they were relatives of MH susceptible individuals or because they developed "complications" during anaesthesia. Therefore, although the in vitro pharmacological assessment of drug induced contractures classified these individuals as "normal", the possibility cannot be excluded that the high incidence of abnormal platelet function in these "normal" individuals reflects incomplete penetrance and variable expression of the MH trait.

These observations on abnormal platelet aggregation in a number of MH susceptible individuals support the hypothesis that the basic abnormality in MH is membrane localised and that it is expressed in a number of different cell types. The different types of aggregatory responses found in this and other studies (Isaacs, 1978; Zsigmond et al., 1978) probably reflect the heterogeneous nature of the syndrome. The occurrence of normal platelet responses in individuals unambiguously identified as being MH susceptible,
together with the occurrence of abnormal responses in normal individuals on a variety of medication (Barrer and Ellison, 1977; Verstraete, 1978), shows that platelet function tests alone cannot be used to identify susceptibility to MH.

8.3 SUMMARY

A variety of evidence favours the E-C coupling process as the primary site of the abnormality predisposing to MH. At the molecular level E-C coupling occurs within the triad, thereby implying that in MH triad structure and/or function is abnormal. The inability to detect structural or functional abnormalities in microsomal membrane fractions is consistent with such a specific localisation of the abnormality. The effects of temperature and TMB-8 on drug-induced contractures in MH skeletal muscle are also consistent with a triadic site for the primary skeletal muscle abnormality. This implies that a more suitable membrane fractionation scheme, designed to isolate an enriched triadic preparation, might lead to a more precise identification of the molecular nature of the skeletal muscle membrane abnormality predisposing to MH.
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