A PILOT STUDY FOR THE DEVELOPMENT OF A DIAGNOSTIC TEST FOR MALIGNANT HYPERTHERMIA

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ABSTRACT

The only definitive diagnostic test for Malignant Hyperthermia, a genetic disease that effects skeletal muscle, is the caffeine-contracture test. Alternative tests are being sought since this test is not totally satisfactory. It requires muscle biopsy, an invasive procedure and often produces results difficult to interpret. A test that could be used for wide spread screening of all patients about to undergo an operation would reduce the incidence of unexpected Malignant Hyperthermic episodes induced by anaesthetics, the most common cause of an episode.

In this project the effect of mild stress induction on skeletal muscle, ischaemia produced by a tourniquet is studied. The tourniquet effect on a sample of five pre-diagnosed Malignant Hyperthermia susceptible subjects is compared to the effect on a sample of twelve normal subjects. The effect was determined by the measurment of serum metabolites before and after tourniquet application.

The variables measured were creatine kinase, lactate dehydrogenase, AMP deaminase, total solids, total protein, potassium, osmolality, inorganic pyrophosphate, creatine and erythrocyte pyrophosphatase.

Between the two groups AMP-deaminase, creatine and osmolality showed no difference in response to tourniquet application. Inorganic pyrophosphate rose in the Malignant Hyperthermia group after tourniquet application but remained unaltered in the normal groups. All other

Malignant Hyperthermia variables moved in a negative direction with respect to the normal levels. That is if the normal metabolites level rose the Malignant hyperthermia metabolites stayed the same, or if the normal levels stayed the same the MH levels dropped.

A measurement of resting metabolite levels showed Creatine kinase was higher in the MH subjects compared to the normal subjects levels but creatine and pyrophosphatase were lower in the MH subjects. These differences may form the basis of a diagnostic test.

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ABBREVIATIONS

Chemicals and Enzymes

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
Ca++	calcium ion
CK	creatine kinase
CO2	carbon dioxide
CP	creatine phosphate
CUSO4	copper sulphate
DHAP	dihydroxyacetone phosphate
EDTA	ethylenediaminetetraacetic acid(disodium salt)
F-6-P	fructose 6 phosphate
F-1,6-P2	fructose 1,6 diphosphate
GAP	glyceraldehyde phosphate
GOT	glutamine oxaloacetic transaminase
GP	glycererol phosphate
GPdH	glycerol phosphate dehydrogenase
G-6-PdH	glucose 6 phosphate dehydrogenase
H+	hydrogen ion
Hg	mercury
HK	hexokinase

H2O	water
H2O2	hydrogen peroxide
H2SO4	sulphuric acid
K+	potassium ion
KCN	potassium cyanide
K3Fe(CN)6	potassium ferrocyanide
LDH	lactate dehydrogenase
Mg++	magnesium ion
MgCl2	magnesium chloride
Na+	sodium ion
NAD+	oxidised nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NaHCO3	sodium bicarbonate
NaOH	sodium hydroxide
NH3	ammonia
(NH4)SO4	ammonium sulphate
O2	oxygen
Pi	inorganic orthophosphate
PPi	inorganic pyrophosphate
Tris	tris(hydroxymethyl)amine methane
TPI	triose phosphate isomerase
TCA	trichloroacetic acid

Units

ug	microgram
mg	milligram
g	gram
kg	kilogram
nm	nanomole
mmol	millimole
mM	millimolar
mol	moles
ul	microlitre
ml	millilitre
l	litre
kU	kilounit
IU	international unit

General

MH	malignant hyperthermia
MHS	malignant hyperthermia susceptibility
PSS	porcine stress syndrome
SR	sarcoplasmic reticulum
TS	total solids

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Chapter 1

INTRODUCTION

1.1 Introduction

For those that have a genetically determined tendency to develop the condition of Malignant Hyperthermia(MH), any operation that involves general anaesthesia is a potentially fatal experience. Anaesthetics used on such a patient can trigger a series of metabolic events that leads to a rise in temperature that the body can not accommodate which results in death.

At present,there is no diagnostic test for MH susceptibility available that is suitable for general screening of all patients about to undergo an operation. Because of this, unless a patient has an obvious family history of problems with anaesthesia or has previously survived a hyperthermic episode while being operated on it is unlikely that anyone could diagnose the susceptibility.It is therefore still common for surgeons to be taken by surprise by a patients adverse reaction to anaesthesia.

What is required is an efficient and reliable diagnostic test for Malignant Hyperthermia Susceptibility (MHS) that can be given to all patients prior to surgery.

In the lower half of the North island there is a family that carries the defective gene(s) that leads to MHS. Because of the proximity of the affected family to Massey this seemed an ideal opportunity to attempt to develope a diagnostic test for MHS that would

forewarn anaethesists to prepare for an attack of MH in susceptible subjects.

1.2 HISTORY

The first published report on MH (Denborough et al, 1970) spoke of accelerated metabolism due to anaesthesia. It resulted from an encounter with a young man who experienced MH while being operated on for a fractured leg. It was subsequently discovered that ten of this patients relatives had infact died as a direct result of ether anaesthesia. As a result of this report and many subsequent reports there grew a gradual awareness of the dangers of genetic susceptibility to certain anaesthetics and stress.

Awareness of a porcine form of MH developed from a report (Herter et al, 1914) that described pork from pigs suffering from a hypermetabolic reaction as unsuitable for making sausage. In 1953 (Ludvigsen, 1953) this was linked to an inherited muscular degeneration. The condition in pigs is termed Porcine Stress Syndrome (PSS) and has proven very useful in providing more information on the human condition with respect to the pathophysiology and identification of susceptible individuals.

1.3 THE REACTION

A reaction can occur in various degrees of severity ranging from mild fever and slight respiratory and metabolic acidosis to a major reaction that will eventually lead to death.

The earliest sign of an episode is an increased respiratory rate with rising carbon dioxide tension monitored by end tidal CO2 analyzers. Respiration is deep and rapid in an attempt to clear the excess CO2. The next most consistent signs are unstable blood pressure, usually moving upwards and increased cardiac output with ventricular arrythmias.

The most characteristic sign of an episode is muscle rigidity. When muscle rigidity occurs there is an acceleration of the metabolic rate and O2 consumption leading to the high CO2 and heat production previously described.

Early in the reaction there is an increase in peripheral blood flow allowing for dissipation of heat but later peripheral vaso-constriction occurs shunting the blood away from the surface.At this stage the skin appears mottled.

With insufficient supply of O2 to the muscle tissues there is an increase in peripheral anaerobic metabolism which results in lactic acid production. The lactic acid together with CO2 produces metabolic acidosis, especially of the venous blood.

With increasing lactic acidosis the membrane becomes leaky leading to multiple electolyte abnormalities. Initially serum Ca++ levels rise but then fall as Ca++ is taken up by the muscle cells. Serum K+ levels rise as K+ leaks across damaged cell membranes as do serum phosphorous levels due to increased breakdown of ATP in the muscle.Severe swelling of the muscle can occur due to the large ion shifts and increased

permeability of the vasculature. Enzymes commonly found in muscle such as Creatine Kinase(CK), Lactic Dehydrogenase (LDH) and Glutamic oxaloacetic transaminase (GOT) also have been observed to be elevated, leaking over damaged membranes. CK tends to be highest about 24 hrs after an episode in surviving patients and has been reported to be as high as 100 000 International units(IU) in some cases, with 10 000 not uncommon. Normal levels in non-MHS people are 10->65 IU.

Myoglobin from the breakdown in muscle tissue results in myoglobinaemia and then myoglobinuria causing red or brown colouring of the urine, followed by oliguria.Unless correctly treated this results in a reduction in renal function.

The cause of death from an MH episode will vary according to the stage in the episode the death occurs . If death is one or two hours after onset of a reaction it will be due to high temperature,anoxia and arrythmia. Later death will be secondary to acute pulmonary oedema, and huge electrolyte and acid/base imbalances.

A patient surviving 2 or 3 days may succumb to renal failure or brain damage from cerebral oedema and hypoxia, leading to decerebration.

Patients who survive an acute episode with rigidity often complain of severe muscle pain for several days or weeks, the muscles being often swollen and tender. Electrolyte imbalances are common for several days after an episode. In bad cases the patients may be left with a neurological defecit evident in mental retardation or sight

loss. A few cases of a fatal recurrence of an episode several days after the initial episode have been reported so patients are carefully monitored for some time.

It is pleasing to note the mortality rate of MH has fallen in recent years. Prior to 1970 the mortality rate was over 70 percent. In 1976 the reported rate was 28 percent and probably would be even lower today.

A drug called Dantrolene has been found to be very effective in treatment of an episode and it no doubt has contributed largely to the decrease in the mortality rate. It apparently (Britt et al, 1984) increases Ca++ uptake into the sarcoplasmic reticulum (SR), preventing the dangerous situation of prolonged elevated myoplasmic Ca++ which appears to be associated with MH episodes.

1.4 GENETICS

Because of the repeated finding that the disease occurs in several members of any family and in successive generations it became obvious that MHS was a genetically inherited disease. In the 1960's two groups of workers (Britt et al, 1969 and Denborough et al, 1962) published large pedigrees of affected families which seemed to indicate MHS was inherited as an autosomal dominant trait. That is, it was not sex linked but only one gene of the pair needed to carry the defect for the condition to show. More recently however, evidence has been produced that suggests MHS inheritance is more complicated, possibly explaining the huge spectrum of severity and the variability of symptoms that

occurs in those with the disease. In some cases there is a pattern of less affected offspring than predicted by dominance patterns (called variable penetrance) and even where there is little variation within a family there are differing susceptibilities between families (called variable expressivity).

Since some people seem to suffer from a form of MHS that produces muscle rigidity during an attack and others do not it has been suggested there is a division of MH susceptibility into phenotypes.These may be inherited by more than one allele, making MHS a multifactorial genetic disorder with various degrees of susceptibilities.

1.5 CAUSE OF DISEASE

The exact defect that leads to susceptibility to MH has not been defined. The current theory is that there is a defect in the control mechanisms that maintain appropriate levels of intracellular Ca++. This has been vaguely described as due to an underlying membrane defect.

Recent papers (Nibroj-Dobosoz et al, 1984 and Do Han Kim et al, 1984 and Nelson , 1983) have looked at various specific components of the muscle membrane structure and most work has pointed to a defect in the channels that release Ca++ in the SR.

Muscle contraction, is normally mediated by Ca++. A nerve impulse will be transmitted from the nerve then down transverse tubules causing

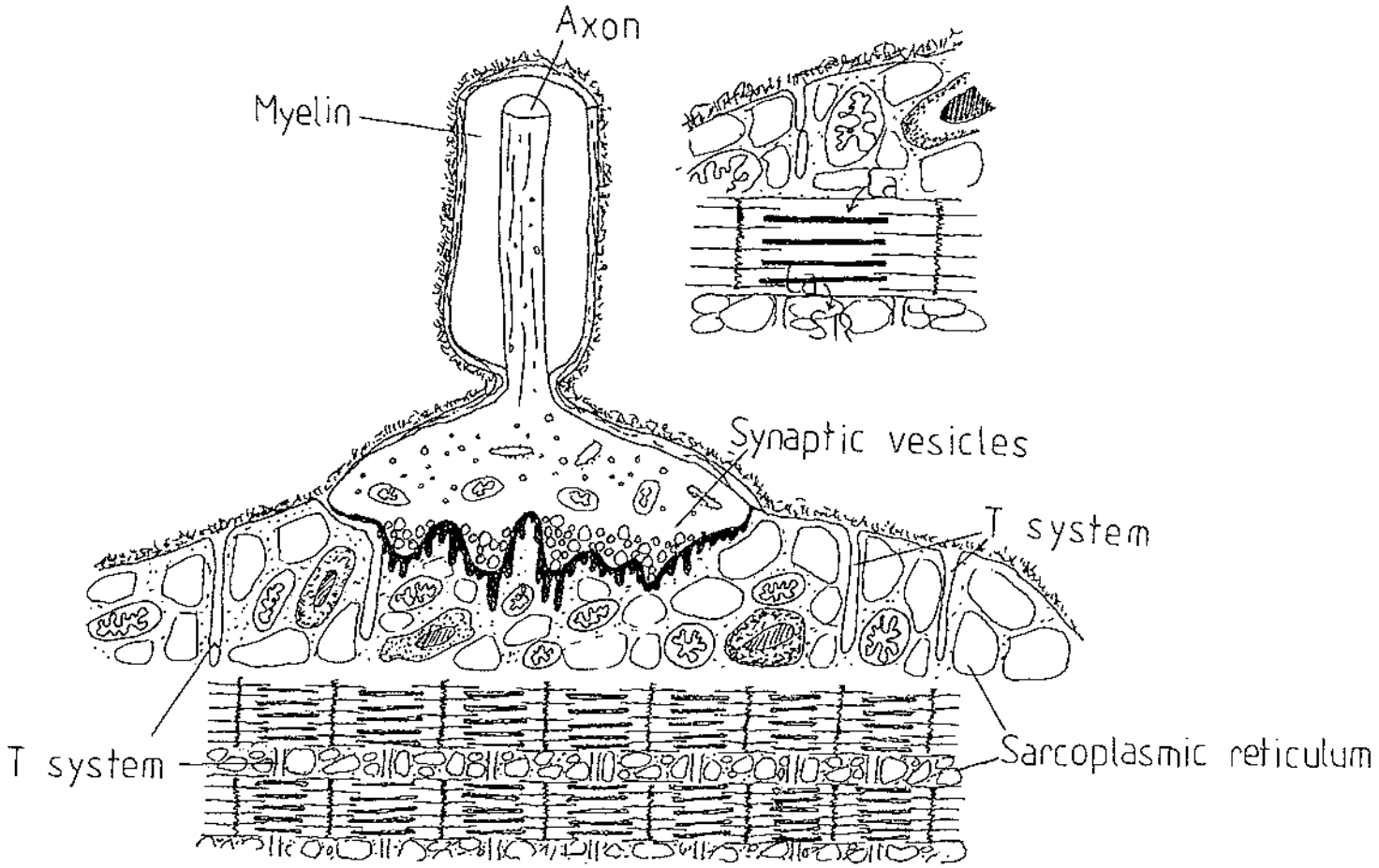

FIG 1.1 Ultrastructure of Motor end-plate on Muscle.

release of the Ca++ stored in the SR into the myoplasm. The Ca++ released acts as a trigger upon myofibrils lying beneath the transverse tubule- SR network resulting in an ATP dependent process involving various ion and protein interactions which causes contraction to occur. This is called excitation-contraction coupling.

In MH muscle there seems to be some defect in the SR structure that leads to a much lower threshold for Ca++ release which ultimately produces a situation of elevated myoplasmic Ca++.

Elevated Ca++ leads to increased ATP utilization by myosin ATPase and phosphorylase kinase. The resulting stimulation of glycolysis and Krebs cycle activity is responsible for the production of Lactic acid, CO2 and heat. Usually the SR would take up the excess Ca++ but in MH muscle this does not seem to occur so the contraction will be maintained until the membrane is damaged leading to leakage of K+, enzymes and myoglobin. Any excess Ca++ may be taken up by the mitochondria which results in the uncoupling of oxidative phosphorylation from the electron transport chain leading to decreased ATP production, accelerated oxygen consumption and output of lactic acid,carbon dioxide and heat.

Catecholamines may also play a role. They may increase heat production by indirectly stimulating several metabolic processes such as gluconeogenesis, ureogenesis, triglyceride synthesis and glycogen synthesis. Their effect of vasoconstriction which inhibits heat loss by radiation probably also induces hyperthermia. These effects of catecholamines may explain stress involvement in inducing an episode.

Succinylcholine, one of the drugs used during the process of anaesthesia induction, is often implicated as a cause of MH induction during an operation. It has been found (McCullogh et al, 1982) that succinylcholine increases the release of catecholamines, specifically noradrenalin, which further supports the belief that catecholamines are involved in the MH syndrome.

When all these events have taken place there is a rise in muscle temperature and decrease in muscle ATP and Creatine Phosphate(CP) which will perpetuate muscle rigor independently of the myoplasmic Ca++. ATP is required for muscle relaxation since it allows separation of the proteins, actin and myosin, responsible for contraction. Low ATP/ADP ratios are a metabolic stimulus leading to heat production.ATP also controls insulin binding to the cells which controls hyperkalemia so K+ control is lowered. ATP is also needed by the SR for the operation of the calcium pumps so since the ATPases are not working the ions will follow their concentration gradient where K+, Mg+, phosphate and enzymes and myoglobin leak out. Ca++ will simultaneously leak in and further disrupt the system.

1.6 TRIGGERING AGENTS

Nearly all potent inhalation anaesthetics and muscle relaxants have been implicated as triggering agents of MH episodes.

Halothane and succinylcholine are the most commonly known triggers but methoxyflurane, diethylether, cyclopropane, ethylene, decamethomium, gallamine and mepivacaine have also been implicated.

In pigs, large intravenous caffeine doses can trigger a reaction and some MH people are known to react badly to coffee. Sympathomimetrics and parasympatholytics will aggravate an already established reaction. Since many anaesthetic agents are used in combination the direct cause of an episode will often be unclear and in some cases it is believed that surgical stress will contribute to the onset of a reaction.

With swine in certain situations where anaesthetics have not been used, MH reactions have been known to occur. For example stress such as exercise, breeding, heat, anoxia apprehension or excitement can be a trigger. In humans, triggering of an episode without anaesthetics has not been proven but susceptible families definitely have a high rate of unexplained deaths. Emotional stress, prolonged exercise or excessive skeletal muscle injury, severe shivering or situations of apprehension have been suggested as non-anaesthetic triggers in humans. The exposure of muscle to excess norepinephrine is believed to be the underlying cause of the stress related reactions.

1.7 OCCURENCE

MH is best known in humans and pigs but has variously been reported to occur in cattle, greyhounds, racehorses and giraffes.

The condition is rare in humans,reported to occur about 1 in 15000 (Britt et al, 1970) although the true incidence is believed to be much higher. Those most susceptible seem to be between the ages of 3 and 30. Above that the incidence gradually declines with no cases being

reported in the over 78 age group. Episodes seem to be more common in teenage males than females but this is believed to be because of the higher admittance of male trauma cases into the operating theatres. When these cases are removed from the statistics the incidence of MH is equally common in both sexes.

About 50 percent of those experiencing an episode have previously undergone anaesthesia with no obvious reaction.The record (Britt et al, 1977 and Britt, 1977) is 12 anaesthesias involving triggering drugs with no effects before a fatal 13th.

All racial groups are effected, but reports from various areas have not been studied closely enough to determine if there are any racial or climatic differences.

1.8 DIAGNOSTIC TESTS

Serum Creatine Kinase(CK) is commonly used as a rough screening test for MHS.

CK has generally been found to be high in MH patients but the usefulness of this finding is limited as some that are known to have MH have normal CK levels. Also several diseases unrelated to MHS feature elevated CK levels.

The CK test has been evaluated in terms of efficiency, sensitivity and specificity (Anaranath et al, 1985). Sensitivity is the frequency of the true positive finding when the individual screened is known to

have MHS. Specificity is the frequency of the true negative findings when the individual screened does not have MHS. An efficient test will establish either the presence or absence of MHS in every individual screened.

The CK test has been found to be efficient; nevertheless, in one study for every positive result there was 100 false-positive results. The predictive value for the negative test is good but does not compensate for the patients who are MH but have normal CK values. A low specificity would be acceptable if the sensitivity was around 100 percent but this was not found to be the case.

In 1970 (Kalow et al, 1970 and Kalow et al, 1977) an assay was developed that screened for MHS by testing the effect of caffeine on skeletal muscle. The test requires biopsy of skeletal muscle, usually that of the quadricep muscle.The muscle is immersed in Ringer in a water bath. One end is tied by silk thread to an electrode which is also immersed in Ringer and the other end tied to a force displacement transducer that records the resulting contracture when certain stimuli are applied to the muscle. After allowing the muscle to stabilize, a tension is applied to the muscle and it is stimulated at regular intervals by electrodes connected to a generator.

The muscle is exposed to a series of concentrations of caffeine beginning at about 2mM and then doubling in sequence, to find the concentration that causes contracture tensions above and below a set value. Once the caffeine measurements are done the muscle is exposed to halothane and the various concentrations of caffeine are applied

again to study the potentiating effects of halothane. The measured parameter is the distance of the recording above the line representing the resting tension.From calibration curves the distance can be converted to grams of tension increase to a given concentration of caffeine. Using graphical means the caffeine concentration which causes an increase of 1g tension is determined.

Although this is the best diagnostic test available at present it has several features that prevent it from being ideal.

1. The biopsy procedure requires patients to be in hospital for two days. For some patients it is a traumatic experience and may leave a permanent scar on the thigh.
2. The execution of the test itself is not easy and requires carefully trained and experienced operators.Muscle tissue can easily be damaged and rendered unresponsive in the process of mounting the muscle in the water bath.
3. Interpretation of the results does not necessarily produce definitive positive or negative cases.That is, the efficiency is not 100 percent.

Because of these problems many researchers are trying to find a more appropriate test. The options explored have been diverse. Some of the main areas studied are as follows:

1. An attempt to find an unusual isoenzyme patterm of CK in MHS muscle has been sought by several groups but no consistent pattern has been uncovered. (Sigmond et al, 1977 and Hassan et al, 1977)
2. Because of the belief that MH syndrome is due to an

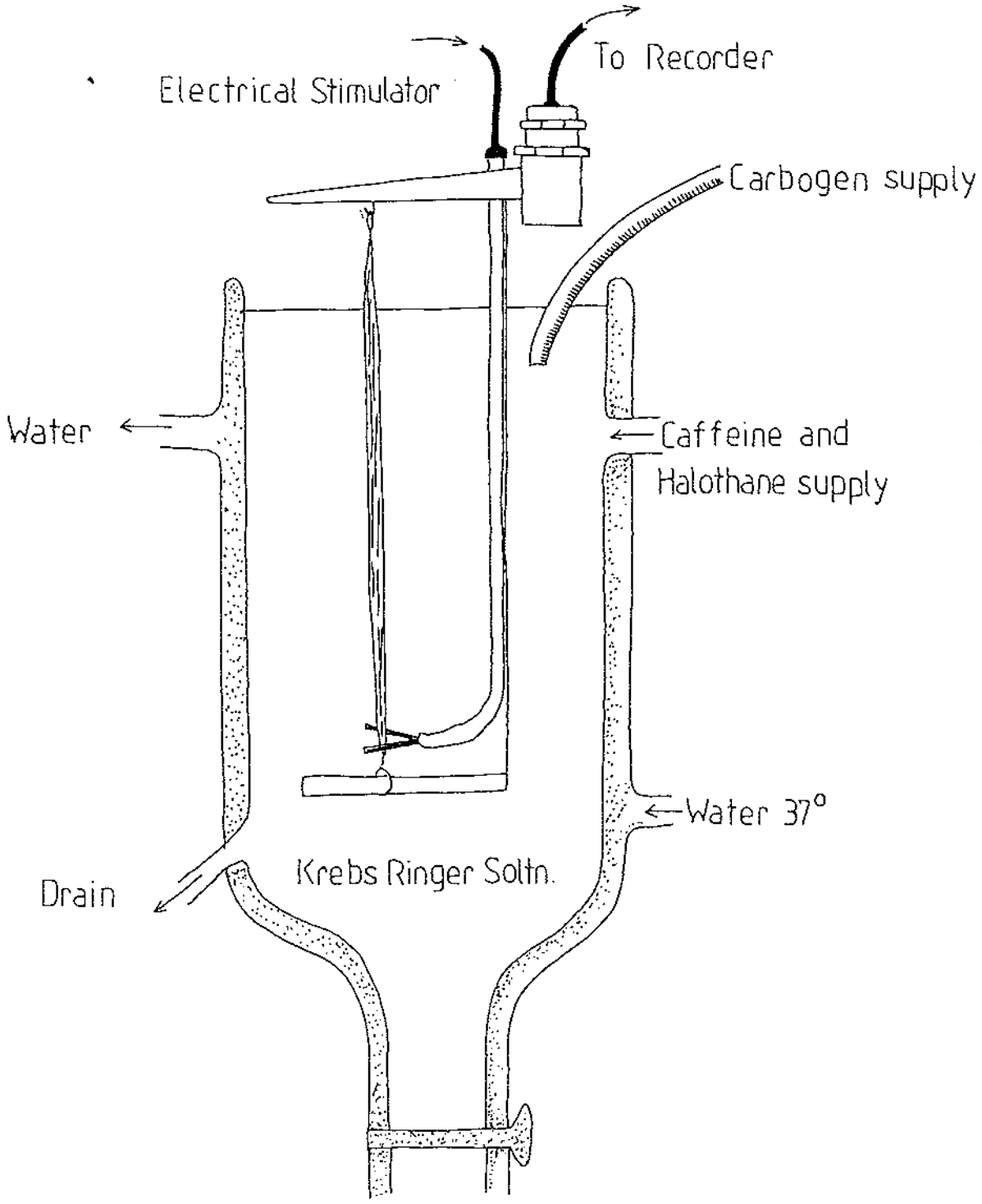

FIG 12 Caffeine Contracture Diagnostic Test.

intrinsic defect in muscle membranes, particularly of the sarcoplasmic reticulum, attempts have been made to identify unusual membrane proteins by electrophoretic techniques.
(Shirooky et al, 1983; Blanck et al, 1984; Fletcher et al, 1984; Fletcher et al, 1985; Marjanen et al, 1986 and Walsh et al, 1986)

3. Some believed HLA typing would identify MH susceptibility (Hutsky et al, 1982) however no such connection was found.

4. Several groups suggested that there was a positive correlation between pyrophosphate and creatine kinase levels. Measurement of pyrophosphate was offered as an alternative and additional screening test to CK measurements.
(Van Wormer et al, 1977; Tan et al, 1977)

5. One group found adenylate kinase to be deficient in MHS subjects.
(Schmidt et al, 1977) however later studies by another group failed to confirm this.(Marjanen et al, 1982)

6. Adenylate cyclase and cAMP of MHS skeletal muscle is reported to be abnormally high. (Willner et al, 1981)

7. Morphological studies of MHS skeletal muscle showed numerous and enlarged mitochondria, more lysozymes and myelin like bodies. (Hull et al, 1978)

8. Motor unit counting was offered as an alternative to the caffeine-contracture test. It was found to be less accurate but had the advantage of being less invasive.
(Britt et al, 1977)

9. An unusually high regional oxygen consumption was

recorded when a tourniquet was applied to an upper arm for ten minutes. (Roberts et al, 1982)

10. In several patients already diagnosed as MHS patients, myoadenylate deaminase was found to be deficient. (Fishbein et al, 1985)

11. Considerable attention has been given to the frequency of the fluoride resistant cholinesterase variants in patients with MHS. Some groups (Evans et al, 1981 and Ellis et al, 1978) have found a high frequency while other workers (Ording et al, 1981) found no such abnormality.

12. Considerable work has also been directed at abnormal erythrocyte fragility and abnormal platelet aggregation in MHS subjects, (Alerner et al, 1977). Use of a platelet nucleotide assay as a test for MHS has been given appreciable attention (Lu et al, 1985). Unusual platelet metabolism has also been studied (Solomans et al, 1977).

1.9 MUSCLE STRESS EFFECT ON NORMAL SUBJECTS

The present project was designed about the possibility that MHS muscle may be abnormally leaky when subjected to stress. Several studies (Haggmark et al, 1981 and Karlsson et al, 1981 and Larsson et al, 1978) have been performed on normal populations looking at the effect of muscle stress on muscle and blood constituents.

One study (Karlsson et al, 1981) looked at muscle ATP, creatine phosphate (CP), glycogen and lactate levels after 30 and 60 minutes of

exercise. Muscle tissue was obtained by needle biopsy. The metabolite changes seen were an increase in muscle glycogen and lactate, a decrease in CP and no change in ATP levels. The CP and ATP response was explained by an apparent lack of oxygen availability required to facilitate resynthesis of both CP and ATP. The maintenance of ATP levels was believed to be at the expense of the CP. Lactate built up in the muscle tissue but measurement of blood lactate showed no change during the exercise. It was proposed the blood lactate level did not change due to the capacity of other tissues to take up and utilise any lactate produced in the muscle cell. This is essential to prevent tissue damage during exercise.

Similar studies of the effect of muscle stress in the form of tourniquet application were performed to determine the response of electrolytes (Larsson et al, 1978) and metabolites (Haggmark et al, 1981).

In the study of electrolyte response tourniquets were applied for about two hours to the upper part of the thigh. Muscle was sampled by using the punch biopsy needle method and analysed for Na, K+, Mg++ and Cl-. The blood was analysed for osmolality, Na+ and K+. After tourniquet release there was a considerable rise in blood flow that did not return to normal until 15 minutes had elapsed. K+ levels were found to be elevated after tourniquet release and continued to be elevated for some time. No correlation was found between K+ levels and the duration of occlusion. Na+ showed a small increase in levels after tourniquet release. Osmolality also rose significantly after tourniquet release.

In the muscle, total Cl- rose after tourniquet release but K+, Mg++ and Na+ showed no significant change. It was suggested the K+ increase in the blood was involved in the increased blood flow as K+ has a vasodilator effect in skeletal muscle. Acidosis may also influence K+ levels causing an increase in extracellular levels and decrease in intracellular levels. The osmolality changes may be due to release of vasoactive agents involved in the hemodynamic response. Release of lactate, and possibly glucose may also contribute to the increase in osmolality.

In the second paper tourniquets were applied for 60 to 120 minutes and muscle biopsies taken every 15 minutes during the course of the ischemia. Muscles were analysed for lactate, ATP and CP. During the course of the ischemia ATP remained unchanged, lactate gradually rose and CP fell. After tourniquet release ATP still showed little change but lactate fell and CP rose back to pre-tourniquet levels.

These studies used prolonged periods of tourniquet application that are not possible on MHS subjects as no anaesthetic can be used. Nevertheless some of the changes observed such as the K+ rise were not dependent on duration of occlusion and therefore probably would also occur after much shorter periods of ischemia.

Since tourniquet application causes detectable changes in metabolite levels in the blood of normal, healthy tissue, changes may also be seen in MHS tissue. Since the MH syndrome is believed to involve structural abnormalaties in the muscle, particularly of cell membranes, these abnormalaties may be reflected in the response to

tourniquet application. Tourniquet application is a particularly appropriate method of stress induction on MHS muscle as it produces ischemia which results in anaerobic metabolism which is what occurs during an MH episode. Tourniquet application is therefore simulating an MH episode and may produce similar metabolite changes. Comparison of the metabolite responses of normal tissue to MHS tissue after tourniquet application may reveal differences that could form the basis of a diagnostic test. This project has been designed to investigate this possibility.