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

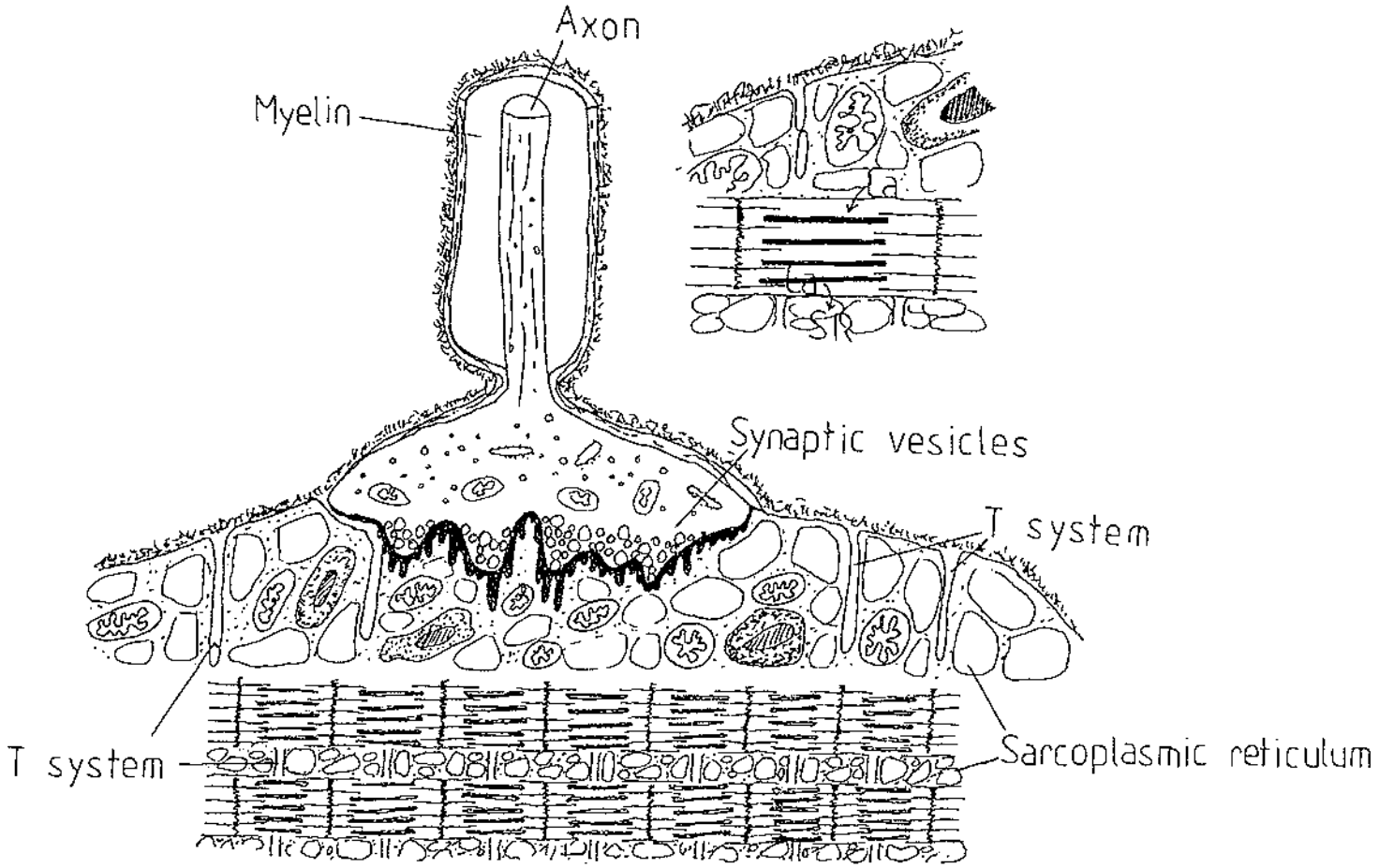


FIG 1.1 Ultrastructure of Motor end-plate on Muscle.

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

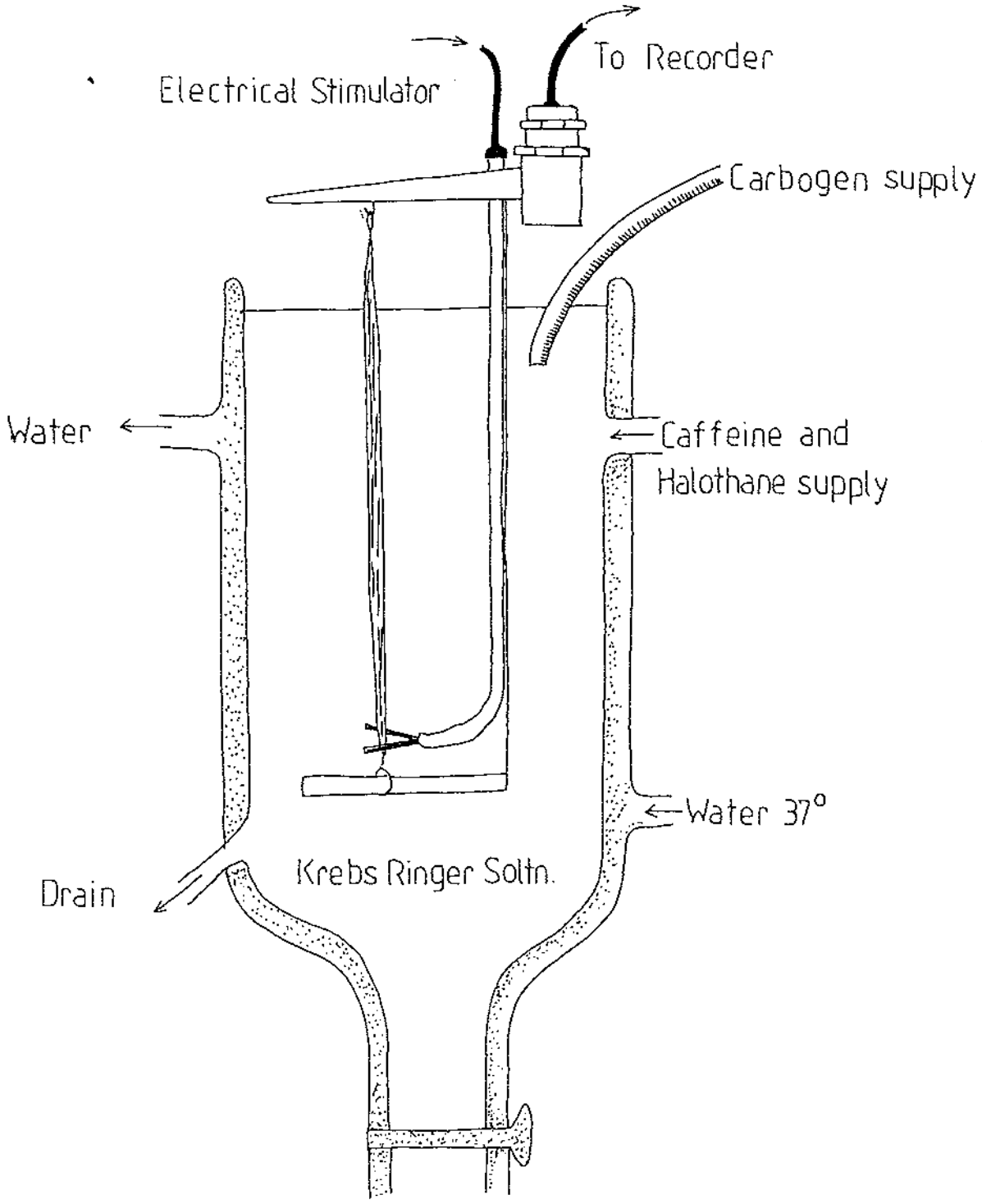


FIG 12 Caffeine Contracture Diagnostic Test.

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.

Chapter 2

METHODS AND MATERIALS

2.1 CHEMICALS

BDH: Brij, Creatine, Creatinine, Cyanomethemoglobin, Imidazole, Potassium sodium tartrate tetrahydrate, Sodium nitroprusside, Triton X-100

Boeringer Mannheim:2,4 Dichlorophenolsulphonate, TRIS

Biochemical Corporation: D-Fructose-6-phosphate

Seelze-Hannover Unilab:EDTA

Sigma: Aldolase, 4-Aminoantipyrine, Ascorbate oxidase, Creatinase, Glycerol-3-phosphate dehydrogenase, Peroxidase, Sarcosine oxidase, Sodium AMP, TES, Tetrasodium pyrophosphate

(All other chemicals were obtained from standard sources.)

METHODS

2.2 Normal samples

The following protocol was designed according to the guidelines and approved by the Massey University Ethics Committee.

A normal database was compiled from a group of 12 volunteers, 7 males and 5 females none of whom had MHS or signs of any other disease. They ranged in age from 18 to 65 with a mean of 42 years.

The process of blood sampling was as follows; Each volunteer was seated, and a surflo, 19G infusion unit inserted into a vein at the antecubitalfosa. In most cases inflation of a blood pressure cuff (SMIC mecurial sphygmomanometer) about the upper arm was necessary to

dilate the vein and make it easier to penetrate. A blood sample was taken immediately, followed by an injection of approximately 1ml of heparinized saline (5 units in 5 mls) to prevent coagulation in the cannula. In the cases where inflation of the cuff was necessary, the first blood sample was discarded and a delay of 5 minutes allowed without stasis on the arm before a sample of 5 mls was taken. This was the pre-tourniquet sample. The tourniquet was then inflated about the arm to a pressure well above that of the systolic pressure of the subject - found usually to be about 220 mmHg. The pressure was designed to completely occlude the blood vessels in the arm and was left on for 15 minutes. After 15 minutes the pressure was released and immediately another blood sample of 5mls was taken. After each sample of blood was taken heparinized saline was injected into the cannula as previously described. Before the next sample was taken a small amount of the fluid was drawn and discarded to ensure there was no dilution of the blood with heparinized saline. A sample of similar volume was taken every 5 minutes until 1 hour after tourniquet application had elapsed, and then every 10 minutes for a further hour.

Once all the samples were drawn, the cannula was removed and pressure applied to the point of needle penetration until the bleeding ceased.

The blood was put into labelled centrifuge tubes,and allowed to stand until the blood had clotted. Once clotted, the blood clot was freed from the side of the tube by an applicator stick and the tubes centrifuged to separate the red cells from the serum. The serum was aspirated from the red cells using a pasteur pipette and placed in 4ml

auto- analyzer cups. All cups were labelled and stored in the freezer at -20 degrees C until required.

2.3 Control samples

For 6 of the volunteers, the same protocol was repeated with ommission of the 15 minute tourniquet application. This provided data to check that pre-tourniquet samples were representative of resting samples in serum.

2.4 MHS samples

Five people, 3 females and 2 males, ranging in age from 22 to 49 with a mean age of 36 years, diagnosed by the Caffeine-contracture test to have MHS were subjected to the same tourniquet test to provide samples for a MHS database.

The blood sampling from the MHS subjects was slightly modified from that of the normal population. A local anaesthetic (1 percent lidocaine without adrenalin from DBL) was used before any attempt to insert the cannula was made. Also a Jelco, 18G catheter placement unit was inserted rather than the shorter cannula to ensure the tube would not move out of the vein during the process.

It is unlikely that either of these changes would have any effect on any of the substances to be assayed. Based on the normal population results it was decided to take only 8 samples: a pretourniquet sample and 7 samples at 5 minute intervals after the tourniquet was

released.This covered the time period that showed the most significant changes in the normal population. The blood samples were processed in the same way as the normal samples.

2.5 ASSAYS

The following assays were performed on all plasma samples from normals and MHS subjests.

2.5.1 ENZYMIC ASSAYS

Assays for the enzymes lactate dehydrogenase(LDH), creatine kinase(CK), and AMP deaminase were done within a week of obtaining the blood samples to ensure minimal loss of activity.

2.5.1.a Lactate dehydrogenase: The assay for LDH was based on the method recommended by the Scandinavian Society for Clinical Chemistry and Clinical Physiology. The assay uses the following reaction

$$\text{Pyruvate} + \text{NAD+} \xrightarrow{\text{LDH}} \text{NADH} + \text{Lactate}$$

The increase in absorbance due to production of NADH was measured as an indication of the activity of LDH present.

Reagents - 1) 57.5 mmol/l Tris buffer pH 7.4
(6.96g of TRIS in 1l of water).
2) 5.58 mmol/l NADH in Tris buffer prepared fresh each day
(.0218g in 5ml of TRIS buffer)
3) 14 mmol/l Pyruvate in Tris buffer
(.154g in 100ml of buffer).

Method - The assay was done on a Flexigem centrifigal analyzer. For each run 1 ml of pyruvate was added to 17 ml of Tris. .72ml of this solution was mixed with 25ul of NADH and 25ul of serum after being equilibrated at 30 degrees for 10 minutes.

The instrument then took 21 absorbance readings at 15 second intervals, at a wavelength of 340 nm . The change in abs/min was converted to International units of enzyme per litre (IU/l) of serum.

2.5.1.b Creatine Kinase: The assay for creatine kinase was also done on the Flexigem centrifigal analyzer. A Merckotest CK kit produced according to the recommendations of the German Clinical Chemistry Association was used for this assay. This assay is based on the following reactions

Phosphocreatine + ADP --> creatine + ATP

(CK)

ATP + D-glucose --> ADP + D-gluconate-6-phosphate

(HK)

D-glucose-6-phosphate + NADP+ --> D-gluconate-6-phosphate

(G-6-PdH) + NADPH + H+

The increase in absorbance due to NADPH production was measured as an indication of the ativity of CK present.

Reagents - The kit provides a buffer solution and a tablet where 10 ml of the solution was added to the tablet plus 1ml of water.

1) The buffer solution contains - 114.4 mmol/l imidazole acetate

buffer pH 6.7

23 mmol/l glucose

11.4 mmol/l Mg acetate

2) The enzyme - coenzyme substrate contains

- 20.8 mmol/l N-acetylcysteine
- 2.08 mmol/l ADP
- 2.08 mmol/l NADP
- 5.2 mmol/l AMP
- 10.4 mmol/l adenosine-5'-pentaphospho-5'-adenosine
- >=1 KU/l glucose-6-phosphate dehydrogenase
- >= 2.6 kU/l hexokinase
- 31.2 mmol/l phosphocreatine
- 2.3 mmol/l EDTA

(where concentrations are those in the ready to use solutions)

Method - 0.5ml of the reaction solution was mixed with 20ul of the sample. Immediately upon mixing the instrument took 21 readings at 340 nm every 7 seconds. The resultant change in abs/min is converted to IU/l enzyme activity.

2.5.1.c AMP deaminase: The assay was based on the method of A.J.Berry and R.C. Pederson. The production of ammonia from

AMP + H2O --> IMP + NH3

(AMP deaminase)

is measured using a colourimetric determination of ammonia as an indication of the amount of AMP deaminase present. Constant amounts of substrate were provided in the assay so the amount of NH3 produced was proportional to the AMP deaminase activity in the sample.

The reagents - 1) phenol-nitroprusside solution-

0.162M crystalline phenol (15.25g)

9.5mM sodium nitroprusside (.708g)

dissolved in 250ml of water

2) alkaline hypochlorite solution-150 mmol of NaOH

(1.5g)

7.2ml of 5.25 percent NaOCl dissolved

in 250ml of water

3)Substrate-1 mmol/l NaAMP in 1M NaOH adjusted to

pH 6.2-.7g in 2ml.

4) Na citrate buffer-

53 mmol/l from dried anhydrous citric acid

(5.5g in 500ml water.)

and 1M NaOH

5)NH3 standard-

1mmol/l dried (100 degrees for 4 hours)

(NH4)2S04 (.132g in 1l of water.)

Method - This assay was performed manually with absorbance readings measured on a Shimadzu spectrophotometer .02ml of serum was added to 1ml of buffer. This mixture was equilibrated for 10 min minutes at 37 degrees C, then .02 ml of substate was added and the mix incubated for 30 minutes at 27 degrees C. 1 ml of phenol reagent was added to stop the reaction, then 1 ml ml of hypochlorite for colour development. Finally the solutions were read at 630nm against a water blank. A sample blank in which the AMP was not added until after the reaction was stopped with phenol, was carried out for each sample. Using the absobances of the standard, blanks and samples the activity of AMP deaminase IU/l was calculated.

2.5.1.d Erythrocyte Pyrophosphatase: The erythrocyte pyrophosphatase assay was based on the method of R.A.Fisher, B.M.Turner, H.L.Dorkin and

H.Harris.

The resting levels of pyrophosphatase in the blood of MHS subjects compared to normal subjects was required, without interference from tourniquet application. The normal samples were obtained from 12 students without haemostasis while the MHS samples were obtained after all the post-tourniquet samples had been taken to minimise the effect of the tourniquet. All samples were centrifuged immediately, the serum removed and the red cells washed several times in isotonic MgCl2. The cells were lysed by adding an equal volume of water to the sample.

The samples were all analyzed 12 hours after the blood drawing. This delay was to accommodate the time taken to transport the samples in the cases where the subject concerned was in their own home rather than the laboratory. This ensured that any loss in activity would be similar in all samples.

Reagents - 1) Substrate-

.0143M tetrasodium pyrophosphate (3.8g in 100ml of water)

.0143M MgCl2 (.291g in 100ml of water)

.0714M Tris HCl pH 8.15 (.865g in 100ml of water)

2) Protein precipitant

.01M CuSO4 in 10 percent trichloroacetic acid

.25g blue CuSO4 in 500ml of TCA.

3) Colour developer

1 volume of .01M ammonium molybdate

(.392g in 200ml of water)

1 volume of 2.5M H2SO4

2 volumes of acetone

Method - 50ul of hemolysate was added to .7ml of substate and incubated at 37 degrees C for 20 minutes. The reaction was stopped by adding 2ml of the protein precipitant. The tubes were allowed to stand at 10 degrees C for 10 minutes to allow the precipitate to form before they were centrifuged. The supernatant was decanted and 2ml of colour developer added . The absorbance of each tube was read at 700nm. Sample blanks were also done in which the hemolysate was not added until after the CuSO4/TCA reagent was added. By measuring the absorbance of a standard phosphate, the amount of phosphate produced could be calculated. The number of units of enzyme was calculated on the basis that 1 unit of enzyme is the amount that converts 1umol of pyrophosphate to phosphate per minute. Since erythrocyte pyrophosphatase levels are usually normalised with respect to haemoglobin, this was assayed in each sample by a cyanomethemoglobin method as follows:

Reagents - 1) Drabkins- 2.05M K3Fe(CN)6 (.2g)

0.768 mMol KCN (.05g)

0.012M NaHCO3 (1g)

diluted to 1000ml with distilled water.

2) Cyanomethemoglobin standard-

57.2 mg/100ml(available ready made from BDH)

Method - A blank was prepared with 6ml of drabkins and 20ul of water. The standard was 6ml of the cyanomethemoglobin. The samples were 6ml of drabkins plus 20ul of lysate. Absorbances were read at 540nm and the mg protein/100ml blood lysate calculated.

Since the number of units of enzyme in the amount of protein is known the number of units in 1mg protein can be determined to convert

the pyrophosphatase activity to IU.

2.5.2 NON-ENZYMIC ASSAYS

Once these assays had been completed the others could be done with rather less urgency as the compounds to be measured were relatively stable. The assays were for total solids, total proteins, K+, osmolality, creatine,and inorganic pyrophosphate.

2.5.2.a Total solids: Total solids (TS) were measured using an American Optical Company TS meter, according to the instructions in the American Optical Company manual. If a solute is added to water the refractive index of the water will be increased in an amount directly proportional to concentration. A drop of serum was placed on the prism of the TS meter, the cover plate pressed down and the instrument pointed to a source of relatively intense light. The light beam enters parrallel to the prism, is refracted by the protein solution and then projected against the eyepiece which contains scales calibrated for both refractive index and grams of protein per 100mls. The refracted rays light up a segment of the field viewed in the eyepiece, where the field is separated by a sharp demarcation line into a light area and a dark area. The reading of the scale at the boundary line separating the two areas gives the serum protein concentration directly.

2.5.2.b Total Protein: The total protein assay is based on the methods of J.G.Rhinhold and D.Seligson.

Total protein content was measured via the following process-

Reagents - 1) Biuret diluent-.03M KI in .25 M NaOH

(5g in 1l.)

2) Stock biuret-.06M CuSO4

(15g in 70-80 ml of water.)

45g of Tartrate was dissolved in approximately 600ml of Biuret diluent then the CuSO4 solution was added. The solution was filtered to remove any insoluble material.

3) Working biuret-dilute stock biuret 5 times with biuret diluent

4) Standard Protein-Bovine serum albumin was dried in 60 degree oven for 4 hours then cooled in a vacuum dessicator .1g was dissolved in 50 ml distilled water to give 2g/dl solution.

Method - .5ml of Biuret was mixed with 20ul of serum in the Flexigem analyzer where the reaction was carried out at 37 degrees. After 3 minutes absorbance was measured at 546nm. The instrument calculated the g/dl of protein using a standard as reference.

2.5.2.c Potassium: Potassium was measured using the Technicon Autoanalyzer flame photometer following the method for potassium and sodium in file N-20b of the Technicon manual.

Reagents - 1) lithium nitrate stock solution

(69g lithium nitrate)

(98g sulphuric acid(concentrated))

dissolved in 1000ml distilled water

125ml of stock lithium nitrate was diluted to 1000ml with distilled water with .5ml of Brij-35 added.

2) stock potassium chloride-.1M potassium chloride

(7.4557g in 1l of distilled water.)

The potassium chloride was dried at 110 degrees overnight before being weighed out.

3)Standard potassium solutions over the range of 2 to 8mM were prepared by appropriate dilutions of the stock solution of KCl.

The chart reading was measured for each serum sample and read from a standard curve to give mmol/l of K+.

2.5.2.d Osmolality:

Osmolality was measured on a Advanced digimatic osmometer and carried out according to the instructions in the Advanced Instruments Osmometer manual.

The osmometer measured the freezing point of the sample since there is a linear relationship between the amount of solvent per unit solvent and the depression of freezing point.

Reagents - High and low standards were used to calibrate the instrument and were prepared as follows.

1) For 100mOs/kg 3.086g of dry sodium chloride is added to 1l of boiled distilled water.

2) For 900mOs/kg 28.596g of dry sodium chloride is added to 1l of boiled distilled water. .2ml of serum were placed in tubes which were placed in the machine. The machine then automatically determined the mOs/kg for each sample.

2.5.2.e Creatine:

The creatine assay is based on the method of M. Suzuki and M.Yoshida.

The reaction is based on the use of 3 enzymes, creatinase, sarcosine oxidase, and peroxidase to yield a quinone monoimine dye.

creatine + H20 --> sarcosine + urea

(creatinase)

sarcosine + H20 + 02 -->glycine + formaldehyde + H202

(sarcosine oxidase)

H202 + 2,4-dichlorophenolsulphonicacid +

4-aminoantipyrine-->quinonemonoimine dye

(peroxidase) +2H20+HCl

The amount of dye produced, which can be measured by reading the absorbance at 500nm, is directly proportional to the amount of creatine present.

Reagents - 1) Reagent A1-1000kU creatinase

-.2 kU ascorbate oxidase

-.035mmol of 4-aminoantipyrine

dissolved in 50ml of .1mol/l

TES buffer pH8-22.93g in 1l of water.

2) Reagent A2-100U sarcosine oxidase

.22kU peroxidase

4umol of potassium ferrocyanide-1.26g

60mmol EDTA-Na2-21.44g

41mg Triton X-100

.114mmol 2,4-dichlorophenosulphonate-

31.16g all in 100ml .1 mol/l TES buffer

3) Reagent B1-same as A1 except no creatinase

4) Reagent B2-same as A2 except no sarcosine oxidase

5) Standard required-.06g/l. (A Jaffe reaction was done to determine the purity of the available creatine. It was found to be 87.5 percent pure therefore .0681g of creatine was measured out into 1l of water to provide a standard of .06g/l.

Method - The assay was performed on the Flexigem centrifugal analyzer. then mixed with 40ul of A1. Absorbance readings were taken 80 minutes after the combination of all solutions. Control assays were run simultaneously where reagents A2 and A1 were replaced by B2 and B1 respectively. To calculate the amount of creatine the B1 and B2 results were subtracted from the A1 and A2 results. The machine calculated the g/l of creatine using the standard .

2.5.2.f Inorganic Pyrophosphate: This assay is based on the method of G.A.Cook, W.E.O'Brien, H.G.Wood, M.T.King, and R.L.Veech. The assay uses the following reactions-

F-6-P + PPi <-> F-1,6-P2 + Pi

F-1,6-P2 <-> GAP + DHAP

GAP <-> DHAP

2DHAP + 2NADH + 2H+ <-> 2NAD+ + 2alphaGP

sum: F6P + PPi + 2NADH + H+ <-> 2alphaGP + Pi + 2NAD+

The decrease in absorbance as NADH is converted to NAD+ is measured as an indication of the amount of PPi present.

Reagents - 1) Buffer-150mmol/l Imidazole buffer pH7.4

(10.21g in 1l of water)

2) Substrate-9mmol/l magnesium chloride (.036g)

7.5mmol/l fructose-6-phosphate (.046g)

all in 20 ml of buffer

3) NADH-.0036g in 20ml of buffer (made fresh each day)

4) Enzyme cocktail-2ml of 9U/ml of Aldolase
1ml of .23mg/ml Triose Phosphate Isomerase (TPI) and Glycerol phosphate dehydrogenase (GPdH).

This gives the following enzyme concentration in the final assay tube:

0.17U/ml of aldolase
0.21U/ml of GPdH
1.73U/ml of TPI

5) Pyrophosphate dependent phosphofructokinase
-10U dissolved in 20ml of buffer.

(NB the aldolase and GPdH/TPI had to be centrifuged out of the ammonium sulphate they were originally suspended in and redissolved in the buffer since ammonium- sulphate was found to inhibit the reaction.)

6) Standard-1umol/l of inorganic pyrophoshate (PPi)
-.256mg per litre.

The serum samples had to be prepared as follows. 100ul of serum was mixed with 100ul of ice cold 3.5 percent HClO4 in micro centrifuge tubes. The tubes were centrifuged for 5 minutes so the precipitate could be easily discarded.To the supernatant was added 20ul of 1M NaOH to adjust the pH of the samples to between pH 6-7. Method - The assay is performed in the Flexigem centrifugal analyzer. Serum, substrate, enzyme cocktail, NADH and PPi are all incubated for 5 minutes at 37 degrees prior to being mixed. Immediately upon mixing the resultant absorbance is read. The decrease in absorbance is converted to IU/l of activity.

2.6 Precision check The precision of each assay was checked by replicate analyses of the same sample. {NB Some protocols were altered from those in the references quoted to enable the assay to be performed on the Flexigem analyzer or to economize on expensive enzymic reagents.}

Chapter 3

RESULTS PRESENTATION AND PROCESSING

In the result tables to follow, data is presented by analyte and compared across subject groups. Comparisons involved application of the following procedures to the data.

3.1 TREATMENT OF DATA

Individual responses were normalised by expressing post-tourniquet values of each subject as a percentage of their pre-tourniquet value. The pre-tourniquet value for any subject therefore is always 100 percent and all post-tourniquet values expressed in relation to that 100 percent.

Within the set of data produced by each assay, three processes of averaging were carried out to produce;

1. The mean of each subjects post-tourniquet values,
across sample times (Xs in results table).
2. The mean response at each sample time,
across subjects (Xt in results table).
3. The overall tourniquet response, across
all subjects and sample times, (Xo in the results table).

3.2 STATISTICAL METHOD USED

All statistical analysis was performed according to the Students t test method of Gosset. The Students t test is appropriate for small

sample test where the t distribution is similar to that of the normal distribution but has a wider sread. (For a detailed description of the statistical methods used, refer to Appendix III.)

3.3 ACCURACY

A 95 percent confidence interval was calculated for all values. A confidence interval is calculated so that if the experiment is repeated the interval within which the value would probably fall 95 percent of the time is known. Precision tests for each assay were performed to provide the confidence intervals for individual values, (given as the method 95 percent CI at the head of each table) and all other confidence intervals were calculated from the standard deviation of the values used to produce the averages. (Ct for the confidence interval of the Xt values, Cs for the confidence interval of the Xs values, and Co for the confidence interval of the Xo values.) Precision test data can be viewed in appendix I.

3.4 TEST OF MEANS

A test of the equality of the means of the normal pre-tourniquet values (before normalisation) and the control values (obtained from subjects where no stasis was applied in the course of the blood drawing) was executed to ensure pre-tourniquet values were infact representative of resting values in a subject. In all assays no significant difference was found. (Data from control samples can be viewed in Appendix II.)

The equality of the means of the MHS pre-tourniquet values and normal pre-tourniquetvalues was also tested to determine if there were any significant differences in the resting levels of the substances measured between the normal and MHS subjects.

3.5 EFFECT OF TOURNIQUET

The following tests were necessary to study the effect of the tourniquet application; The overall post-tourniquet value for each sample population assay was tested for equality with the pre-tourniquet value (of 100 percent). The level of significance of difference is given as 'tourniquet effect significance' (TESo in the results table).

The value at each sample time was also tested for equality with the pre-tourniquet value to obtain an indication of the levels of the substance assayed with respect to time once the tourniquet was released (TESt in the results table). Finally, mean post-tourniquet values for each subject were tested for any significant difference compared to the pre-tourniquet value (TESs in the results table). This provided a measure of the efficiency of the tourniquet application to produce an effect, that is the fraction of the subject population who showed a difference.

3.6 GRAPHS

Graphs have been plotted of the post-tourniquet levels at each sample time. Normalised values were used for the plot but to show the effect of MHS relative to normal subjects, the pre-tourniquet MHS value

was plotted as a percentage of the normal pre-tourniquet value (before normalisation) and all the post-tourniquet normalised values of the MHS were adjusted accordingly. The same y axis scale was used for all analytes so a comparison of the tourniquet effect among analytes could be made.

3.7 CORRELATION COEFFICIENT

To test the relationship between certain compounds, correlation coefficients were calculated. Of the various combinations studied, that is, PPi versus AMP deaminase, creatine versus CK, PPi versus CK, and PPi versus pyrophosphatase only the MHS pre-tourniquet PPi and pyrophosphatase, and normal post-tourniquet creatine and CK data showed any significant correlation.

The MHS PPi/pyrophosphatase had a significant negative correlation coefficient of -.774, where .1>a>.05.

The normal post-tourniquet creatine/CK had a positive correlation coefficient of .69 where .025>a>.01. These results are given as scattergrams at the end of the graph section.

RESULTS TABLES

CREATINE KINASE

Normal subjects - absolute values in IU

method 95 percent CI=5

		SUBJECT PK	JK	AB	MM	BG	JN	DK	CG	JM	DM	PV	GN	Xt	Ct
	pre	35	32	36	55	28	21	56	34	14	29	88	46	40+10	
	0	32	84	42	57	36	26	53	31	35	34	140	45	-	
TIME	5	24	109	51	64	34	20	54	40	32	40	102	30		
AFTER	10	29	101	41	61	29	18	52	33	9	38	84	64		
TOURN	15	32	86	49	60	30	17	31	29	8	42	117	62		
RELEASE	20	33	90	33	46	29	19	45	36	12	40	122	63		
	25	29	72	32	50	33	24	52	38	4	25	116	71		
	30	24	103	33	62	28	12	57	24	13	32	82	58		

MHS subjects- absolute values in IU

method 95 percent CI=5

		SUBJECT SC	MT	EC	RT	ZM	Xt	Ct
	pre	127	324	104	80	61	100 +	100
	0	111	322	78	79	52	-	
TIME	5	92	348	89	70	44		
AFTER	10	103	317	76	78	42		
TOURN	15	101	290	89	77	62		
RE-	20	100	225	85	75	62		
LEASE	25	104	373	72	75	84		
	30	103	355	85	81	45		

RESULTS TABLES

CREATINE KINASE

Normal subjects-values in relation to pre-tourniquet values

method 95 percentCI=5

	SUBJECT RK	JK	AB	MM	BG	JN	DK	CG	JM	DM	PV	GN	Xt	Ct	TESt
pre	100	100	100	100	100	100	100	100	100	100	100	100	100		
0	91	263	117	104	129	95	95	91	254	117	159	98	134	24	>
TIME 5	69	340	142	116	121	95	96	118	238	138	116	65	138	31	-
AFTER10	83	316	114	111	104	86	93	97	66	131	95	139	120	26	-
TOURN15	91	894	136	109	107	81	91	85	62	145	133	135	172	68	-
RE- 20	94	281	92	84	104	90	80	106	87	138	139	137	119	22	-
LEASE25	83	224	89	91	118	114	93	112	32	86	132	154	111	18	-
30	69	322	92	113	100	57	102	71	96	110	93	126	113	27	-
													Xo	Co	TESo
Xs	124	377	112	104	112	88	93	97	119	124	124	122	130	50	-
Cs	10	213	20	11	10	16	6	15	26	19	22	28			
TESs	<<<<	>>>	---	---	---	---	<<	---	---	>>	>>	---			

MHS subjects - values in relation to pre-tourniquet values

method 95 percent CI=5

	SUBJECT SC	MT	EC	RT	ZM	Xt	Ct	TESt
pre	100	100	100	100	100	100		
0	87	99	75	99	85	89	7	<
TIME 5	72	107	86	88	72	85	10	<
AFTER 10	81	99	73	98	69	84	10	<
TOURN 15	80	90	86	95	102	91	6	<
RE- 20	78	69	82	94	102	85	9	<
LEASE 25	82	115	69	94	138	106	19	-
30	81	110	82	101	74	90	10	-
						Xo	Co	TESo
Xs	80	98	79	96	92	89	11	<<
Cs	4	14	6	4	23			
TESs	<<<<	--	<<<<	<<	--			

RESULTS TABLES

INORGANIC PYROPHOSPHATE

Normal subjects - absolute values in umol/l

method 95 percent CI=.1

	SUBJECT RK	PK	MK	PM	AB	MM	BG	JN	DK	CG	PV	GN	Xt	Ct
pre	2.0	2.0	1.6	2.0	1.4	2.1	2.1	2.3	2.5	2.0	2.1	1.7	2.0+.2	
0	1.9	2.0	1.9	1.7	1.3	2.1	2.3	2.2	2.5	2.1	2.2	1.5	-	
TIME 5	2.0	1.9	2.1	2.5	1.0	2.1	2.3	2.2	2.4	2.2	2.3	1.8		
AFTER10	1.8	1.9	2.1	1.3	1.4	2.2	2.2	2.4	2.4	2.2	2.4	1.4		
TOURN15	1.9	1.9	2.1	2.1	1.4	2.1	2.2	2.3	2.4	2.2	2.4	1.8		
RE- 20	1.8	1.8	2.1	2.2	1.3	2.1	2.2	2.3	2.2	2.2	2.1	1.6		
LEASE25	2.0	2.0	2.8	2.2	1.3	2.0	2.2	2.5	2.1	2.3	2.3	1.7		

MHS subjects - absolute values in umol/l

method 95 percent CI=.1

	SUBJECT SC	MT	EC	RT	ZM	Xt	Ct
pre	2.2	1.8	1.7	1.8	2.1	1.9 + .3	
0	2.1	1.8	1.9.	2.0	2.2	-	
TIME 5	2.7	2.1	1.5	1.7	2.3		
AFTER 10	2.6	2.4	1.8	1.8	2.2		
TOURN 15	2.2	1.3	1.7	1.9	2.1		
RE- 20	2.1	1.9	2.1	2.0	2.4		
LEASE 25	2.5	1.8	1.8	2.1	2.2		
30	2.6	1.7	2.1	1.8	2.2		

RESULTS TABLES

INORGANIC PYROPHOSPHATE

Normal subjects - values in relation to pre-tourniquet values

method 95 percent CI=1

	SUBJECT RK	PK	MK	PM	AB	MM	BG	JN	DK	CG	PV	GN	Xt	Ct	TESt
pre	100	100	100	100	100	100	100	100	100	100	100	100	100		
0	97	97	117	86	93	105	93	98	99	102	107	93	100	3	-
Time 5	98	94	128	128	74	107	105	96	98	106	109	105	104	6	-
after10	90	93	127	65	102	100	84	105	96	110	114	84	99	6	-
tourn15	97	91	131	105	104	103	107	102	95	109	115	107	105	4	-
re- 20	91	81	126	112	92	104	96	101	90	109	102	96	101	4	-
lease25	99	82	140	115	59	101	105	118	87	107	106	105	101	8	-
30	100	97	171	114	91	105	102	111	85	113	108	102	108	9	-
													Xo	Co	TESo
Xs	96	91	134	104	88	104	99	104	93	108	109	99	102	8	-
Cs	4	6	16	20	15	2	8	7	5	3	4	8			
TESs	<<<<	<<<<	>>>>	--	<	>>>>	--	--	<<<<	>>>>	>>>>	--			

MHS subjects - values in relation to pre-tourniquet values

method 95 percent CI=1

	SUBJECT SC	MT	EC	RT	ZM	Xt	Cs	TESs
pre	100	100	100	100	100	100		
0	96	102	111	108	106	105	4	>
TIME 5	122	120	90	96	108	107	10	-
AFTER 10	117	132	108	101	104	112	9	>
TOURN 15	101	74	97	106	99	95	9	-
RE- 20	96	107	120	113	115	108	6	>
LEASE 25	112	100	107	115	104	108	4	>>>
30	117	96	121	99	104	107	8	-
						Xo	Co	TESo
Xt	109	104	108	107	106	107	3	>>>>
Ct	10	17	10	7	5			
TESt	>	--	>	>>	>>>			

RESULTS TABLES

POTASSIUM

Normal subjects - absolute values in mmol/l

method 95 percent CI=.1

	SUBJECT RK	PK	MK	JK	MM	BG	JN	DK	CG	DM	PV	GN	Xt	Ct
pre	4.5	3.8	3.6	4.2	4.2	4.0	4.7	6.2	4.3	3.5	4.0	3.2	4.2+.5	
0	4.3	4.0	3.4	4.4	4.5	4.4	5.0	5.8	5.7	4.1	3.5	3.7	-	
Time 5	4.5	3.9	3.1	4.3	4.4	4.3	5.2	6.3	6.1	3.9	3.5	3.7		
after10	4.2	4.2	3.0	4.4	4.4	4.2	4.8	5.8	5.0	3.8	4.0	4.0		
tourn15	4.2	4.2	3.2	4.6	4.5	4.3	5.5	5.0	5.3	5.5	4.3	3.5		
re- 20	4.4	4.0	3.0	4.2	4.2	4.4	4.9	6.0	6.0	5.1	3.5	3.7		
lease25	4.2	4.3	3.1	4.0	4.3	4.2	4.9	6.2	5.8	4.2	3.6	3.9		
30	4.3	4.2	3.3	4.4	4.6	4.2	5.9	6.2	5.0	4.0	3.5	3.8		

MHS subjects - absolute values in mmol/l

method 95 percent CI=.1

	SUBJECT SC	MT	EC	RT	SM	Xt	Ct
pre	4.2	4.1	4.36	4.6	4.6	4.4 + .3	
0	4.3	4.4	4.6	4.8	4.9	-	
TIME 5	4.2	4.1	4.3	4.9	4.8		
After10	4.0	4.0	4.8	4.5	4.5		
Tourn15	3.9	4.3	4.6	4.8	5.0		
Re- 20	4.1	4.0	4.5	4.6	4.9		
lease25	4.0	4.2	4.8	4.8	4.7		
30	3.9	4.5	4.8	4.8	4.5		

RESULTS TABLES

POTASSIUM

Normal subjects - values in relation to pre-tourniquet values

method 95 percent CI=2

	SUBJECT RK	PK	MK	JK	MM	BG	JN	DK	CG	DM	PV	GN	Xt	Ct	TESt
pre	100	100	100	100	100	100	100	100	100	100	100	100	100		
0	96	117	94	105	107	110	106	94	133	117	88	116	106	5	-
Time 5	100	111	86	102	105	108	111	102	142	111	88	116	106	6	-
after10	93	109	83	105	105	105	102	94	116	109	100	125	104	14	-
tourn15	93	157	89	110	107	108	117	97	123	157	108	109	111	7	>>
re- 20	98	146	83	100	100	110	104	100	140	146	88	116	108	7	-
lease25	93	120	86	95	102	105	126	100	135	120	90	122	107	6	-
30	96	114	92	105	110	105	121	97	116	114	88	119	106	4	>
													Xo	Co	TESo
Xs	96	125	103	88	105	107	112	97	129	125	93	118	107	8	>
Cs	3	17	4	4	3	2	8	3	10	17	7	5			
TESs	<<<<	>>>	<<<<	--	>>>>	>>>>	>>>>	--	>>>>	>>	<<	>>>>			

MHS subjects - values in relation to pre-tourniquet values

method 95 percent CI=2

	SUBJECT SC	MT	EC	RT	ZM	Xt	Ct	TESt
pre	100	100	100	100	100	100		
0	102	107	100	105	107	104	2	>>
Time 5	100	104	93	107	104	101	3	--
After10	95	98	104	105	98	99	2	--
Tourn15	93	109	100	95	109	102	4	--
Re- 20	98	107	98	100	107	100	3	--
lease25	95	102	104	105	102	102	3	--
30	93	98	104	105	98	102	5	--
						Xo	Co	TESo
Xs	97	103	100	104	104	102	4	--
Cs	3	4	14	3	4			
TESs	<	--	--	>>>	>			

RESULTS TABLES

TOTAL PROTEIN

Normal subjects - absolute values in g/dl

method 95 percent CI=1

	SUBJECT RK	PK	JK	PM	AB	MM	JN	DK	CG	DM	PV	GN	Xt	Ct
pre	27	28	28	36	30	36	33	54	42	57	51	36	38+7	
0	24	25	36	42	27	40	46	45	29	54	34	38	-	
Time 5	29	31	28	33	17	32	33	37	21	50	58	24		
After 10	22	21	23	27	18	32	33	38	35	50	42	21		
Tourn 15	23	21	19	31	20	27	32	37	36	47	51	21		
Re- 20	21	28	27	29	22	31	39	41	17	36	54	25		
lease 25	21	29	20	46	17	30	34	37	20	43	38	21		
30	19	18	24	30	17	24	21	35	19	33	45	13		

MHS subjects - absolute value in g/dl

method 95 percent CI=1

	SUBJECT SC	MT	EC	RT	ZM	Xt	Ct
pre	23	20	28	20	59	30 +	20
0	20	19	25	19	40	-	
Time 5	19	20	25	19	58		
After 10	23	20	24	19	52		
Tourn 15	19	20	25	19	64		
Re- 20	10	20	23	10	44		
lease 25	23	20	18	18	52		
30	21	20	22	19	--		

RESULTS TABLES

TOTAL PROTEIN

Normal subjects - values in relation to pre-tourniquet values

method 95 percent CI=3	SUBJECT													
	RK	PK	JK	PM	AB	MM	JN	DK	CG	DM	PV	GN	Xt	CtTESt
pre	100	100	100	100	100	100	100	100	100	100	100	100	100	
0	87	91	126	116	88	113	139	83	69	95	67	107	98	9 ---
Time 5	106	110	100	92	55	88	98	69	50	88	115	67	87	8 <<
After10	80	76	80	75	60	91	98	71	82	87	82	59	78	4 <<<<
Tourn15	85	76	66	85	65	74	97	68	85	82	101	59	79	5 <<<<
Re- 20	76	102	96	82	73	86	116	75	39	62	105	70	82	8 <<<<
lease25	78	103	69	127	56	78	102	69	48	75	75	59	78	9 <<<<
30	69	66	86	85	58	66	62	65	46	58	88	36	65	6 <<<<
													Xo	CoTESo
Xs	83	89	89	93	79	85	102	71	60	78	90	65	82	8 <<<<
Cs	11	15	19	18	11	14	21	5	17	13	16	20		
TESs	<<<<	--	--	--	<<<<	<<	--	<<<<	<<<<	<<<<	--	<<<<		

MHS subjects - values in relation to pre-tourniquet values

method 95 percent CI=3	SUBJECT							
	SC	MT	EC	RT	ZM	Xt	Ct	TESt
pre	100	100	100	100	100	100		
0	89	98	90	97	69	87	10	<
Time 5	83	100	87	94	99	93	5	<
After 10	100	101	83	94	89	93	5	<
Tourn 15	83	101	88	97	110	96	7	-
Re- 20	43	101	80	95	76	79	15	<
lease 25	103	99	64	93	89	90	10	-
30	93	101	76	98	--	92	8	-
						Xo	Co	TESo
Xs	85	100	81	95	88	89	10	<<<<
Cs	18	1	8	2	16			
TESs	<	-	<<<<	<<<<	--			

RESULTS TABLES

LACTATE DEHYDROGENASE

Normal subjects - absolute values in IU

method 95 percent CI=6

	SUBJECT RK	PK	JK	PM	AB	MM	BG	JN	DK	CG	DM	PV	Xt	Ct
pre	272	331	242	1070	109	198	125	116	153	262	233	179	300+	200
0	986	158	498	685	133	996	686	163	184	217	141	133	-	
Time 5	776	166	528	629	155	223	250	167	180	201	062	134		
After10	596	202	453	620	124	347	283	130	192	201	234	196		
Tourn15	1067	222	1045	611	155	856	375	139	154	223	238	156		
Re- 20	464	155	655	207	147	361	266	149	158	229	194	175		
lease25	579	175	659	954	123	486	178	162	160	231	261	189		
30	891	142	1196	653	140	982	168	98	138	275	205	167		

MHS subjects - absolute values in IU

method 95 percent CI=6

	SUBJECT SC	MT	EC	RT	ZM	Xt	Ct
pre	158	234	336	232	201	230 +	80
0	243	233	239	296	283	-	
Time 5	267	242	252	231	255		
After10	197	274	244	222	280		
Tourn15	251	253	241	253	205		
Re- 20	205	251	247	324	241		
lease25	163	225	215	252	286		
30	171	229	240	266	250		

RESULTS TABLES

LACTATE DEHYDROGENASE

Normal subjects - values in relation to pre-tourniquet values

method 95 percent CI=3	SUBJECT													
	RK	PK	JK	PM	AB	MM	BG	JN	DK	CG	DM	PV	Xt	CtTESt
pre	100	100	100	100	100	100	100	100	100	100	100	100	100	
0	363	48	206	487	122	503	549	141	120	83	61	74	230	19 >>
Time 5	285	49	218	311	142	113	200	111	118	77	70	75	147	9 >
After10	219	61	187	286	114	176	226	112	125	77	100	109	149	7 >>
Tourn15	392	67	432	282	142	462	300	120	101	85	120	87	213	14>>>
Re- 20	171	47	217	278	135	182	213	128	103	87	83	98	150	8 >>
lease25	213	53	272	94	118	247	142	140	105	89	112	106	141	7 >>
30	328	43	492	434	128	496	134	84	90	105	88	93	210	17 >>
													Xo	CoTESo
Xs	282	53	297	310	129	307	252	119	109	86	91	92	177	65>>>
Cs	77	8	109	117	10	152	131	18	12	9	20	13		
TESs	>>>>	<<<<	>>>>	>>>>	>>>>	>>>	>>	>>	--	<<<<	--	--		

MHS subjects - values in relation to pre-tourniquet values

method 95 percent CI=3	SUBJECT							
	SC	MT	EC	RT	ZM	Xt	Ct	TESt
pre	100	100	100	100	100	100		
0	154	100	71	128	141	119	23	-
Time 5	169	103	75	100	127	115	24	-
After 10	125	117	72	96	139	110	18	-
Tourn 15	159	108	72	109	102	110	21	-
Re- 20	130	107	74	140	120	114	18	-
lease 25	103	96	64	109	142	103	19	-
30	108	98	71	115	124	103	14	-
						Xo	Co	TESo
Xs	135	104	71	114	128	110	31	-
Cs	23	7	3	14	13			
TESs	>>>	--	<<<<	>	>>>>			

RESULTS TABLES

PYROPHOSPHATASE

Normal subjects

method	95 percent CI=20
1	370
2	400
3	320
4	420
5	440
6	420
7	350
8	420
9	400
10	430

$Xn = 400 \pm 30$

MHS subjects

method	95 percent CI=20
1	140
2	280
3	320
4	360
5	290

$Xmh = 300 \pm 100$

MHS <<<< Normal

RESULTS TABLES

OSMOLALITY

Normal subjects - absolute values in mOsmol/kg

method 95 percent CI=5	SUBJECT												
	RK	PK	MK	PM	AB	MM	JN	DK	CG	DM	PV	GN	Xt Ct
pre	352	234	256	251	215	322	294	307	256	297	179	263	270+30
0	319	254	293	281	235	362	232	329	264	242	271	216	-
Time 5	328	316	283	237	262	271	226	294	256	250	188	231	
After 10	256	364	304	289	204	328	228	339	207	280	208	228	
Tourn 15	248	236	218	290	234	305	235	309	259	204	236	219	
Re- 20	258	258	286	297	289	400	207	318	243	184	210	190	
lease 25	264	293	299	176	271	367	154	319	179	237	214	208	
30	310	278	277	290	187	302	194	299	239	285	182	217	

MHS subjects - absolute values in mOsmol/kg

method 95 percent CI=5	SUBJECT					
	SC	MT	EC	RT	ZM	Xt Ct
pre	247	271	296	285	239	270+30
0	244	256	295	290	235	-
Time 5	279	257	255	283	239	
After10	261	263	270	284	243	
Tourn15	256	237	273	283	267	
Re- 20	274	260	267	287	260	
lease25	271	253	260	286	255	
30	249	250	256	280	260	

RESULTS TABLES

OSMOLALITY

Normal subjects - values in relation to pre-tourniquet values

method 95 percent CI=3	SUBJECT													
	RK	PK	MK	PM	AB	MM	JN	DK	CG	DM	PV	GN	Xt	CtTESt
pre	100	100	100	100	100	100	100	100	100	100	100	100	100	
0	91	109	114	112	109	112	79	107	103	81	151	82	104	8 -
Time 5	93	135	111	94	122	84	77	96	100	84	105	88	99	7 -
After10	73	156	119	115	95	102	78	110	81	94	116	87	102	9 -
Tourn15	70	101	85	116	109	95	80	101	101	69	132	83	95	7 -
Re- 20	73	110	112	118	134	124	70	104	95	62	117	72	94	14 -
lease25	75	125	117	70	126	114	52	104	70	80	120	79	94	10 -
30	88	119	108	116	87	94	66	97	93	96	102	83	96	6 -
													Xo	CoTESo
Xs	80	122	109	106	112	104	72	103	92	81	120	82	99	11 -
Cs	9	17	11	16	16	13	9	5	11	11	15	5		
TESs	<<<<	>>>	>	--	--	--	<<<<	>	--	<<<<	>>>	<<<<		

MHS subjects - values in relation to pre-tourniquet values

method 95 percent CI=3	SUBJECT							
	SC	MT	EC	RT	ZM	Xt	Ct	TESt
pre	100	100	100	100	100	100		
0	99	94	100	102	98	99	4	-
Time 5	113	95	86	99	100	99	12	-
After10	106	97	91	100	102	99	7	-
Tourn15	104	87	92	99	112	9	12	-
Re- 20	111	96	90	101	109	101	11	-
lease25	110	93	88	100	107	100	11	-
30	101	92	86	98	109	97	11	-
						Xo	Co	TESo
Xs	106	93	90	100	105	99	9	-
Cs	5	3	4	1	5			
TESs	>>	<<<	<<<	--	>>			

RESULTS TABLES

TOTAL SOLIDS

Normal subjects - absolute values in g/dl

method 95 percent CI=.1

	SUBJECT RK	PK	JK	PM	AB	MM	BG	JN	CG	DM	PV	GN	XtCt
pre	8.4	7.5	6.6	10.2	6.2	10.0	9.4	3.5	7.5	5.6	9.0	8.6	8+1
0	9.0	8.0	6.5	9.4	6.5	8.6	9.2	5.4	6.2	3.0	5.5	9.8	-
Time 5	9.8	6.0	8.0	9.2	8.5	9.0	9.0	4.5	4.4	2.8	8.6	9.0	
After10	9.0	6.0	6.8	9.2	6.4	9.2	8.6	3.5	8.0	4.4	4.0	9.0	
Tourn15	9.6	6.7	9.0	9.2	8.5	9.0	9.0	5.0	7.4	3.8	4.2	8.8	
Re- 20	9.2	6.5	8.6	9.2	6.0	9.2	9.2	4.6	5.0	5.0	8.8	8.8	
lease25	9.0	8.0	7.3	9.2	5.5	9.2	7.4	5.2	7.8	4.0	8.2	8.0	
30	9.0	7.5	8.6	8.6	5.4	8.6	10.2	4.5	8.0	4.2	9.0	8.8	

MHS subjects - absolute values in g/dl

method 95 percent CI=.1

	SUBJECT SC	MT	EC	RT	ZM	Xt	Ct
pre	7.8	8.0	7.2	8.0	7.8	7.7	+0.4
0	6.4	7.8	7.0	7.6	7.0	-	
Time 5	7.0	7.4	6.2	7.5	8.0		
After10	7.2	7.2	6.8	7.4	7.0		
Tourn15	7.0	4.8	7.0	7.5	7.6		
Re- 20	7.2	7.6	6.8	7.4	6.8		
lease25	7.2	7.7	6.8	7.2	6.6		
30	7.2	7.9	7.0	7.4	6.6		

RESULTS TABLES

TOTAL SOLIDS

Normal subjects - values in relation to pre-tourniquet values

method 95 percent CI=1

	SUBJECT RK	PK	JK	PM	AB	MM	BG	JN	CG	DM	PV	GN	Xt	Ct	TESt
pre	100	100	100	100	100	100	100	100	100	100	100	100	100		
0	107	107	98	92	105	86	98	154	83	54	61	114	97	10	-
Time 5	117	80	121	90	137	90	96	129	59	50	96	105	98	10	-
After10	107	80	103	90	103	92	91	100	107	79	44	105	92	7	-
Tourn15	114	89	136	90	137	90	96	143	99	68	47	102	101	11	-
Re- 20	110	87	130	90	97	92	98	131	67	89	98	102	97	6	-
lease25	107	107	111	90	89	92	79	149	104	71	91	93	99	8	-
30	107	100	130	84	87	86	109	129	107	75	100	102	101	7	-
													Xo	Co	TESo
Xs	110	93	118	89	108	90	95	134	89	69	77	103	98	11	-
Cs	4	11	14	2	19	2	8	17	18	13	23	6			
TESs	>>>>	--	>>>	<<<<	--	<<<<	--	>>>>	--	<<<<	<	--			

MHS subjects - values in relation to pre-tourniquet values

method 95 percent CI=1

	SUBJECT SC	MT	EC	RT	ZM	Xt	Ct	TESt
pre	100	100	100	100	100	100		
0	82	98	97	95	90	92	5	<<<
Time 5	90	93	86	94	103	93	4	<<
After10	92	90	94	93	90	92	1	<<<<
Tourn15	90	60	97	94	97	88	11	<
Re- 20	92	94	94	93	87	92	2	<<<<
lease25	92	96	94	90	85	91	3	<<<<
30	92	99	97	93	85	93	3	<<<
						Xo	Co	TESo
Xs	93	94	90	91	90	92	3	<<<<
Cs	2	4	3	6	13			
TESs	<<<<	<<<<	<<<<	<<<	<			

RESULTS TABLES

AMP-DEAMINASE

Normal subjects - absolute values in IU

method 95 percent CI=1

	SUBJECT RK	PK	MK	PM	AB	MM	BG	JN	CG	DM	PV	GN	XtCt
pre	10	6	8	2	15	2	5	11	2	16	5	2	7+3
0	4	11	5	7	10	1	6	4	4	10	4	4	-
Time 5	4	2	2	6	6	1	6	6	2	8	3	2	
After10	4	19	2	4	3	1	4	5	3	10	3	2	
Tourn15	2	11	1	4	1	1	4	5	2	5	4	4	
Re- 20	2	2	2	3	0	1	11	10	3	8	1	7	
lease25	2	23	5	1	13	1	5	14	2	14	9	4	
30	2	13	2	1	5	3	6	11	2	8	6	2	

MHS subjects - absolute values in IU

method 95 percent CI=1

	SUBJECT SC	MT	EC	RT	ZM	Xt	Ct
pre	1	15	4	11	8	8 + 7	
0	3	23	0	7	1	-	
Time 5	2	22	1	1	17		
After10	3	26	12	3	16		
Tourn15	6	22	12	5	11		
Re- 20	3	15	4	5	12		
lease25	7	13	12	2	0		
30	7	13	13	0	2		

RESULTS TABLES

AMP DEAMINASE

Normal subjects - values in relation to pre-tourniquet values

method 95 percent CI=10

	SUBJECT RK	PK	MK	PM	AB	MM	BG	JN	CG	DM	PV	GN	Xt	Ct	TESt
pre	100	100	100	100	100	100	100	100	100	100	100	100	100		
0	40	170	70	340	60	60	120	30	220	60	80	230	120	40	-
Time 5	40	30	30	300	450	70	140	60	110	50	60	100	90	30	-
After10	40	300	30	200	20	60	90	40	160	60	60	110	100	30	-
Tourn15	20	170	20	200	10	60	20	60	90	30	70	210	80	30	-
Re- 20	60	30	30	160	0	10	80	90	170	50	20	370	90	40	-
lease25	20	40	60	60	80	70	230	120	100	90	190	210	100	30	-
30	20	20	30	50	30	160	110	100	100	50	130	110	80	20	<
													Xo	Co	TESo
Xs	40	100	40	200	40	70	120	70	140	60	90	190	95	35	-
Cs	20	100	20	100	30	40	60	30	50	20	50	90			
TESs	<<<<	--	<<<<	>	<<<<	--	<<<<	<	>	<<<<	--	>>			

MHS subject - values in relation to pre-tourniquet values

method 95 percent CI=10

	SUBJECT SC	MT	EC	RT	ZM	Xt	Ct	TESt
pre	100	100	100	100	100	100		
0	300	150	0	60	10	160	88	-
Time 5	170	140	230	10	220	160	60	-
After10	300	170	270	30	210	200	70	-
Tourn15	570	140	280	40	150	200	100	-
Re- 20	300	100	100	40	160	140	70	-
lease25	720	80	270	20	0	200	200	-
30	650	80	290	0	160	200	200	-
						Xo	Co	TESo
Xs	400	120	200	30	150	195	200	-
Cs	200	32	100	20	80			
TESs	>>>	--	--	<<<<	--			

RESULTS TABLES

CREATINE

Normal subjects - absolute values in g/dl

method 95 percent CI = .003

	SUBJECT RK	PK	MK	PM	AB	BG	JN	DK	CG	DM	PV	GN	Xt	Ct
pre	.052	.056	.077	.099	.050	.095	.049	.049	.051	.075	.087	.085	.069	+.007
0	.047	.014	.031	.072	.094	.091	.029	.084	.061	.046	.084	.088		-
Time 5	.050	.050	.041	.087	.075	.067	.038	.094	.059	.049	.099	.068		
after10	.037	.068	.078	.094	.065	.083	.048	.060	.060	.062	.075	.068		
tourn15	.039	.065	.075	.089	.064	.017	.044	.077	.060	.073	.067	.070		
re- 20	.040	.068	.059	.129	.063	.052	.036	.062	.052	.080	.099	.095		
lease25	.036	.060	.059	.082	.053	.032	.057	.055	.040	.061	.073	.096		
30	.036	.049	.052	.076	.069	.044	.040	.052	.110	.071	.064	.059		

MHS subjects - absolute values in g/dl

method 95 percent CI = .003

	SUBJECT SC	MT	EC	RT	ZM	Xt	Ct
pre	.078	.045	.039	.017	.043	.044	+.03
0	.010	.034	.075	.009	.019		-
Time 5	.050	.014	.055	.002	.052		
After10	.091	.034	.087	.022	.027		
Tourn15	.103	.032	.059	.021	.001		
Re- 20	.097	.060	.047	.004	.021		
lease25	.092	.030	.072	.032	.016		
30	.087	.019	.042	.002	.004		

RESULTS TABLES

CREATINE

Normal subjects - values in relation to pre-tourniquet values

method 95 percent CI = 5

	SUBJECT RK	PK	MK	PM	AB	BG	JN	DK	CG	DM	PV	GN	XtCtTESt	
pre	100	100	100	100	100	100	100	100	100	100	100	100	100	
0	90	25	40	73	188	96	59	171	120	61	97	104	94	27 -
Time 5	96	89	53	88	150	71	78	192	116	65	114	80	99	22 -
After 10	71	121	101	95	130	87	98	122	118	83	86	80	99	11 -
Tourn 15	75	116	97	90	128	18	90	157	118	97	77	82	95	19 -
Re- 20	77	121	77	130	126	55	73	127	102	107	114	112	102	14 -
lease 25	69	107	78	83	106	34	116	112	78	81	84	113	88	13 <
30	69	88	68	77	138	46	82	106	216	95	74	69	94	25 -
													XoCoTESo	
Xs	78	95	73	91	138	58	85	141	124	84	92	91	96	5 -
Cs	10	31	20	17	24	26	17	30	40	16	16	17		
TESs	<<<<	--	<<<	--	>>>>	<<<<	<	>>>	--	<<	--	--		

MHS subjects - values in relation to pre-tourniquet values

method 95 percent CI = 5

	SUBJECT SC	MT	EC	RT	ZM	Xt	Ct	TESt
pre	100	100	100	100	100	100		
0	13	76	192	53	44	76	86	-
Time 5	64	31	141	12	121	74	78	-
After 10	117	76	223	130	63	122	78	-
Tourn 15	132	71	151	124	2	96	74	-
Re- 20	124	133	121	24	49	90	70	-
lease 25	118	67	185	188	37	119	83	-
30	112	42	108	129	57	50	62	<
						Xo	Co	TESo
Xt	97	71	160	76	46	90	53	-
Ct	40	30	38	64	36			
TESt	--	<<	>>>>	--	<<<			

RESULTS TABLES

SUMMARY TABLE

	RESTING NORM CF RESTING MH	NORMAL POST CF PRE	TIMESn	PEOPLEn	MH POST CF PRE	TIMESmh	PEOPLEmh
CREATINE KINASE	norm<<<<MH	NSD	immed post greater pre	2/12post higher pre 3/12post less than pre 7/12 NSD	post<<pre	immed,5,10,15,20 post less than pre	3/5post less than pre 2/5 NSD
INORGANIC PYRO-PHOSPHATE	NSD	NSD	-	4/12post higher pre 4/12post less pre 4/12 NSD	post>>>>pre	immed,10,20,25 post higher pre	4/5post higher pre 1/5 NSD
POTASSIUM	NSD	post>pre	15,25post greater pre	3/12post less pre 7/12post higher pre 2/12NSD	NSD	immed post higher pre	2/5post higher pre 1/5post less pre 2/5NSD
TOTAL PROTEIN	NSD	post<<<<pre	5,10,15,20,25 30post greater than pre	7/12post higher pre 5/12 NSD	post<<<<pre	immed,5,10,20	3/5post higher pre 2/5 NSD
LACTATE DEHY-DROGENASE	NSD	post>>>pre	all post times greater than pre	7/12post higher pre 2/12post less pre 3/12 NSD	NSD	-	3/5post higher pre 1/5post less pre 1/5 NSD
OSMOLALITY	NSD	NSD	-	4/12post higher pre 4/12post less pre 4/12 NSD	NSD	-	2/5post higher pre 2/5post less pre 1/5 NSD
TOTAL SOLIDS	NSD	NSD	-	3/12post higher pre 4/12post less pre 5/12 NSD	post<<<<pre	all post less pre	5/5 post less pre
AMP DEAMINASE	NSD	NSD	30post greater then pre	6/12post higher pre 3/12post less pre 3/12 NSD	NSD	-	1/5post higher pre 1/5post less pre 3/5 NSD
CREATINE	MH<<<NORM	NSD	25post less pre	5/12post less pre 2/12post higher pre 2/12NSD	NSD	30post less pre	2/5post less pre 1/5post higher pre 2/5NSD
PYROPHOSPHATASE	MH<<<<norm						

SUMMARY TABLE DESCRIPTION

Columns:

1).Resting norm cf resting mh - statistical comparison of the MHS subjects resting levels to that of the normal subjects resting levels, where

i. NSD means not significantly different

ii. <<<< or >>>> means less or greater with significance a<.005

iii.<<< or >>> means less or greater with significance .01>a>.005

iv. << or >> means less or greater with significance .025>a>.01

v. < or > means less or greater with significance .05>a>.025

(a is the probability that the value will be greater or less than the tabulated t value)

2).Normal post cf pre - statistical comparison of the overall post-tourniquet value to the pre-tourniquet value for the normal subjects, where for example 3/12 post less pre means three of the twelve subjects post-tourniquet values were significantly lower than the pre-tourniquet values.

3).TIMESn - The sample times that the post-tourniquet values are different from the pre-tourniquet values for the normal subjects where,

i. immed is sample taken immediately after tourniquet release

ii. 5 is the sample taken 5 minutes after tourniquet release

iii.10 is the sample taken 10 minutes after tourniquet release

iv. 15 is the sample taken 15 minutes after tourniquet release

(and so on.)

4).PEOPLEn - The number of people out of the total sample that show

different post-tourniquet values from the pre-tourniquet values, for the normal subjects.

5).MH POST CF PRE - as for normal subjects but applying to MHS subjects.

6).TIMESmh - as for normal subjects but applying to the MHS subjects.

7).PEOPLEmh - as for normal subjects but applying to the MHS subjects.

FIG 31

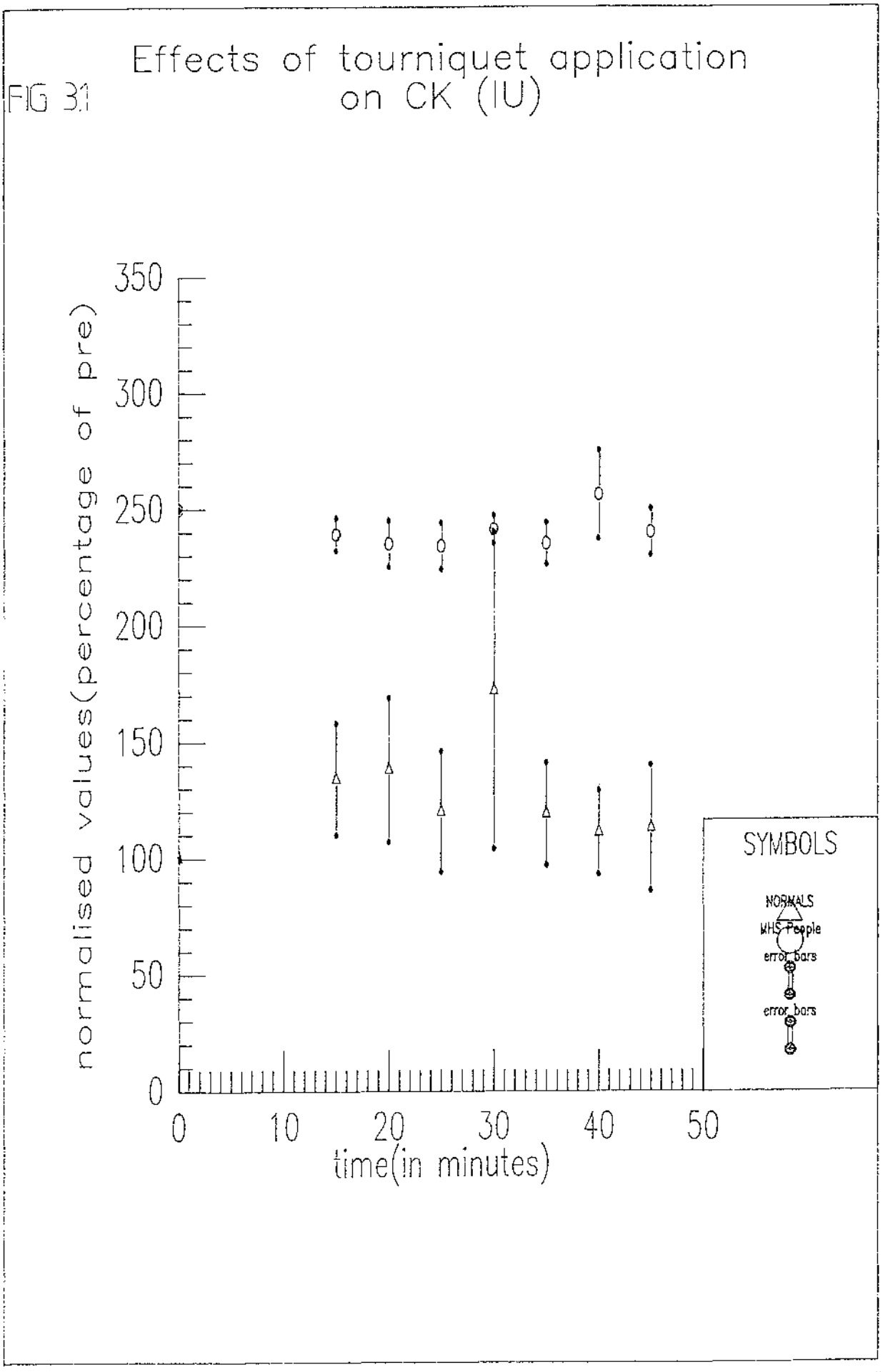

Effects of tourniquet application on CK (IU)
normalised values(percentage of pre)
350
300
250
200
150
100
50
0
0
10
20
30
40
50
time(in minutes)
SYMBOLS
NORMALS
WHS People
error bars
error bars

FIG 32 Effects of tourniquet application on inorganic pyrophosphate(umol/l)

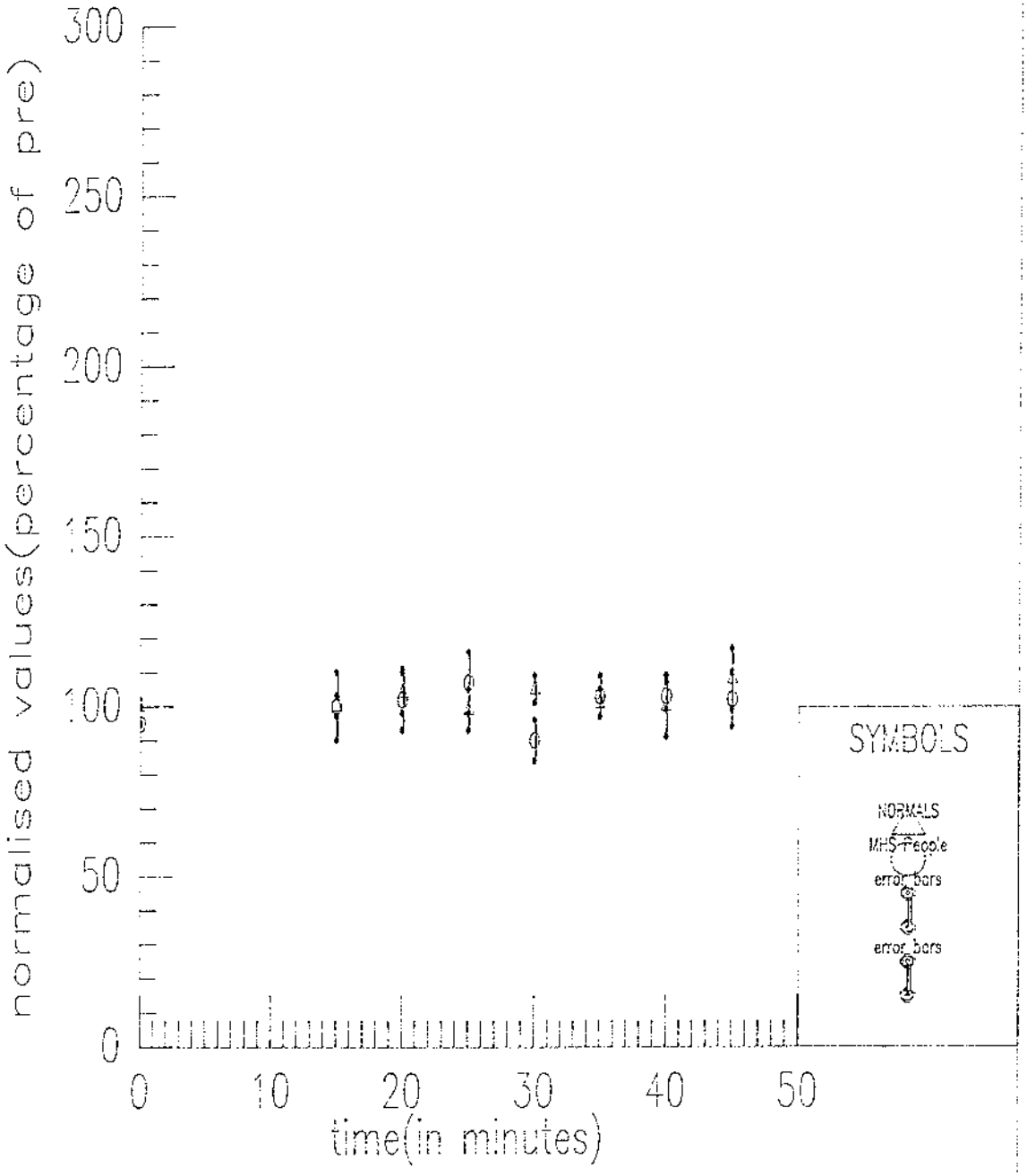


FIG 33

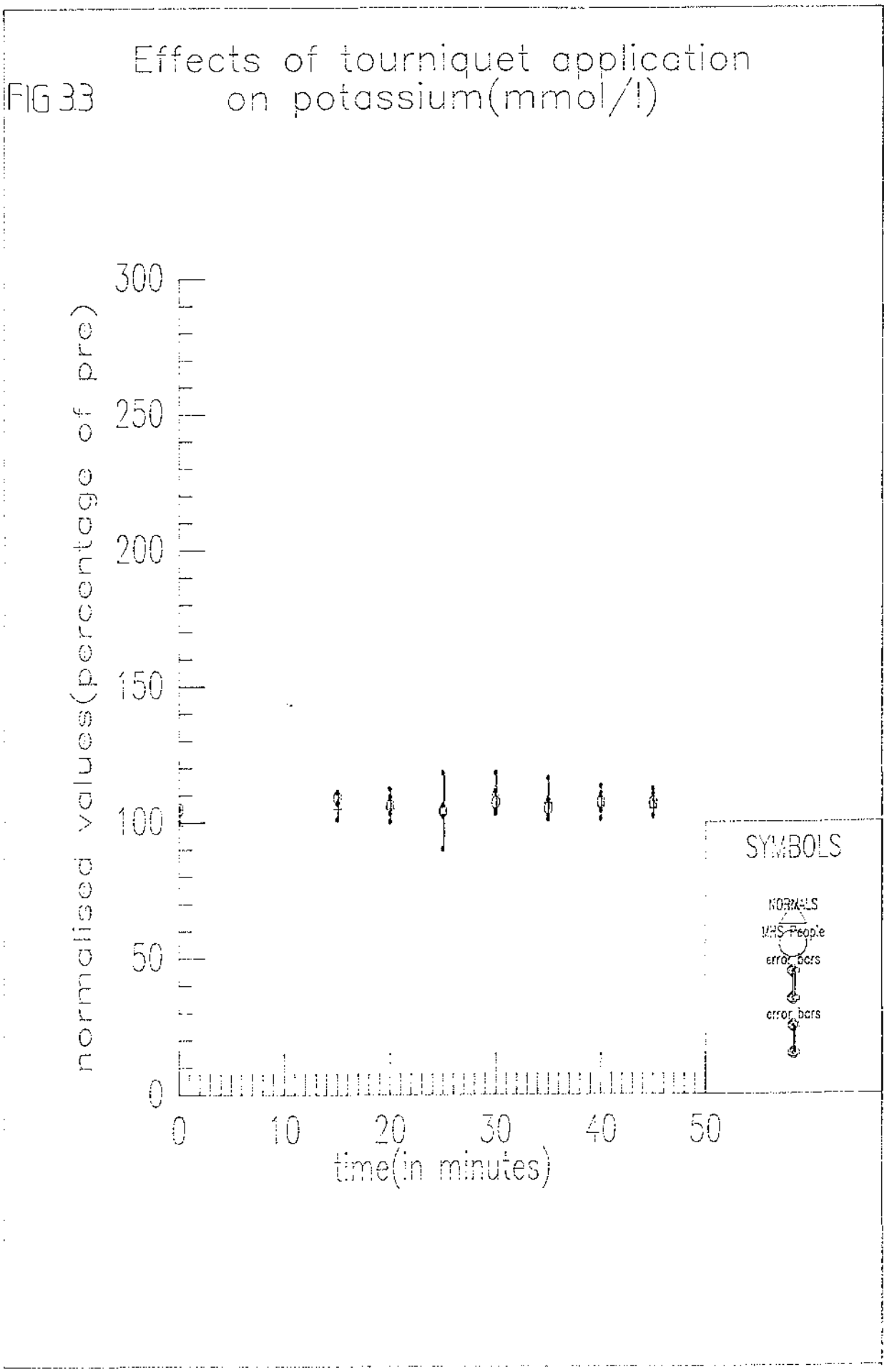

Effects of tourniquet application
on potassium(mmol/l)
normalised values(percentage of pre)
300
250
200
150
100
50
0
0
10
20
30
40
50
time(in minutes)
SYMBOLS
NORMALS
MHS People
error bars
error bars

FIG 34

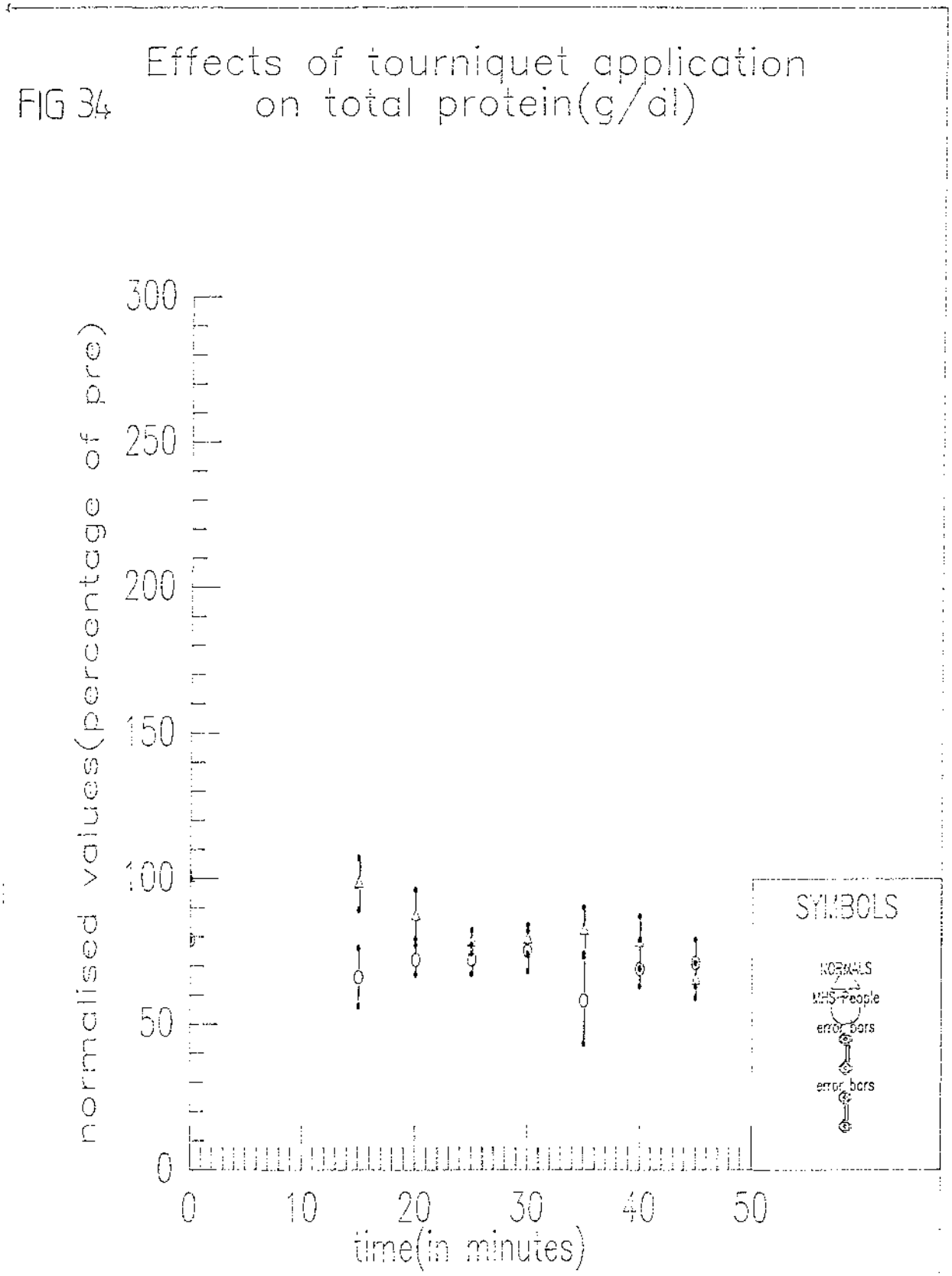

Effects of tourniquet application
on total protein(g/dl)
normalised values(percentage of pre)
300
250
200
150
100
50
0
0
10
20
30
40
50
time(in minutes)
SYMBOLS
NORMALS
MHS People
error bars
error bars

FIG 35

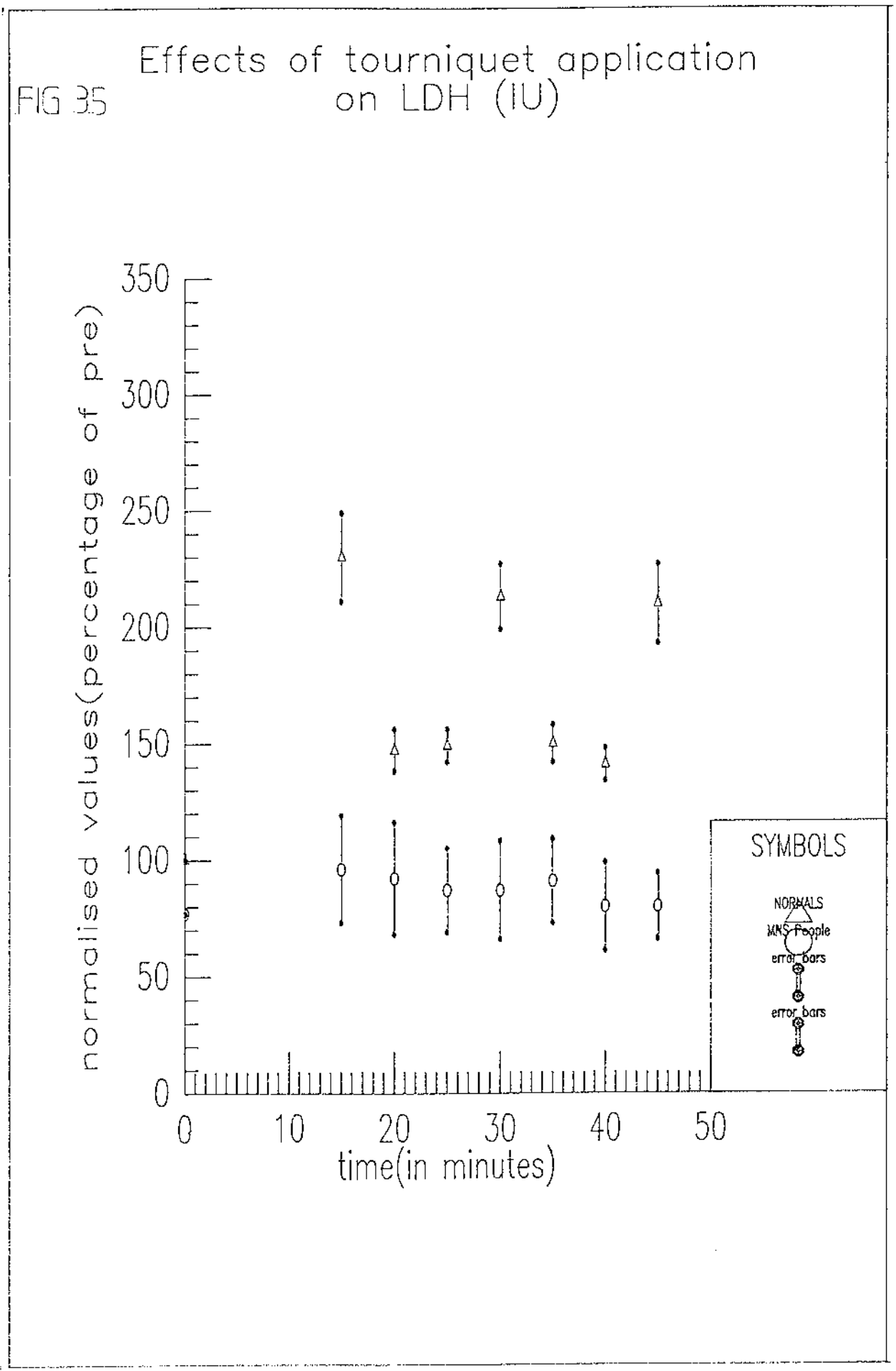

Effects of tourniquet application
on LDH (IU)
normalised values(percentage of pre)
350
300
250
200
150
100
50
0
0
10
20
30
40
50
time(in minutes)
SYMBOLS
NORMALS
MNS People
error bars
error bars

FIG 36

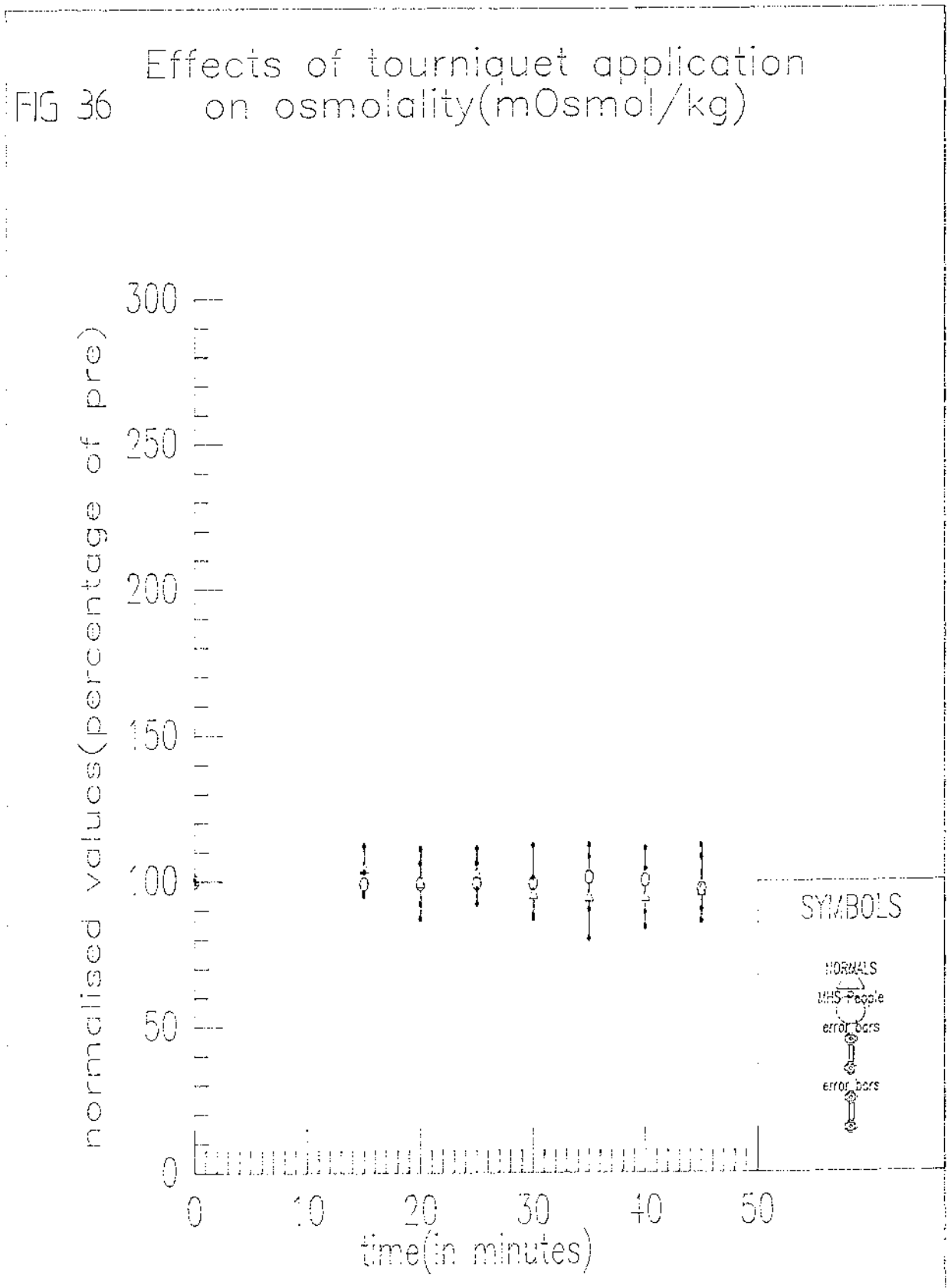

Effects of tourniquet application on osmolality(mOsmol/kg)
normalised values(percentage of pre)
300
250
200
150
100
50
0
0
10
20
30
40
50
time(in minutes)
SYMBOLS
NORMALS
MHS People
error bars
error bars

FIG 3.7 Effects of tourniquet application on total solids(g/dl)

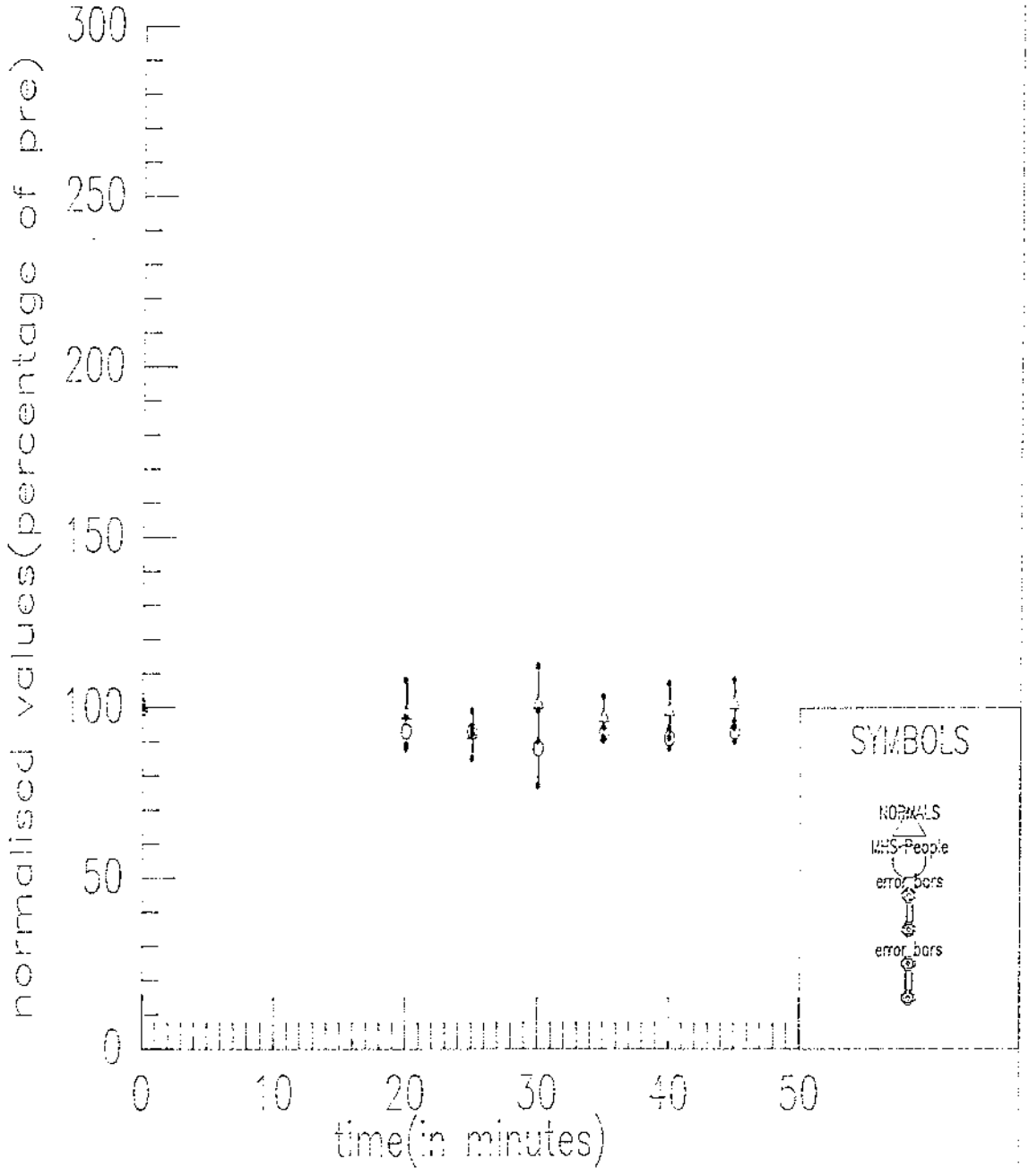


FIG 38

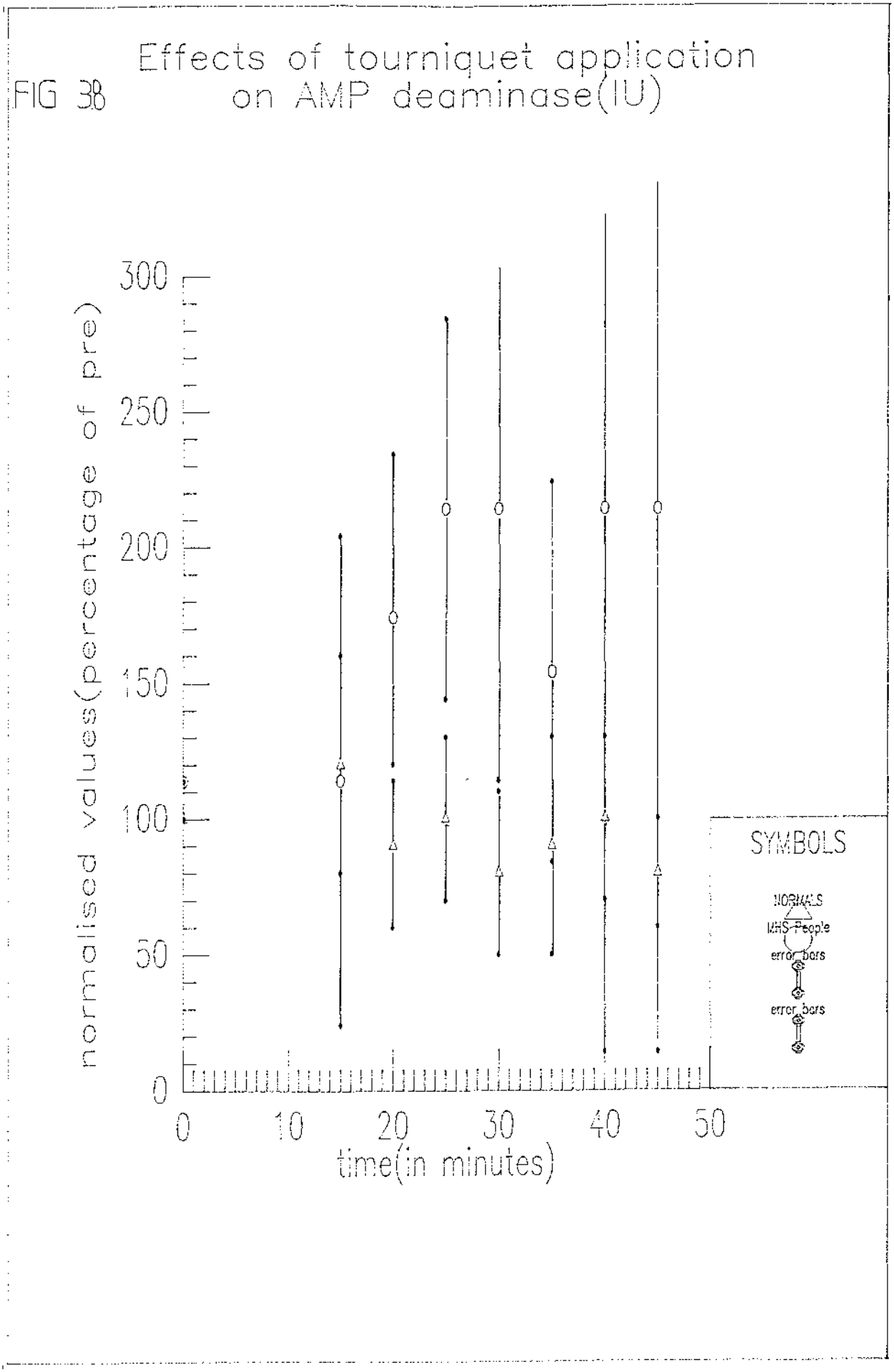

Effects of tourniquet application
on AMP deaminase(IU)
normalised values(percentage of pre)
300
250
200
150
100
50
0
0
10
20
30
40
50
time(in minutes)
SYMBOLS
NORMALS
MHS People
error bars
error bars

FIG 39

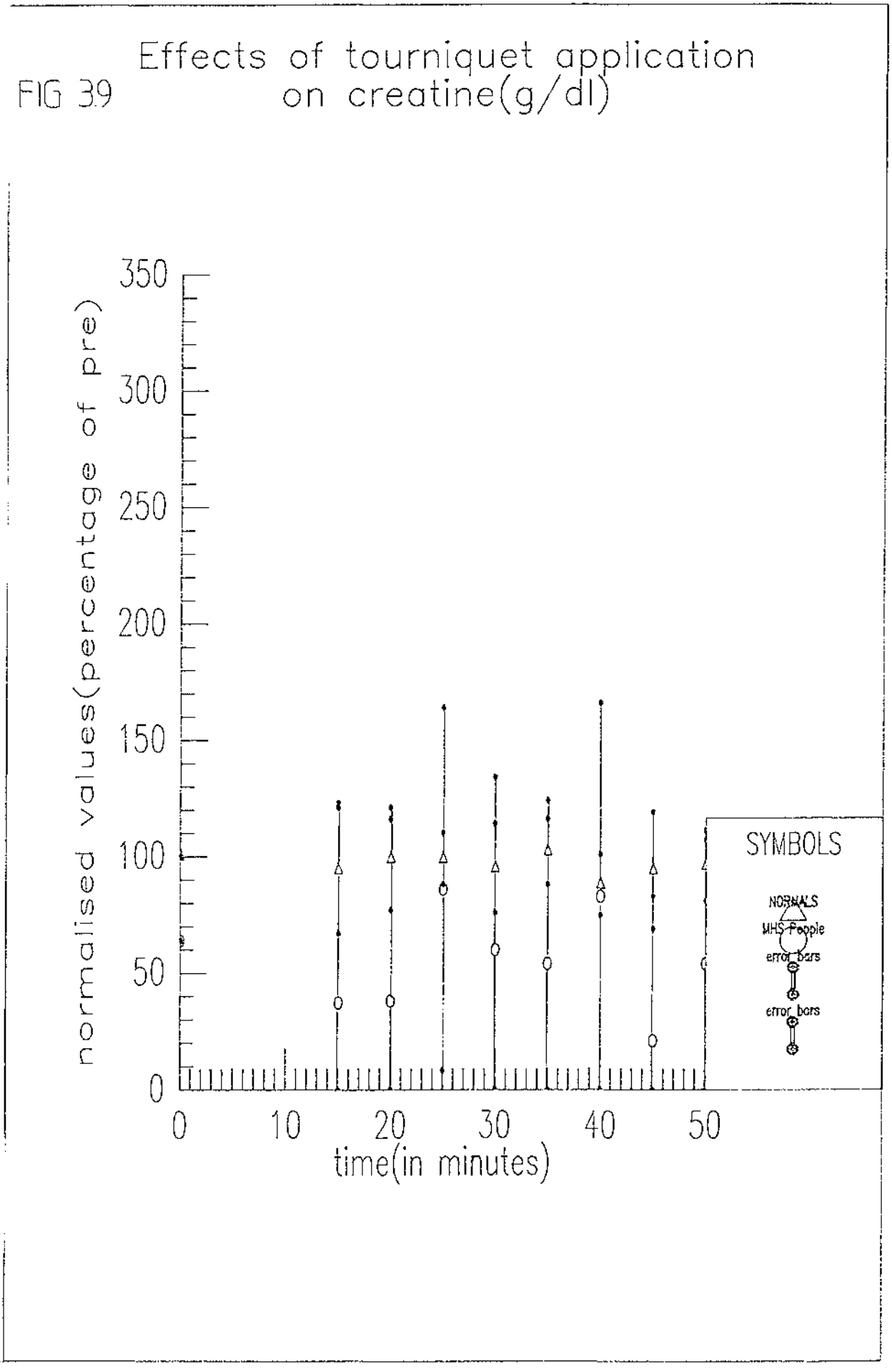
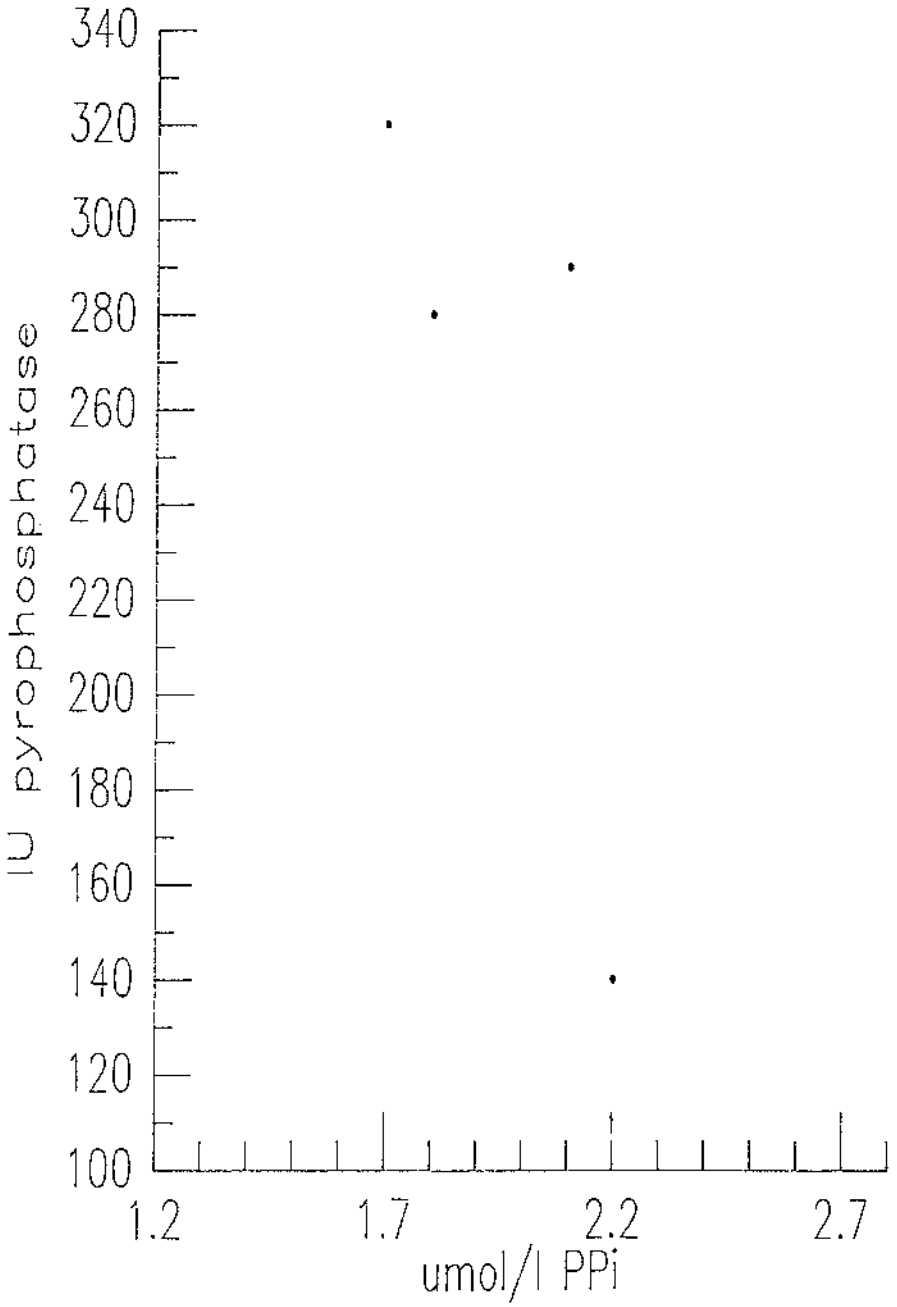

Effects of tourniquet application
on creatine(g/dl)
normalised values(percentage of pre)
350
300
250
200
150
100
50
0
0
10
20
30
40
50
time(in minutes)
SYMBOLS
NORMALS
MHS People
error bars
error bars

FIG 3.10 Scattergram of PPi vs PPase.
MHS subjects before tourn. application.



Chapter 4

DISCUSSION

4.1 INTRODUCTION

Malignant hyperthermia susceptibility is a genetic syndrome that particularly affects the skeletal muscle. Susceptible patients most often experience an MH episode after being exposed to anaesthetic agents such as halothane or succinylcholine. An episode involves increase in temperature, tachycardia and cardiac arrythmias together with severe metabolic acidosis. Without intervention by appropriate drug administration or physical cooling, an episode can culminate to heart failure and death. Preoperative awareness of the susceptibility is necessary so that proper precautions can be taken during an operation and an MH episode avoided altogether.

Since the MHS subject is otherwise symptomless preoperative awareness obviously relies on available diagnostic methods. At present diagnosis of MHS relies on a patients family history with respect to previous anaesthesias, serum CK levels and the result of a caffeine-contracture test. The definitive diagnosis depends on the contracture test. This test requires muscle biopsy so a series of procedures to check the contractile response of muscle fibres when exposed to halothane and caffeine can be made. Because of the invasive nature of the biopsy, the laborious procedures required and the difficulty in interpreting some results an alternative diagnostic test is being sought.

The options explored have been diverse. Although several

alternative tests have been proposed the caffeine contracture test has not yet been replaced. Promising tests are usually rejected because of lack of confirmation of findings by subsequent study or the test is inappropriate for use in a routine clinical setting.

Most studies have attempted to find abnormalities in the resting MHS patient. Because abnormal findings have seldom been consistent in all patients except after an MH episode has occurred, an alternative approach where a very mild stress is applied locally to muscle was used in this project.Because of the general belief that MH muscle cell membranes have some intrinsic abnormality it follows that if stress was applied to MHS muscle in the form of tourniquet induced ischemia, as experienced during an MH episode, certain metabolites may leak from the muscle cells into the plasma where they could be detected. For this to be useful in diagnosis the observed changes in analyte levels would have to be quite distinct from those observed in normal subjects.

4.2 METABOLITES MEASURED

The metabolites chosen for measurement were those known to be specifically associated with skeletal muscle and its activity.

Lactate dehydrogenase (LDH) is an enzyme that catalyses the conversion of lactate to pyruvate using NAD+ as the oxidising agent. When muscle is in an ischemic state anaerobic metabolism occurs which results in production of high levels of lactate. To prevent tissue damage lactate must be converted by LDH to pyruvate. In normal tissue during ischemia, lactate will rise and then fall once the stress is

removed. The fall in lactate is due to the increased activitly of LDH. LDH is found in much higher levels in heart, liver, kidney and skeletal muscle than in serum so any damage to these tissues usually leads to leakage of the enzyme into the serum. Measurement of LDH and its various isoenzymes are commonly used in aid of diagnosis of liver, renal or various muscle diseases. LDH is one of the enzymes found to be markedly elevated after an MH episode. (Aldrete et al, 1981): this could be due to leakage of the enzyme or the increased activity required for removal of lactate that builds up during an episode.

Creatine kinase (CK) catalyses the phosphorylation of creatine using ATP. CK is found with high activity in skeletal muscle, brain and heart tissues. Increased CK activity is one of the most sensitive tests for diagnosis of skeletal muscle disease, specifically the muscular dystrophies. It can also be an indication of acute cerebrovascular disease and hypothyroidism. CK is already used as a screening test for MHS since most MHS patients exhibit elevated CK levels.(Britt et al, 1976) However, since not all MHS patients have elevated CK levels and some non-MHS people have elevated CK levels, the test can not be used definitively. Evaluation of the test (Amaranath et al, 1983) concluded it has a high overall efficiency but poor predictive value from the positive test. Because of this the CK test can not be used as a replacement for the contracture test, but rather as an indication of whether the latter should be performed.

Creatine is the precursor of the high energy reservoir, creatine phosphate. The conversion of creatine to creatine phosphate occurs predominently in the skeletal muscle. In diseases where extensive

skeletal muscle damage features serum creatine levels have been found to be elevated. Creatine was chosen for analysis in this project to compare to the CK response, and provide information on the behaviour of the low molecular weight molecules.

AMP deaminase (or myoadenylate deaminase) catalyses the conversion of AMP to IMP with the release of ammonia. Researchers involved with diagnosis of Duchenne muscular dystrophy,(a disease often associated with MHS ,Shigehiro Oka et al, 1982 and Ellis et al, 1984) who had previously used CK measurement for aid in the disease's diagnosis found several reasons why use of this assay was no longer ideal.(Rosenberg et al, 1982)

CK can rise after exercise, enzyme inhibitors may be present in serum of patients with muscular disorders and CK activity is found to fall with increasing age. AMP deaminase was suggested as an alternative analyte as it is even more specific to skeletal muscle than CK. Recently, results of a study of previously diagnosed MHS subjects were published that found AMP deaminase was low in these patients. It was suggested that measurement of this enzymes activity could serve as an indicator of MHS (Fishbein et al, 1985).

Several assays were chosen to give a general indication of the muscle cell leakiness. Osmolality was measured to determine the solute concentration in the serum. Leakage of compounds such as Ca++, K+, Na+, glucose and lactate may be reflected in osmolality changes.

Total solids (TS) and total proteins (TP) were also measured as a

general indication of muscle leakiness.

Potassium, the major intracellular ion, was chosen as the electrolyte most likely to indicate the extent of muscle cell leakiness. Potassium has been found to rise after tourniquet release in studies of the effect of ischemia on normal skeletal muscle (Larsson et al, 1978). It may be involved in producing vasodilation and the increase in blood flow observed immediately after the ischemia inducer is released. This electrolyte is also another substance that is commonly found to elevated in the serum after an MH episode. (Aldrete et al, 1981)

Inorganic pyrophosphate was chosen for more specific reasons. Several groups of researchers have suggested that PPi is elevated in MHS serum in a similar manner to CK. (Van Wormer et al, 1977 and Tan et al ,1977). PPi is high in serum and urine of patients with Osteogenesis Imperfecta, another myopathy often referred to in relation to MHS (Armstrong et al, 1975 and Russel etal, 1971). The origin of PPi is likely to be the adenylate cyclase reaction:

$$\text{creatine phosphate} + \text{ADP} \xleftrightarrow[\text{CK}]{\text{Mg}^{++}} \text{creatine} + \text{ATP}$$

$$\text{ATP} \xrightarrow[\text{adenylate cyclase}]{} \text{c-AMP} + \text{PPi} + \text{heat}$$

PPi has also been implicated as an inhibitor of AMP deaminase (Fishbein et al, 1979).

Since blood PPi is hydrolysed to inorganic ortho-phosphate by

erythrocyte pyrophosphatase, the latter enzyme was also measured in all blood samples to aid interpretation of the PPi data.

4.3 RESULT TRENDS

Contrary to our expectations that metabolite levels would be noticeably elevated as a result of tourniquet induced muscle cell leakiness, levels in MHS subjects were generally unaltered or diminished with respect to pre-tourniquet levels.

Analytes which showed no changes in either normal or MHS groups between pre-tourniquet and post-tourniquet levels were osmolality, AMP deaminase and creatine. The change in osmolality levels observed in previous studies (Larsson et al, 1978) may not be seen in this study because the period of ischemia was relatively short. In the Larsson study tourniquets were applied for up to 3 hours. Resting osmolality levels and AMP deaminase activity showed no difference between groups either.

The analytes that did respond to tourniquet application were LDH, CK, TS and K+. When a comparison of the response of these analytes to tourniquet application was performed across the two groups a noticeably uniform pattern was seen. Where the normal post-tourniquet levels were elevated the MHS post-tourniquet levels were unchanged and when the normal post-tourniquet levels were unchanged the MHS post-tourniquet levels were decreased. That is , the MHS response was always negative in relation to the normal response. For example in the normal group, tourniquet application had no effect on CK or TS , while in the MHS

group tourniquet application produced a significant drop in the levels of these analytes. The tourniquet effect in the normal group on K+ and LDH produced a rise in these analytes in the serum but in the MHS group no change was observed. The increase in K+ and LDH in the normal subjects reflects the changes seen in the Larsson and Haggmark studies. Total protein fell in both groups.

The exception to this pattern is the response of PPi. After tourniquet application the PPi levels increased in the MHS subjects whereas it remained unchanged in the normal subjects.

The analytes differing in resting or pre-tourniquet levels between the two groups were CK, creatine and pyrophosphatase. CK levels were significantly elevated in the MHS subjects, supporting early research findings.(Britt et al, 1976) Creatine levels were significantly lower in the MHS subjects. It would be interesting to carry out further study to determine if this is a secondary effect of the high circulatory CK levels. Pyrophosphatase levels were significantly lower in the MHS subjects.

Graphical plots of the analyte levels for the MHS group and normal group with respect to time gives some indication of the different responses of the two groups. The most striking graphs are produced by the assays for CK, LDH and AMP deaminase. AMP deaminase did not show a statistically significant response to tourniquet application but this is probably due to the large confidence intervals. The main contribution to this variance apart from experimental error was the huge range of responses by different subjects. This was most marked in

the MHS group, suggesting that further investigation of factors affecting the level of this enzyme is warranted.

4.4 PUTATIVE EXPLANATIONS

Two explanations present themselves as to why most MHS metabolites showed a negative response to tourniquet application as compared to the normal response.

The first is that MHS muscle cell membranes are less effected by stasis, perhaps due to stabilisation by a higher membrane potential or altered membrane structure. This would explain why levels in MHS subjects were unchanged at times when normal subjects levels were raised, but does not explain the occassions when the MHS analytes were reduced.

A more likely explanation is that the capillaries in MHS muscle are exhibiting greater permeability after tourniquet application than their normal equivalents, so that even if leakage from muscle cells is occurring the metabolites are passing from the serum across the capillary walls into the interstitial fluid. The degree of capillary permeability depends on four major factors. The hydrostatic pressure in the vessel, the osmotic pressure exerted by the serum proteins, the extracellular or tissue tension, and the colloid osmotic pressure of the extracellular fluid. Fluid will leave the serum and cross the capillaries into the extracellular fluid when the colloid osmotic pressure of the extracellular fluid is greater than the serum protein osmotic pressure or the hydrostatic pressure in the vessel overcomes

the tissue tension.

Capillaries contain within their wall structural discontinuities so that molecules pass through the vessel walls. Small molecules can pass through gaps between endothelial cells and larger molecules can pass through pores that can enlarge on capillary lumen dilation. Small dissolved molecules and smaller proteins such as albumin or the gamma globulins can pass across the capillaries relatively easily while larger molecules such as fibrinogen tend to find the capillaries impermeable.

Capillary permeability can be additionally influenced by several factors. In situations of prolonged ischemia the pO2 of the muscle falls, the pCO2 rises and the pH falls due to accumulation of lactic acid. In normal muscle the ramifications of short periods of ischaemia are slight: however, it is possible that MHS muscle is much less resistant to periods of ischaemia, resulting in an increase in capillary permeability.

After tourniquet release there is a sudden rise in vascular supply. Increased vascular supply would increase venous pressure and therefore increase capillary blood pressure. In combination with the altered pO2, pCO2 and pH this would also increase capillary permeability in MHS muscle.

Ca++ is known to cause venous constriction. If the tourniquet application does indeed simulate an MH episode it is likely Ca++ would be released from the sarcoplamic reticulum. Venous constriction would

raise the pressure in the small vessels, increasing capillary permeability. Furthermore, the endothelial cells lining capillaries are believed to contain contractile elements that behave as muscle when acted upon by Ca++. Ca++ would cause contraction of the cells so the inter-endothelial cell gaps would widen providing escape channels for plasma metabolites.

Confirmation of these suggestions is difficult because interstitial fluid is not easy to sample. Much later blood sampling after tourniquet release may be helpful as much of the interstitial fluid is eventually reabsorbed into the venous system. This could also explain why CK levels tend to be found most elevated at least 12 hours after an MH episode.

Further study of the permeability state of capillaries in patients suffering from conditions such as McArdles disease or Duchenne Muscular Dystrophy would also be beneficial. These diseases involve an abnormality in Ca++ homeostasis in a similar way to the MHS syndrome. In this way whether the findings of this project are unique to MHS patients or to muscular disorders in general could be determined.

Because of the negative response of most MHS analytes to tourniquet application, the increase in PPi levels in MHS subjects after tourniquet application is particularly remarkable. Calculation of the correlation coefficient of pre-tourniquet pyrophosphatase to PPi in MHS patients,produced a significant negative correlation with PPi levels falling as pyrophosphatase levels increased.

No obvious explanation presents itself for these findings though several tentative suggestions may be made. PPi has been described as a Ca++ seeking compound. Studies of the action of PPi in McArdles syndrome (Swift and Broon, 1981) showed there was abnormal sarcoplasm Ca++ accumulation occurring during exercise and radioactively labelled PPi was localised to these areas. This could be due to the inhibitory action of PPi on Ca++ precipitation, preventing soft tissue calcification. After tourniquet application a similar series of events may be occuring which would account for the rise in PPi seen after tourniquet application.

Pi is also required for Ca++ release from the sarcoplasmic reticulum. If there is a defect in the SR membrane that allows low threshold release of Ca++, possibly less Pi would be required.

Low levels of PPase in these situations would be advantageous as high PPi levels would be maintained and Pi levels that could encourage unwanted Ca++ precipitation would be suppressed.

Calculation of correlation coefficients failed to confirm findings of the groups that suggested PPi is elevated along with CK in MHS subjects or that endogenous PPi acts as an inhibitor of AMP deaminase. Because the MHS sample is so small these calculations are of very limited value: to explore the suggestions more thoroughly a project would need to be more specifically directed.

This project can be regarded as a pilot study of the effect of tourniquet application on MHS muscle. Fifteen minutes of tourniquet

application definitely does cause changes in metabolite levels in the serum of MHS subjects that are distinct from the normal subjects response. These responses, however, are not universal for all subjects and are probably not of a substantial enough magnitude to be useful diagnostically. Application of the tourniquet for greater than ten to fifteen minutes might well produce more dramatic metabolite changes such as seen in the Haggmark and Larsson studies. A tourniquet can be left on for over two hours without induction of permanent damage (Larsson et al, 1978) but the comfort of the volunteer must be of prime importance. Most subjects were at their limit of discomfort after fifteen minutes. One of the reasons an alternative test to the caffeine-contracture test is being sought is that it should be less of an ordeal. Furthermore, prolonging tourniquet application to the point where emotional stress is induced is unethical. Other methods such as severe exercise or electrical stimulation may be an alternative to the tourniquet method.

Numerous attempts to find a single fully reliable diagnostic test for MHS have failed in the past: Possibly an approach such as undertaken in this project, which looks at a whole battery of tests may succeed in picking MHS subjects where any single test fails. MHS might best be predicted when a certain number of abnormal analyte levels out of a whole series measured are found. In this way the expression of a variety of indicators of the disease could be catered for.

Alternatively a completely different approach may be required such as the development of a genetic probe that could detect an abnormal gene sequence in the patients DNA. Obviously there is no easy answer

and after study of the available literature it still seems it will be some time before the caffeine-contracture test is replaced by another.

APPENDIX I

PRECISION DATA

	AMP deaminase		Total Solids		Creatine Kinase		Lactate Dehydrogenase	
	IU	REL	g/dl	REL	IU	REL	IU	REL
1	12.36	118	9.0	101	108	109	217	105
2	12.36	118	9.0	101	107	108	216	104
3	12.23	116	9.0	101	106	107	215	104
4	10.61	101	9.0	101	102	103	212	102
5	10.49	100	9.0	101	98	99	210	101
6	10.24	98	9.0	101	97	98	208	100
7	10.11	96	9.0	101	96	97	205	99
8	9.49	90	8.8	99	94	95	199	95
9	8.86	84	8.8	99	91	92	196	95
10	9.60	82	8.8	99	91	92	193	93
CI	1	10	0.1	1	5	5	6	3

	Total Protein		Inorganic Pyrophosphate		Pyrophosphatase	
	g/dl	REL	umol/l	REL	IU	REL
1	27.98	108	1.996	108	343.30	117
2	27.54	106	1.948	105	321.77	110
3	27.01	104	1.912	103	294.26	100
4	26.87	103	1.866	101	294.26	100
5	26.26	101	1.854	100	288.28	98
6	25.69	101	1.848	100	288.28	98
7	25.45	98	1.830	99	281.10	96
8	25.25	97	1.796	97	275.12	94
9	24.90	96	1.748	94	275.12	94
10	24.62	95	1.712	93	275.12	94
CI	1	3	0.1	1	16	1

	Potassium		Osmolality		Creatine	
	mmol/l	REL	mOsM/kg	REL	g/dl	REL
1	5.25	105	212	105	.0620	110
2	5.25	105	209	103	.0609	108
3	4.95	99	207	102	.0604	107
4	4.95	99	207	102	.0591	105
5	4.95	99	205	101	.0576	102
6	4.95	99	203	100	.0562	99
7	4.95	99	202	100	.0492	87
8	4.90	98	197	98	.0462	82
9	4.90	98	190	94		
10	4.80	96	189	94		
CI	.1	2	5	3	.003	5

Abbreviations:

1.REL-Absolute values expressed as a percentage of the mean absolute

(Gives confidence intervals for normalised data in results tables)

2.CI-Ninety five percent confidence interval.

APPENDIX II

CONTROL DATA

INORGANIC PYROPHOSPHATE-umol/l

95 percent CI=.1 for individual values

	SUBJECT								Pretourn Normals
	1	2	3	4	5	6	x	CI	cf Controls(2+2)
0	1.9	2.5	2.1	1.9	2.2	1.5	2.0	0.3	- -
5	2.2	2.5	2.2	2.1	2.3	1.7	2.2	0.3	-
Time 10	2.0	2.4	2.3	2.0	2.4	1.8	2.2	0.3	-
Sample15	2.1	2.3	2.2	2.0	2.5	1.8	2.2	0.3	-
Taken 20	2.2	2.4	2.3	2.0	2.2	1.8	2.2	0.2	-
25	2.1	2.3	2.3	2.0	2.3	1.7	2.1	0.2	-
30	2.4	2.1	2.1	2.2	2.5	2.0	2.2	0.2	-
35	1.9	2.4	1.9	2.1	2.4	2.1	2.1	0.2	-

AMP DEAMINASE-IU

95 percent CI=1 for individual values

	SUBJECT								Pretourn Normals
	1	2	3	4	5	6	x	CI	cf Controls(7+3)
0	7	4	4	5	7	2	5	2	- -
5	5	5	6	6	7	4	5	2	-
Time 10	6	2	4	4	2	4	4	2	-
Sample15	8	5	2	2	11	2	5	4	-
Taken 20	9	4	3	2	1	1	3	3	-
25	19	6	5	6	10	3	8	6	-
30	14	3	4	3	8	1	6	5	-
35	9	4	5	7	6	1	5	3	-

POTASSIUM-mmol/l

95 percent CI=.1 for individual values

	SUBJECT								Pretourn Normals
	1	2	3	4	5	6	x	CI	cf Controls(4+.5)
0	3.8	3.4	3.9	3.9	3.4	4.0	3.7	0.3	- -
5	4.0	3.8	3.9	4.0	4.0	4.3	4.0	0.2	-
Time 10	4.1	3.9	3.7	4.0	2.7	4.1	3.8	0.6	-
Sample15	4.3	4.0	3.4	4.0	3.6	3.9	3.9	0.3	-
Taken 20	4.0	4.0	3.8	4.0	3.4	3.8	3.8	0.3	-
25	4.2	4.1	3.7	4.0	4.1	4.7	4.1	0.3	-
30	4.5	3.8	4.3	4.0	3.6	4.3	4.1	0.4	-
35	4.0	3.9	4.1	3.9	4.2	3.9	4.0	0.1	-

TOTAL SOLIDS-g/dl

95 percent CI=.1 for individual values

	SUBJECT								Pretourn Normals
	1	2	3	4	5	6	x	CI	cf Controls(8+1)
0	8.8	8.8	8.0	5.5	5.0	4.8	6.8	2	- -
5	8.6	8.8	8.6	8.0	7.5	5.6	7.9	1	-
Time 10	6.8	8.8	8.2	5.0	7.4	6.2	7.1	1	-
Sample15	8.8	9.0	6.8	5.0	6.0	6.0	6.9	2	-
Taken 20	8.8	8.8	8.5	6.5	5.2	5.6	7.2	2	-
25	7.0	9.0	9.0	4.0	5.2	5.0	6.5	2	-
30	9.0	8.6	8.0	5.5	4.5	7.0	7.1	2	-
35	8.5	8.6	8.5	5.5	4.5	6.4	7.0	2	-

LACTATE DEHYDROGENASE-IU

95 percent CI=6 for individual values

	SUBJECT								Pretourn Normals
	1	2	3	4	5	6	x	CI	cf Controls(300+200)
0	156	201	219	169	106	160	169	41	- -
5	77	165	224	157	139	206	161	55	-
Time 10	124	118	196	144	138	233	159	48	-
Sample15	168	120	320	143	114	235	183	84	-
Taken 20	141	188	220	178	111	282	187	63	-
25	158	139	232	210	138	139	174	41	-
30	89	106	280	186	123	270	176	88	-
35	107	156	213	156	114	242	165	56	-

OSMOLALITY-mOsmol/kg

95 percent CI=5 for individual values

	SUBJECT								Pretourn Normals
	1	2	3	4	5	6	x	CI	cf Controls(270+30)
0	327	272	233	273	315	217	273	45	- -
5	246	312	227	264	305	180	256	53	-
Time 10	336	243	204	265	321	149	253	75	-
Sample15	407	257	261	279	259	200	277	72	-
Taken 20	313	319	206	220	285	243	264	50	-
25	299	317	201	230	352	220	270	64	-
30	261	301	181	240	270	182	239	51	-
35	243	233	139	384	315	226	257	88	-

CREATINE KINASE-IU

95 percent CI=5 for individaul values

	SUBJECT								Pretourn Normals
	1	2	3	4	5	6	x	CI	cf Controls(40+10)
0	35	38	28	89	50	35	46	23	- -
5	29	38	31	85	46	38	45	22	-
Time 10	31	35	26	83	36	33	41	20	-
Sample15	22	33	26	88	46	46	44	25	-
Taken 20	31	34	30	84	52	36	45	22	-
25	38	42	30	85	51	35	47	21	-
30	36	52	28	73	51	39	47	17	-
35	35	37	25	85	47	32	44	23	-

TOTAL PROTEIN-g/dl

95 percent CI=1 for individual values

	SUBJECT								Pretourn Normals
	1	2	3	4	5	6	x	CI	cf Controls(38+7)
0	40	54	42	54	32	28	42	11	- -
5	21	26	35	58	37	29	34	14	-
Time 10	18	34	40	53	31	28	34	13	-
Sample15	22	31	26	61	37	27	34	15	-
Taken 20	22	32	29	51	37	18	31	12	-
25	28	20	29	59	40	21	33	15	-
30	29	27	30	47	37	26	33	8	-
35	19	14	38	47	42	26	31	14	-

CREATINE-G/DL

95 percent CI = .003

	SUBJECT								Pretourn Normals
	1	2	3	4	5	6	x	CI	cf Controls(.069+.007)
0	.039	.098	.061	.067	.067	.055	.065	.02	- -
5	.073	.054	.090	.068	.099	.041	.071	.02	-
Time 10	.060	.097	.091	.063	.084	.074	.078	.02	-
Sample15	.082	.090	.098	.078	.087	.074	.085	.009	>>
Taken 20	.061	.120	.091	.051	.089	.052	.077	.028	-
25	.075	.115	.086	.072	.087	.056	.082	.021	-
30	.058	.099	.022	.043	.090	.050	.060	.030	-
35	.062	.065	.114	.067	.098	.046	.075	.03	-

APPENDIX III

Statistical Method Description

Students t test Reference: Mendenhall/Ott

Understanding Statistics
Wadsworth International Students Edition

Small sample confidence interval - 95 percent

$$\bar{x} + ts/\text{sqr } n$$

x = mean of sample

t = t value corresponding to a = .025 and

degrees of freedom(df)=n-1

n = number in sample

s = standard deviation

a = probability that the calculated t value will exceed

the tabulated t value with n-1 degrees of freedom

sqr = square root

Small sample test of the hypothesis about the sample mean

Null hypothesis: u = u'

Alternative hypothesis: For a one tailed test 1. u>u'

2. u<u'

Test statistic - t = x - u'/(s/sqrn)

s = standard deviation of the sample

n = sample size

x = sample mean

u = population mean (100 percent)

u'= sample mean

For a one tailed test reject Null hypothesis if

t>ta where a is specified, (a is the probability

that the null hypothesis is rejected when it is true),

and the degrees of freedom (df)=n-1

Small sample test for comparing two sample means

Null hypothesis: u1-u2 = 0

Alternative hypothesis: For a one tailed test 1. u1-u2>0

2. u1-u2<0

Test statistic - t = x1-x2/(s*sqr(1/n1+1/n2)

u1 and u2 are population means for population 1 and 2

x1 and x2 are sample means for sample 1 and 2

n1 and n2 are sample sizes for sample 1 and 2

sq(s1) and sq(s2) are sample variances for sample 1 and 2

s=sqr((n1-1)*sq(s1)+(n2-1)*sq((s2))/(n1+n2-2)

Rejection region: For specified value of a and df=(n1+n2-2)

and one tailed test

1.Reject null hypoth if t > ta

2.Reject null hypoth is t <-ta

Correlation Coefficient

p' = Sxy/sqr(SxxSyy)

where Sxy = sumxy - (sumx*sumy)/n

Sxx = sum(x*x) - (sumx*sumx)/n

Syy = sum(y*y) - (sumy*sumy)/n

x = x coordinate values

y = y coordinate values

Possible values for p'

-1----------0----------1

perfect negative correlation	some negative correlation	no correlation	some positive correlation	perfect positive correlation

Test of correlation significance

Null hypothesis: p = 0

Alternative hypothesis: p>1 or p<0

Test statistic - t = ((n-2)/(1-(p'*p'))

n = number of points

Rejection region for specified value of a for n-1 degrees

of freedom and one tailed test:

1.Reject null hypothesis if t>ta

2.Reject null hypothesis if t<-ta

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