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LA THÈSE A ÉTÉ
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If a man would pursue philosophy, his first task is to throw away conceit, for it is impossible for a man to begin to learn what he has a conceit that he already knows.

Epictetus

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Abstract

The purpose of this study was to investigate the relative importance of several biochemical factors involved in muscle fatigue, since it has been shown that during fatigue, tissue stores of ATP and CP decline, lactate levels increase and intracellular pH decreases.

In order to examine this problem, a method to maximize intracellular lactate concentration was hypothesized. The production of pyruvate was increased by inducing thiamine deficiency, and glucose loading of the isolated tissue. Thiamine deficiency was produced by feeding rats a thiamine-free diet, and isolated diaphragm strips were glucose loaded by elevating the glucose content of the bathing medium to 55 mM, in the presence of 10 mU/ml insulin.

Pyruvate and lactate levels, as well as ATP and CP concentrations of tissues in the rested and fatigued states were measured. In addition, the rates of lactate, pyruvate and H^+ efflux were studied.

In the resting state, tissue pyruvate and lactate concentrations were not increased more in thiamine deficient tissues than in control tissues. Muscle contractility was also not significantly affected by thiamine deficiency.

Following fatigue, tissue pyruvate levels were significantly greater in the thiamine deficient tissues than the control tissues. The efflux rate of both lactate and H^+ was significantly greater from the thiamine deficient tissues than the control tissues throughout all the conditions tested.

The results of this study indicate that the effect of thiamine deficiency on pyruvate metabolism is most apparent during higher levels

of activity when pyruvate production is accelerated. However, even in these conditions the measured concentration of pyruvate remained lower than anticipated.

It is also suggested that in thiamine deficiency, the permeability of the skeletal muscle membrane is altered because glucose influx increased, lactate and H^+ efflux increased, and glucose uptake did not respond to insulin.

Introduction

The biochemical basis of muscle fatigue

Muscle fatigue is a term used to describe the decreasing tension generated by a muscle during prolonged or intense activity. As this word describes a particular phenomenon, it offers no implicit explanation of the events causing the decline in response.

In isolated nerve muscle preparations where the nerve is stimulated at a high frequency and muscle contractions recorded, the observed response has often been attributed to an actual fatigue at the neuromuscular junction (Mashima et al, 1962), ie. a quantitative decrease of transmitter release. However, in isolated muscle preparations where the nerve is not intact, fatigue can not be explained in terms of the quantity of neurotransmitter released.

It has been noted for some years that prolonged stimulation of isolated muscle, particularly under anaerobic conditions, can result in an almost total loss of the mechanical response, even though the action potential remains near normal and the contractile mechanism is apparently unimpaired. Several investigators have suggested that this may be due to a failure of excitation-contraction coupling, which reverses only very slowly (Eberstein and Sandow, 1963; Mashima et al, 1962; Grabowski et al, 1972), and some evidence for this type of fatigue occurring in Man has been presented by Edwards et al (1977).

In the conditions employed in isolated muscle studies, two theories of muscle fatigue predominate, and both attribute the fatigue to a biochemical origin. More directly, fatiguing may be due directly to depletion

of high energy phosphate stores such as adenosine triphosphate (ATP) and creatine phosphate (CP), or indirectly to a building of metabolic products from glycolysis such as lactate and/or hydrogen ion (H^+), which then cause a shut down of energy producing processes. It is generally accepted that muscle contraction is not possible without the high energy phosphates, ATP and CP. During periods of relative inactivity, ATP and CP are produced by catabolic processes where free fatty acids and carbohydrates serve as substrates for the tricarboxylic acid cycle (TCA). The TCA cycle is an oxidative process requiring oxygen, and is the primary energy producer under aerobic conditions. TCA substrates are provided by β-oxidation of free fatty acids in vivo (Randle et al, 1963, 1966; Lessers et al, 1971, 1972), with glycolysis being an important, but secondary pathway. There is some evidence however that hyperlactacidaemia is associated with a decrease in the concentration and peripheral utilization of circulating free fatty acids (Bjorntorp, 1965; Diertle et al, 1969; Boyd et al, 1974; Issekutz et al, 1975; Gorski, 1977). Conditions of work requiring ATP expenditures which surpass the capabilities of the oxidative systems, or conditions where the oxidative systems are inhibited, rely more heavily on glycolytic ATP production.

The metabolism of glucose and other hexose carbohydrates to pyruvate and lactate is the only known process of anaerobic ATP production. Glycolysis may be considered a two stage process. The first stage catalyzes the metabolism of several different hexoses to glyceraldehyde-3-phosphate. This process involves phosphorylation, at the expense of ATP, and cleaving to

form the three carbon sugar, which is then converted into lactic acid in the following stage (Krebs, 1957). In the second stage, all sugars follow a common pathway of oxido-reduction reactions and energy conserving mechanisms which result in the phosphorylation of adenosine diphosphate (ADP) to form ATP. The energy released (or potentially stored) by the glycolytic process actually represents only a fraction relative to the total chemical energy available in the structure of the glucose molecule; the overall $\Delta G^{o} = -47.0$ kcal following glycolysis, as opposed to $\Delta G^{o} = -686$ kcal when the molecule is further metabolized to CO_2 and H_2O (Lehninger, 1972). Thus work being performed with energy of predominantly anaerobic origin requires much greater amounts of carbohydrate substrates (about 14.5-fold) and results in about a 12-fold increase in rate of pyruvate production. Costin et al (1971) and Hirche et al (1970) demonstrated that there is a net production of lactate by skeletal muscle at rest, and that its production increases on stimulation. The production of lactate itself is not important except as an indication of anaerobic energy production, as lactate passively diffuses out of muscle tissue into the extracellular fluids and blood, movement being governed by concentration gradients. The important biochemical parameter in the process is the oxidation of NADH to NAD^{+}. The NAD^{+} can then be reduced, allowing the oxidation of glyceraldehyde-3-phosphate during the metabolism of other glucose molecules, providing for the phosphorylation of further amounts of ADP to replenish depleted stores. However, several experimental preparations have been used to demonstrate lactate oxidation in skeletal muscle (Corsi et al, 1972;

Jorfeldt, 1970), and the ability of skeletal muscle to extract lactate when arterial lactate was elevated has been demonstrated in dogs (Dunn and Critz, 1975) and in Man (Carlson and Pernow, 1959; Freychuss and Strandell, 1967).

The step controlling the production of energy in the form of ATP appears to be the activity of the enzyme phosphofructokinase (PFK), (Mansour, 1963; Danforth, 1965; Trivedi and Danforth, 1966). This enzyme catalyzes the overall rate limiting reaction of energy production by limiting the amount of substrate (pyruvate) made available from the glycolytic pathway for the TCA cycle. Aside from being temperature and pH dependent, the activity of this enzyme is governed by the concentration of ATP and ADP such that if the concentration of ATP or the ATP/ADP ratio increases within the tissue, then the enzyme is inhibited, while conversely an increase in ADP concentration results in an increase of PFK activity. In anaerobic conditions, where the levels of ATP usually drop and are often critically depleted, glycolytic activity has been reported to increase several fold (Karlsson, 1971_{ab}; Knuttgen and Saltin, 1972; Graham et al, 1976), presumably to recover from the ATP deficit. The fact that glycolytic activity increased when ATP levels fell would tend to suggest that in normal conditions some factor other than the concentration of glycolytic metabolites, must be limiting energy production via the TCA cycle. In vivo, it has been proposed that the availability of oxygen to the muscle from the blood may become the limiting factor of energy production at higher work loads, (Kajser, 1970), however, this has

been refuted by several investigators (Kajser, 1973; Neill et al, 1969). Studies of the effects of other factors on aerobic energy production, such as PO_2 (Kajser, 1973), pH (Danforth, 1965) and PCO_2 (Hirche, 1973) have all been inconclusive.

Muscle fatigue in Man

Hill and his coworkers (Hill et al, 1924; Hill and Lupton, 1923) were probably the first to observe an increased formation of lactic acid in exercising humans. Their report, together with an earlier paper (Krogh and Lindhard, 1920) which suggested that oxygen consumption remained elevated in the period following exercise; led to the proposal of an "oxygen debt". This proposal of an oxygen debt stemmed from an idea that there may be a connection between the increased oxygen uptake following exercise and the accumulation of lactic acid (as lactate) during exercise, indicating that a certain amount of energy was being produced from non-aerobic sources, and must be replenished when oxygen becomes available (Hill et al, 1924).

Over the next 45 years, many developments occurred in the fields of muscle biochemistry and physiology. However, the question remained as to whether anaerobic respiration was simply a part of the total energy production at all times, or whether it only became important under certain circumstances. Reports of resting skeletal muscle lactate concentrations have varied from 0.6mmoles/kg wet muscle weight to 1.6mmoles/kg wet muscle weight (Knuttgen and Saltin, 1972, Karlsson, 1971, Diamant et al, 1968, Graham et al, 1976). Work loads represented by 50-70% VO_{2max} do not

result in a significant increase of muscle lactate concentration in Man (Karlsson, 1971_a , Knuttgen and Saltin, 1972) or in dogs (Graham et al, 1976). At these submaximal work loads (where VO_2 is 50-70% maximum) there also appeared to be no difference in muscle lactate concentration between trained and untrained subjects. Although muscle lactate concentration did not increase with lower work loads, work loads of 70-95% VO_{2max} result in very large and often linear increases in muscle lactate concentrations (Karlsson, 1971_b). Karlsson (1971_b) reported an increase from 2mmoles/kg wet tissue weight to 16.9mmoles/kg wet weight and 20mmoles/kg wet weight in untrained and trained subjects respectively. When exercises were of short one minute bursts of maximum effort (on the bicycle ergometer), with short rests, muscle lactate appeared to peak following the first one minute period (23.2mmoles/kg wet weight) and to plateau at a slightly lower concentration of 22.5mmoles/kg wet weight after the fifth one minute burst of exercise. Muscle lactate values between the first and fifth bursts followed a linear path. In other studies in the same report, lactate concentration increased with time if workload was continuous.

Extracellular pH and muscle fatigue

If indeed tissue and blood lactate concentrations do significantly increase during heavy work loads, what is the effect on acid-base balance?

As early as 1942, Turrell and Robinson suggested that changes in lactate content definitely could affect the homeostatic acid-base equilibrium. This being the case, one may speculate that concomitant to the increased acid load due to increased concentration of CO_2 and lactate during physical

exertion, a ventilation rate synchronized with the production of these metabolites might be required, to minimize changes in blood pH (particularly arterial blood). Keul et al (1967_a) were able to demonstrate during and after exercise that 95% of the decrease in plasma HCO_3^- content was attributable to the simultaneous increase in lactate and pyruvate concentrations. They suggested that the remaining 5% might be associated with the uptake of free fatty acids and their conversion to acetoacetate and β-hydroxybutyrate. In a second study (Keul et al, 1967_b) plasma pH changes were affected by the ability of subjects to eliminate CO_2. Also, very athletic individuals did not show a lower pH under maximum stress than did non-athletes. A more quantitative study was reported by Wasserman et al (1967). They noted a linear relationship between plasma lactate and bicarbonate concentration such that for each milliequivalent (meq) increase in lactate concentration a corresponding milliequivalent decrease of plasma HCO_3^- was observed, but these observations were only noted at very heavy work loads. Under these conditions, plasma lactate concentrations increased from approximately 1 meq/L at rest, to 8 meq/L during heavy exercise, while plasma HCO_3^- concentration fell from 23 meq/L to 17 meq/L respectively. Determination of blood pH, HCO_3^- concentration and PCO_2 changes indicated that no changes in acid-base balance could be observed during moderate exercise; that a compensated metabolic acidosis occurred with heavy exercise, and an incompletely compensated metabolic acidosis occurred with very heavy exercise. Compensated acidosis was observed as an altered venous pH or HCO_3^- concentration but at normal arterial levels, the compensation occurred as a result of a sufficiently increased gas

exchange. At very heavy work loads only partial compensation was possible, as ventilation was already at a maximum.

These changes in plasma pH suggest that a similar or greater change in tissue intracellular pH (pH_i) may occur concomitantly. Using various techniques, pH_i has been calculated to be between 7.1 and 6.7 (Sahlin, 1978; Ellis and Thomas, 1976; Wiggins, 1973; Aickin and Thomas, 1977). Sahlin (1975) reported that in human skeletal muscle pH_i decreased from a mean resting value of 7.00 to 6.56 when tension development declined to 68% maximum. In this same study, it was noted that a linear relationship was evident between declining pH_i and an increasing total lactate plus pyruvate concentration. Similarly, Furasawa and Kerridge (1927) reported that in fatigued cat hearts, pH_i fell to a mean of 6.56 from a resting value of 7.07. Under the same conditions, pH_i fell from 7.04 at rest to 6.26 following fatigue of the cat gastrocnemius (Furasawa and Kerridge, 1927). These alterations of pH_i following muscle contraction may lend support to the hypothesis that the decline of muscle contractility observed as fatigue may be associated with intracellular changes of pH.

However, the involvement of the concentration of the high energy phosphate compounds ATP and CP in muscle fatigue can not be ignored. Sahlin (1978) and Harris et al (1977), working with muscle biopsy samples of human subjects, reported that ATP concentration declines markedly in fatiguing muscle. Furthermore, Harris et al (1977) observed that the decline in CP content in tissue from human subjects following exercise may be correlated with increased lactate concentration. The relationship was

not linear, and in fact suggests that CP stores deplete more rapidly than lactate accumulates.

The evidence from these biochemical studies clearly suggests that high energy phosphate depletion and/or increased lactate and H^+ levels may be principal factors causing fatigue. In fact, these two phenomena may be related. Since lactate is actually produced as lactic acid, concomitant with the increased lactate levels, H^+ concentration is also increased. An increased intracellular H^+ concentration (i.e. decreased pH) could possibly exert effects on several parameters of muscle function: a) metabolism, by reducing the activity of certain enzymes. b) excitation-contraction coupling. c) calcium release from the sarcoplasmic reticulum and d) signal propagation along the membrane.

Intracellular H^+ concentration and muscle function

Some disagreement exists as to whether intracellular or extracellular pH changes have a more prominent effect on muscle contractility (Vaughan Williams and White, 1967; Cingolani et al, 1970). It is generally agreed that an external pH beyond the range of 6 to 8 does have a large effect. Direct evidence for this goes back to the report of Daly and Clark in 1921, which provided evidence that acidic solutions had a negative inotropic effect on the heart. However, more recent results tend to suggest that pH_i may play a significant role as well. McElroy et al (1958) reported that increasing pH_i of guinea pig heart muscle would result in contractility decreasing to 70% of that observed before pH alteration. Lorkovic (1966) observed the same tendency using strips of

ventricular muscle from frog hearts. Both of these studies employed a method whereby the muscle tissue was exposed to an altered PCO_2 while the external pH was maintained constant by altering the bicarbonate concentration. Only CO_2 was assumed to cross the cell membrane, thus producing a change in pH_i.

The introduction of the recessed tip design of glass pH microelectrodes (Thomas, 1974) has allowed direct measurement of pH_i. Using this relatively new technique, Ellis and Thomas (1976) reported that alteration of the bubbling gas from 0 to 5% CO_2 resulted in pH_i decrease from 7.2 to 7.14 in rat ventricle preparations. They noted that stimulation of rat Purkinge fibres at a rate of 120/minute for 4 minutes resulted in no measurable change in pH_i.

Although extrapolation of the results from heart muscle to infer what may be happening in skeletal muscle may be a questionable practice, the results published by Clancy and Brown (1966) may still provide some insight. They suggest that due to the lower intracellular buffering capacity of skeletal muscle relative to cardiac muscle, variations of intracellular pH caused by CO_2 should not appear to differentially affect the contractile responses. According to these authors, the difference in pH_i between the 2 muscle types would result in an H^+ concentration graded effect on intracellular organelles involved in contraction and/or excitation-contraction coupling, i.e. the myofilaments and the sarcoplasmic reticulum. This hypothesis was further supported by the results of Fabiato and Fabiato (1978). In this paper, evidence was provided to illustrate that if the intracellular pH of frog semitendinosus fell to

6.6, the maximum tension that the muscle could generate was only 90% of that observed at pH 7.0.. Further, if the calcium level was not increased concomitantly, but was maintained at a pCa of 5.5., then tension generated at pH 6.6 was only 80% of that observed at pH 7.0. An even more dramatic decline of maximum tension was observed when rat ventricular muscle was exposed to the same treatment.

Thus, increasing the concentration of H^+ resulted in a decreased ability to generate tension. This effect could be lessened by increasing calcium availability, suggesting that H^+ concentration may have an effect on calcium-activated contraction. This hypothesis is supported by the results of Donaldson et al (1978). Porzehl et al (1969) also reported similar observations with fibrils extracted from rabbit and crab muscle, where they found that it was necessary to maintain pH above 6.0 and calcium concentration at about 5×10^{-5} M in order to produce maximal activation of muscle fibrils. In this same paper a second, perhaps equally important observation was made. As the pH of the extracted fibres was lowered from 8.0 to 6.0, a marked decline in ATPase activity was observed, as recorded by the percent of ATP splitting and the μmoles P_i produced/minute/mg protein. These results suggest a direct link between pH and muscle contraction in that the activity of ATPase decreases dramatically as pH falls, and thus not only is less ATP produced, but also the tissue is incapable of utilizing the ATP available to it. This may also help to explain the involvement of calcium in fatigue.

Following depolarization of the membrane, the sarcoplasmic reticulum, and in particular the terminal cisternae, liberates calcium,

whose concentration in the myofibrillar medium rises from 10^{-7}M to 10^{-5}M (Ebashi and Endo, 1968; Ebashi et al, 1969). According to the theory of Huxley (1969), when calcium ions penetrate into the contractile system following liberation by the sarcoplasmic reticulum they interact with the tropomyosin-troponin system. Whatever change this interaction elicits in this system, it results in actin activating the ATPase activity of myosin which in turn permits the binding necessary for contraction (Huxley, 1969). Thus if an increase in H^+ does reduce calcium release, then muscle contractility may indeed be affected.

The evidence presented thus far tends to suggest that concomitant with a decreased muscle tension development observed during fatigue, intracellular lactate and H^+ concentrations also increase. Whether this is a causal or resultant relationship is still a matter of question.

ATP and CP concentration in fatigued muscles

A review of the literature published regarding concentrations of high energy phosphates in muscle tissues at rest and following exercise does not help to clarify the question of the biochemical basis of muscle fatigue. As discussed earlier, high energy phosphate, in the form of ATP, is used as the immediate source of energy for muscle contraction (Cain and Davies, 1962). ATP is metabolized to both ADP and AMP, and inorganic phosphate (P_i) during muscle contraction. ATP levels are replenished with phosphate stored in the form of creatine phosphate (CP), (Needham and von Heyningen, 1935; Needham and Pillai, 1937; Meyerhof et al, 1937; Innes, 1937). If ATP levels can not be maintained by stores of CP, then

ATP must be generated via glycolysis and oxidative processes. However, one may suggest that the glycolytic pathway may be inhibited, or at least reduced in activity, during fatigue, because the associated drop in intracellular pH would decrease the activity of the rate limiting enzyme of this process, PFK (Danforth, 1965; Ui, 1966). The CP concentration in skeletal muscle cells is high compared to other cells (Mommaerts, 1950). Hohorst et al (1962) were the first to report a depletion of CP stores with fatigue. Their report of in situ experiments in rat muscle subjected to tetanic stimulation illustrated that whereas ATP concentration in the muscle tissues declined only 15% from resting levels, CP declined towards 0. Similarly Mainwood et al (1972) reported that in frog sartorius muscles stimulated to fatigue, CP levels declined significantly, but ATP concentration did not. Spande and Schottelius (1970) observed a direct linear correlation between the concentration of CP and the development of tension.

Thus, evidence indicates that H^+ and lactate accumulate in fatiguing muscle tissue, and CP is depleted, leaving the question of the biochemical basis of muscle fatigue still unanswered. Nuclear Magnetic Resonance (NMR), a new technique being utilized in molecular biology (Dawson et al, 1977), has presented great hope in solving this mystery. This technique was employed by the same authors in 1978 to study muscle fatigue. Their results, although exciting, unfortunately did not clarify the question. Using frog gastrocnemius muscle, the results with NMR corroborate the papers previously discussed, in that concomitant with fatigue, an increased concentration of ADP, P_i, and H^+ occurs, as well as an increased lactic acid concentration.

They suggested that the decrease in force development was approximately proportional to the rise in ADP and H^+ concentrations. One extremely interesting observation was a shift of the ATP peak to the left, which indicates that a small amount of magnesium (from the MgATP complex) may have been displaced by hydrogen ions. It is known that many enzymes involved with ATP and ADP react as if the true substrates were MgATP and MgADP: e.g. hexokinase (Melchior and Melchior, 1958), pyruvate kinase (Melchior, 1954), phosphoglycerate kinase (Larsson-Raznikiewics, 1967), PFK (Lowry and Passoneau, 1966), adenylate kinase (Rose, 1968), and creatine kinase (Watts, 1973). Thus observations with ^{31}P-NMR tend to suggest that biochemical changes involved in fatigue may be initiated by H^+ altering the concentration of MgATP and/or the MgATP:MgADP ratio.

In light of the fact the measurement of the concentrations of these various components of the muscle has provided information about muscle fatigue, but has as yet not provided an indication of the principal causative factor, perhaps an alternative approach to the problem is worthy of consideration. Since the main question of concern is whether an increased concentration of lactate and/or H^+ or a decreased availability of ATP is ultimately the cause of skeletal muscle fatigue, accentuating either one of these factors may provide some insight into the fatigue process. If the rate of production of intracellular lactate, and therefore H^+ could be enhanced in a particular muscle, then comparing the fatigue characteristics of this tissue with a control tissue may facilitate some understanding.

Pyruvate metabolism in thiamine deficiency

The first step in pyruvate metabolism, via the TCA cycle, is the conversion of pyruvate to 2-carbon fragments to be used for acetyl-CoA anabolism. This step is catalyzed by the pyruvate dehydrogenase enzyme complex, of which one co-factor is thiamine pyrophosphate (TPP), also known as thiamine diphosphate and cocarboxylase (Peters, 1936; Green et al, 1942; Stumpf et al, 1947; Wright and Scott, 1954; Franklen and Stapert, 1954; Thompson and Johnson, 1935; Platt and Lu, 1939). Thiamine deficiency can be induced by restriction of vitamin B_1 dietary intake, or by the use of the thiamine antagonists oxythiamine and pyrithiamine (Gubler, 1961). Although these substances chemically induce thiamine deficiency, several authors have reported differences between this and dietary avitaminosis, and that they may incorporate different modes of action (Gubler, 1961; Bitter et al, 1968; Meghal et al, 1977). Many of these differences have been thoroughly discussed in a review article by Gubler (1976). Two prominent symptoms of thiamine deficiency are anorexia and growth failure, or weight loss (Bai et al, 1971; Bitter et al, 1968, 1969). Several biochemical changes have also been reported in thiamine deficiency. Calf liver pyruvate dehydrogenase activity decreased to 30% of control values after 40 days of vitamin B_1 deficient diet (Benevenga et al, 1967). Similar decreases have been observed in the livers of thiamine deficient rats (Gubler 1961; Oshima, 1960; Framken, 1954) and in pigeon breast and heart muscle (Montfoort, 1955). However, the results of a large number of diverse experiments have led to serious questioning of the concept that the ability of tissues to decarboxylate pyruvate can be altered by thiamine deficiency (Meghal et al, 1977). Interestingly,

Benevenga et al (1966) reported that the most marked changes occurring in thiamine deficient calves were the increases in blood lactate and pyruvate. Blood pyruvate increased 5-fold from a normal of 1mg/100ml, and lactate 6.5-fold from a normal of 15mg/100ml in thiamine deficiency. Even though Meghal et al (1977) reported no change in brain pyruvate metabolism during B_1 avitaminosis, the increased blood lactate and pyruvate concentration observed in these other studies suggests pyruvate metabolism in muscle may be affected by this condition, particularly when the work of Peters (1936) and many others is recalled.

More recently, Davies and Jennings (1970) reported that thiamine deficiency results in specific morphological changes in rat heart tissue. Amongst observations was the presence of material within individual mitochondria, which Hruban et al (1963) had earlier suggested is an indication of cytoplasmic degradation and mitochondrial destruction. In the report of Davies and Jennings (1970), the mitochondria of individual muscle fibres showed severe individual disruption in later stages of thiamine deficiency. Suzuki (1967) noted contraction bands which have been associated with severe mitochondrial damage, in thiamine deficient rats. Suzuki (1967) related the appearance of these bands to an effect of thiamine deficiency on the sarcoplasmic reticulum causing abnormal relaxation, and Gilev (1962) suggests that the bands represent uncoordinated relaxation and contraction of myofibrils in different areas of individual muscle fibres. Indeed Cohen et al (1976) observed that left ventricular muscle preparations from thiamine deficient rats exhibited a decrease in performance associated with a decrease in the duration of contraction and

rate of tension development. These studies indicate that the decrease in pyruvate metabolism induced by thiamine deficiency may ultimately have an influence on muscle contractility.

Glucose loading of muscle tissue

Another approach to increasing the lactate and/or H^+ concentration in muscle tissue would be to drive glycolysis at a rate that would produce quantities of pyruvate that were beyond the capacity of the oxidative system. However, this would only be possible if increased amounts of substrate were made available to the glycolytic enzymes.

Parks et al (1955) demonstrated that glucose uptake into the rat diaphragm could be enhanced if the bathing medium contained a high concentration of glucose in the presence of insulin. Mainwood et al (1977) quantified this phenomenon, when they observed that perfusing the rat diaphragm with 55mM glucose and 10mU/ml insulin results in a dramatic increase in intracellular glucose concentration. The increased availability of glucose, under these conditions, could be used by the tissue in conditions of increased activity resulting in a greater production of pyruvate.

Statement of the Problem

It is clear from the studies reviewed in the previous pages that certain biochemical changes correlate to muscle fatigue. Tissue and blood levels of glycolytic metabolites increase, while concomitantly the tissue concentration of creatine phosphate decreases. The parameter that apparently may link these changes together is the intracellular H^+ concentration, which changes following various levels of activity. However, which of these parameters if any, is/are the primary causes of muscle fatigue remains elusive.

The Aims of the Project

The aims of the present study were rather straightforward. It was hypothesized that intracellular levels of lactate could be dramatically increased by depressing pyruvate metabolism (through thiamine deficiency). The resulting buildup of pyruvate could be accentuated by perfusing an isolated tissue with a high glucose-insulin solution. Increasing the activity of the isolated tissue could also increase the need for ATP, which would only be produced via glycolysis, thus further increasing pyruvate production. By monitoring the fatigue profile of these isolated tissues, and enzymatic assay for lactate, pyruvate ATP and CP content of the tissue during basal and high level of activity, any relationship between these parameters could be observed (Figure 1).

Specifically, this study was designed to investigate a) whether or not pyruvate metabolism is impaired in thiamine deficient tissues, and b) if a) is positive does this affect muscle contractility. The answer to these questions may elucidate whether H^+ affects muscle contractility directly, or by inhibiting energy production.

Figure 1. Scheme for developing lactacidosis. The diagram on the left illustrates the normal course of glucose metabolism. Glucose loading (centre) increases the amounts of pyruvate formed, which in turn is converted to lactate, if the production of pyruvate exceeds the capacity of the oxidative metabolism system. Thiamine deficiency should further decrease the amount of pyruvate being oxidatively metabolised, resulting in an even greater production of lactate.

glyc - glycogen

G - glucose

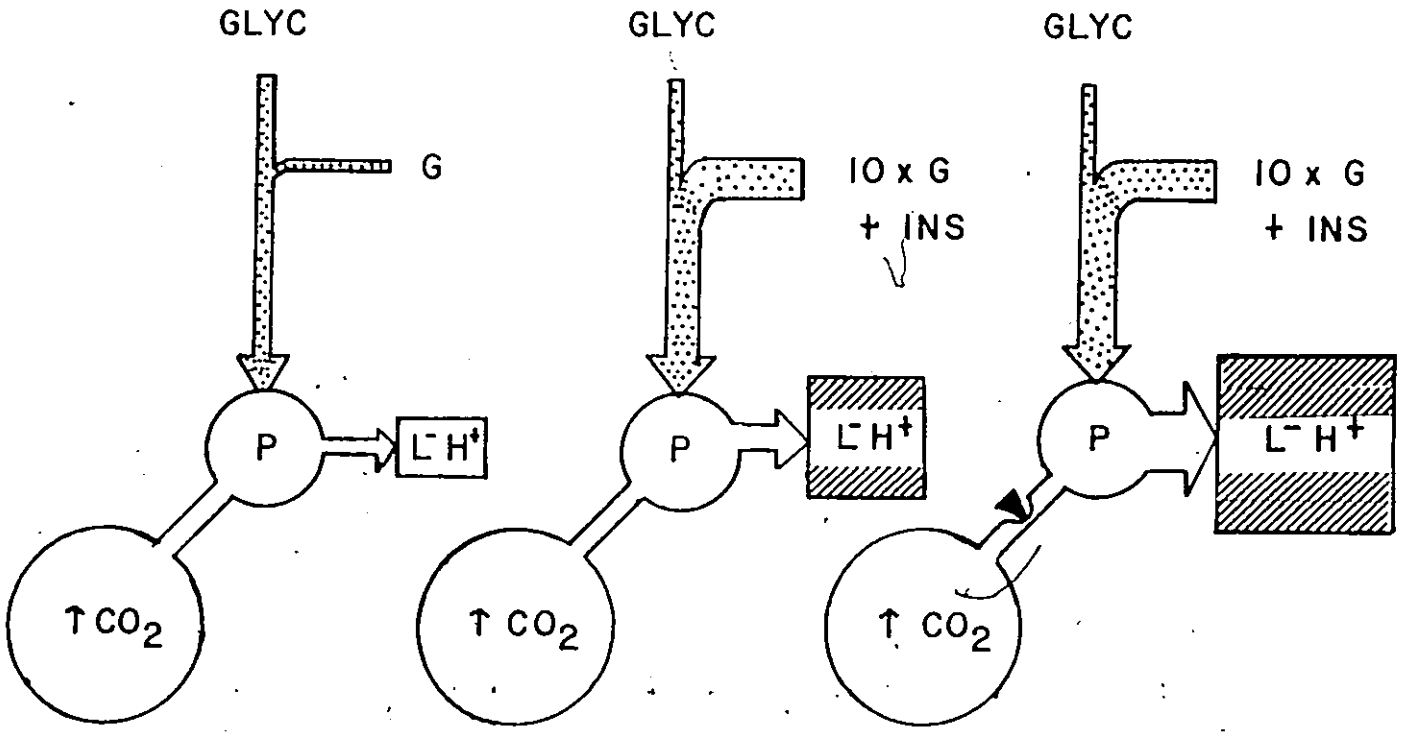
P - pyruvate

L^- - lactate

H^+ - hydrogen ion

ins - insulin

TPP - thiamine pyrophosphate


GLYC
G
P
L⁻H⁺
↑ CO2
NORMAL
GLYC
10 x G
+ INS
P
L⁻H⁺
↑ CO2
GLUCOSE
LOADED
GLYC
10 x G
+ INS
P
L⁻H⁺
↑ CO2
GLUCOSE
LOADED
-TPP

Materials and Methods

General

Male Sprague-Dawley rats obtained from Bio-Breeding Laboratories (Ottawa) were used for these studies. All animals were housed in individual cages, those on the thiamine deficient (TD) or pair fed (TP) diets were kept in metabolic cages, the weight (WC) and age (AC) controls in regular plastic rat cages. Conditions were controlled for humidity and temperature, with lights on in the room from 0730h to 1730h. Plastic cages were changed twice weekly and water freshened daily. All animals received water ad libitum. Rats of the weight and age control groups received the standard Purina Rat Chow in pellet form, ad libitum.

The thiamine deficient and pair fed animals received a special diet prepared by ICN Pharmaceuticals Inc., Cleveland. The powder form of this diet was chosen as it could be weighed more accurately. Two batches were prepared, with matched analysis of ingredients, excepting the thiamine content. The thiamine deficient diet contained no measurable traces of thiamine, while the control diet (for the pair fed animals) was supplemented with 1.0g/45.4kg thiamine hydrochloride.

The animals were fed normal laboratory chow for two to three days upon their arrival at our animal quarters. After this accomodation period they were randomly divided into groups and put into individual cages. The mean weight of the rats was about 68g.

Housing the special diet rats in metabolic cages served at least two functions. The wire bottom cages provided for immediate removal of organic waste, eliminating the possibility of reconsumption of food and/or vitamins. Secondly, the design of the metabolic cages was such

that the food was placed in a glass jar which was suspended from a trough protruding from the front of the cage. A plastic cup was fitted inside this jar, which facilitated removal of the food for weighing. At day one of the experiment, the total weight of the cup and food for both the thiamine deficient and pair fed group was 30g, constituting about 22g of food. Each day after this, the food cups were removed from the cages of the thiamine deficient animals and weighed. By subtracting this amount from the 30g, the food consumption of the previous 24 hours was calculated. The difference was the exact amount of special control food placed in the cups of the pair fed animals. The animals were specifically paired such that the rat of cage TP-1 always received the gram equivalent amount of food eaten the day before by rat TD-1. After this the thiamine deficient food cup was refilled each day to a total of 30g. This regime was strictly maintained as long as possible. However, after about three weeks on the thiamine deficient diet, this group of animals became anorexic and began spilling tremendous amounts of food from the special cups. Observation of the rats not spilling their food indicated their food consumption was approaching nil. When any individual animal of the thiamine deficient group reached this point, its pair fed control was given 3.0g of the special control diet daily. This was done to prevent causing the animal undue harm by starvation, yet little enough food was provided to maintain "control" status.

All rats were weighed every second day.

Plasma Samples

Approximately one hour before sacrifice, rats were injected with 36 mg/kg sodium pentobarbital (Abbott Laboratories Limited, Montreal). After allowing 25 minutes for the anaesthetic to take effect, the tail of the animal was cleansed with alcohol, and a small, approximately 1mm, section of the tail tip clipped off. Blood was then milked from the tail directly into a heparinized hematocrit tube (American Hospital Supply Company, Miami), which was then stoppered and placed on ice. A total of three samples approximately 80µl each were drawn from each rat. The sample tubes were spun for five minutes in a model MB micro hematocrit centrifuge (International Equipment Company, Needham Heights, Mass.), at $4^{o}C$. The hematocrit tubes were maintained at cold temperature until used for assays, usually one to two hours. Multiple samples from the same rat obtained in this fashion and stored for periods of five minutes to 120 minutes did not show significant variation of lactate or pyruvate concentration.

Following collection of blood samples, the animals were sacrificed by cervical dislocation.

Fatigue Experiments

Immediately after sacrifice, the entire diaphragm was removed and rinsed in oxygenated Tyrode solution. The final ionic concentration of this solution was: Na, 145.5mM; Ca, 2.5mM; K, 4.7mM; Mg, 3.1mM; SO_4^-, 3.1mM; Cl, 129.0mM; HCO_3^-, 25mM; HPO_4^-, 1.2mM; and glucose, 5.5mM. After rinsing, the entire diaphragm was transferred to a paraffin dissection bath, where removal of extraneous tissues and excess rib was

performed. At this point an incision was made along the centre of the central ligament to the sternum, and the sternum cut in half. One hemidiaphragm was mounted such that a portion of the central ligament remaining attached to the diaphragm tissue was clamped into the teflon bracket (figure 2-A). The portion clamped into the bracket was that portion of muscle where the individual fibres attached perpendicular to the ribs. Tightening of the clamp was executed carefully so as to ensure only the central ligament and no part of the muscle fibre was pinched. The muscle width was trimmed, including the rib, the final width being 9-11mm. The other end of the diaphragm was secured to a plexiglass bracket (figure 2-B) with three sutures, ensuring the ribs would not move during muscle contraction. The whole preparation was then transferred to the 100ml muscle bath (figure 2-E), containing Tyrode solution continuously aerated with 5% CO_2 in O_2. The entire apparatus was maintained at 37^{o}C with a heating/circulating pump (Precision Scientific). Isometric tension was transferred from the muscle via a silver chain (figure 2-C), connected to a model FT03C force-displacement transducer (Grass Equipment Company, Quincy, Mass.) (figure 2-D). The signal was traced on a model 7 Polygraph, employing a model 7P1A low-level DC preamplifier and model 7DAB DC driver amplifier (all from Grass Equipment Company). Resting length of the muscle was set to produce 2 grams tension.

Field stimulation of the muscle was induced by charging platinum plates (figure 2-F). Pulse trains were generated by a Digitimer type 3290 in series with a Logic Unit type 3080, Gated Pulse Generator type 2521 and an Isolated Stimulator MKIII, all from Devices Instruments Ltd., Welwin.

Figure 2. Apparatus for studying muscle fatigue.

A - teflon bracket

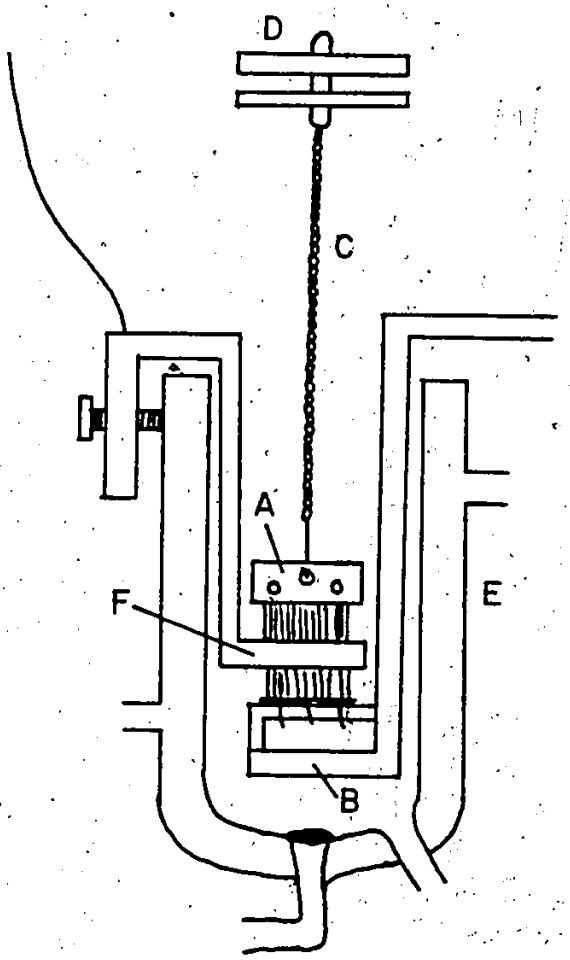
B - plexiglass bracket

C - silver chain

D - force-displacement transducer

E - 100 ml muscle bath

F - platinum plates


D
C
A
F
E
B

The output of this was modified by a model S4G stimulator (Grass Equipment Co.) and fed into a homebrew current booster before being applied to the platinum plates. Tetanic muscle contraction was stimulated by a 20-50 volt pulse train at a frequency of 150 per second, for a period of 200 milliseconds, once every 180 seconds. Immediately after the muscle preparation was in place in the muscle bath, a series of procedures was followed to elicit maximum isometric tension. The stimulator voltage was varied from 10 to 70 volts, and was reset at the voltage which generated maximum tension, (usually 50 to 55 volts). In addition, the resting length of the muscle was adjusted in small increments to obtain the optimal resting length of the muscle, judged as that length at which maximal response to the electrical stimulus occurred. Fatigue of the isolated diaphragm tissue was induced by altering the cycle length from 180 seconds to 2 seconds, for a period of 5 minutes. If on a given day a thiamine deficient animal was sacrificed, the matched animal of the pair fed group was also, and the two studied concomitantly. In order to freeze the muscles instantaneously at the end of the experiment a pair of Wollenburg clamps with faces of 25mm by 12mm was prepared. The muscle was blotted and frozen by holding it between the plates of the Wollenburg clamps which were precooled in liquid nitrogen. The dimensions of the frozen muscle tissue were measured using a caliper and storage for future biochemical analysis was provided at -60°C.

Estimation of muscle thickness and normalization of tension

Diaphragm thickness was estimated by dividing the weight of the muscle sample by the product of the length, width and a factor of 1.05, to take into account muscle density.

Normalization of muscle tension was somewhat more complicated. First, gram tension was converted to the force equivalent in Newtons. Secondly, the cross sectional area of the muscle was estimated by dividing the muscle sample weight by the product of the length and density factor (1.05). Normalized tension was calculated as the tension divided by the cross sectional area.

Efflux experiments

Immediately following sacrifice, the animal's abdomen was exposed and the descending aorta isolated. The aorta was cannulated with size PE90 polyethylene tubing (Clay Adams). A 30ml syringe was used to flush out the diaphragm, a total of 90ml Tyrode solution being used for this purpose. The entire diaphragm, still intact on the ribs, was removed from the animal, and rinsed in Tyrode solution. Following rinsing, the whole preparation was transferred to a paraffin bath filled with fresh Tyrode, where excess ribs and extraneous tissues were removed. An incision bissecting the diaphragm from the central ligament to the sternum was performed. One hemidiaphragm was secured by three pins through the central ligament, and the diaphragm was carefully peeled off the remaining rib bone with a razor blade. Great care was taken to cause as little tissue damage as possible. The central ligament of the diaphragm was positioned in a teflon clamp (figure 3A-B), so that a section of

Figure 3a and 3b

Apparatus for efflux experiments.

A - gas equilibration unit

B - teflon clamp

C - stainless steel bar

D - custom muscle bath

E - 5% CO_2 in O_2 gas cylinder

F - polyethylene tubing

G - coiled tubing

H - scintered glass

K - 37°C constant temperature bath

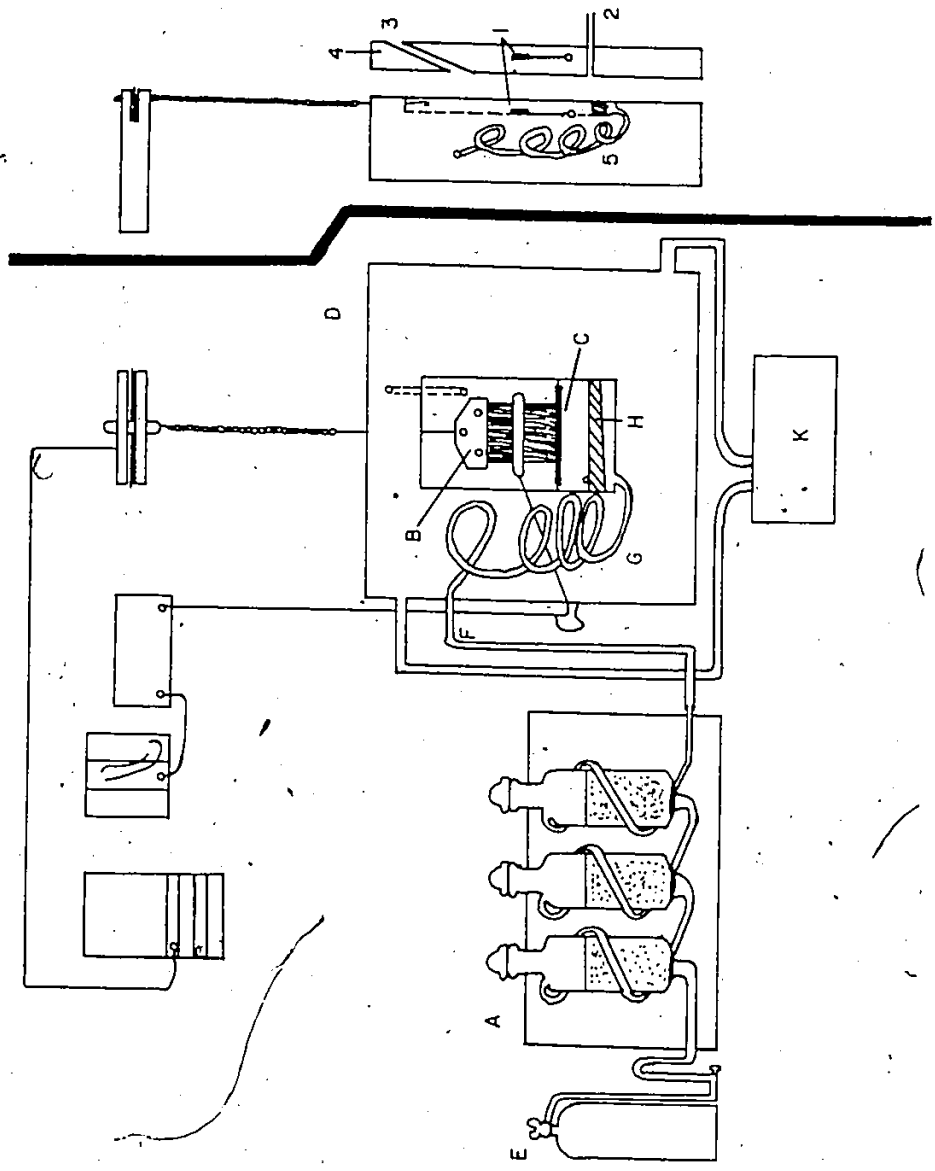
1 - platinum electrodes

2 - drain tube

3 - injection tube

4 - front piece

5 - coiled tubing


3B
3A
1
2
3
4
5
A
B
C
D
E
F
G
H
K

muscle fibres all the same length and totaling 9mm wide was central. The muscle was then secured in this position by tightening of the clamp. Excess muscle was trimmed away, leaving only the 9mm central portion secured in the clamp.

The free end of the diaphragm was secured to a stainless steel bar (figure 3A-C) designed specifically for the custom made bath (figure 3A-D). The bar was 19mm wide, 15mm of which were usable for muscle attachment, with 2mm provided off each side to secure the bar in the muscle chamber. In securing the muscle to this bar, care had to be taken not to damage any muscle fibers by pinching them between the bar and sutures. To prevent this, a second bar was employed, to be used as a spacer. Eight 000 silk, black braided, silicone treated; style 1013-40 sutures, (American Cyanamid Company, Pearl River) were tied through the muscle and around both stainless steel bars. After all eight were knotted, the second bar was removed, and the suture loops remained around the custom designed bar. In this fashion, all the loops were of equal size, ensuring the base of the teflon clamp and the stainless steel bar were parallel and that tension generated by the muscle would be distributed equally along the clamp.

The entire preparation was transferred to a custom designed muscle bath chamber (figure 3A-D). The clamp was attached via a bar, which exited the muscle chamber, and a silver chain to a Grass model FT03C force-displacement transducer. The transducer input to a Grass model 7P1A low level DC preamplifier and the model 7DAB polygraph DC driver amplifier in series. Recordings were made with a Grass model 7 Polygraph. The front piece (figure 3B-4) was fitted into place and secured with four wingnuts,

and the muscle flushed with 150ml Tyrode solution which was preheated to 37°C and aerated with 5% CO_2 in O_2. The whole rinsing process lasted about 45 minutes.

At the beginning of the rinse process, the muscle was set to optimum length for maximal contraction response to an electrical stimulus, as described for the fatigue studies.

The electrical stimulus was applied as a field stimulation by supplying a DC pulse train to platinum electrodes (figure 3B-1) mounted on the plexiglass surface of the muscle chamber and front piece. This stimulating current was generated by a Grass model S4C stimulator, with gating provided by a type 160A power supply, 161 pulse generator, and type 162 waveform generator, all from Grass.

Five per cent CO_2 in O_2 equilibrated for temperature and water vapour content was bubbled through the chamber. This was produced by bubbling the gas from a cylinder through a custom designed equilibration chamber with three chambers in series (figure 3A-A). The chambers contained 117mM NaCl which was maintained at 37°C by enclosing all three chambers with an outer jacket and circulating heated water with a heating bath/ circulating pump (Haake model FJ). The equilibrator outlet was connected to the muscle chamber gas inlet by a small piece of polyethylene tubing (figure 3A-F). From the bath inlet to the scintered glass of the bath (figure 3A-H), a coiled tubing was used (figure 3A-G and figure 3B-5). This provided a final temperature equilibration of the gas before it reached the muscle tissue.

To start the experiment, the remaining rinse fluid was drawn out of the bath chamber with a syringe, through drain tube (figure 3B-2), and .75ml fresh Tyrode injected by a second syringe, through a small hole in the front piece (figure 3B-3).

Throughout the experiment, samples were collected in this fashion, every 20 minutes. The sample was immediately placed in a second glass bath (figure 4) where H^+ concentration was measured.

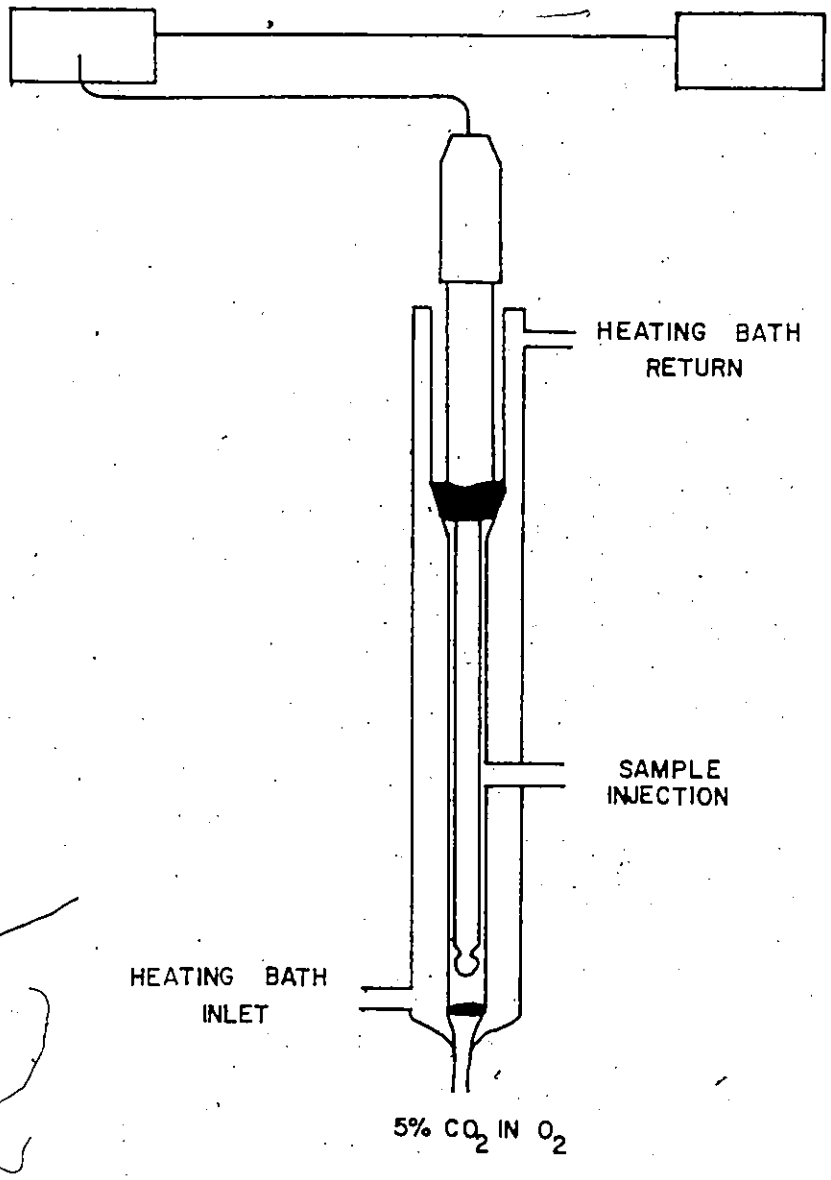
One inherent problem of the sampling method was the residual volume of bathing fluid in the muscle bath. The fluid remaining in the chamber after the sample was considered withdrawn, would obviously have an effect on the H^+, lactate and pyruvate concentration of the next sample. The actual residual volume, as determined using sucrose-C^{14}, was approximately 10% of the total, Or 75µl. This residual bathing fluid may tend to somewhat buffer any acute changes of H^+, lactate or pyruvate concentration in the successive sample.

The values calculated as the efflux rates can be corrected for this inaccuracy. Letting V represent the amount of fluid injected and withdrawn from the muscle bath; C_1, the concentration of the substance from the last sample, C_2, the concentration of the substance in the present sample; and E_2, the amount of substance which will efflux from the muscle tissue into the present sample; and x the residual volume, then:

$$C_2 = \frac{E_2 + C_1 x}{V + x}$$

Using this calculation, it can be shown that the difference between the measured concentration of the substance, and the actual

Figure 4. Apparatus for measurement of pH.


HEATING BATH
RETURN
SAMPLE
INJECTION
HEATING BATH
INLET
5% CO_2 IN O_2

concentration due to efflux from the muscle into the present sample is represented by $x(C_2-C_1)$.

The use of this formula illustrated that the end effect of the residual volume on the H^+, lactate and pyruvate concentration of the next sample would introduce a maximal error of 5%, and thus no correction factor was applied.

At the end of the experimental period, the front piece was removed and the resting muscle dimensions recorded. The muscle was then removed from the clamp, the sutures cut, blotted and frozen with liquid nitrogen. The frozen muscle weight was recorded. Tissues for biochemical analysis were stored at -60°C until assayed.

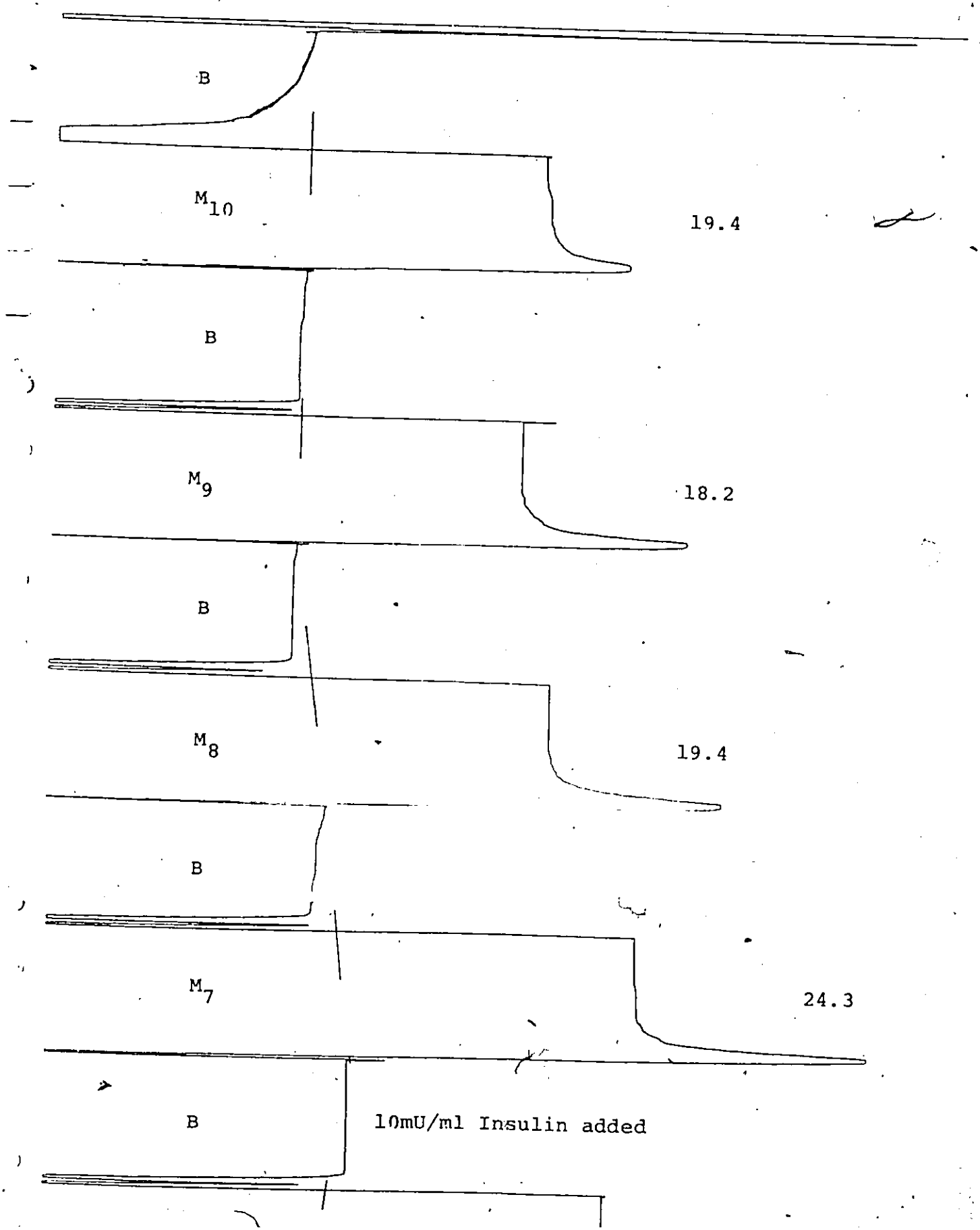
Measurement of H^+ concentration

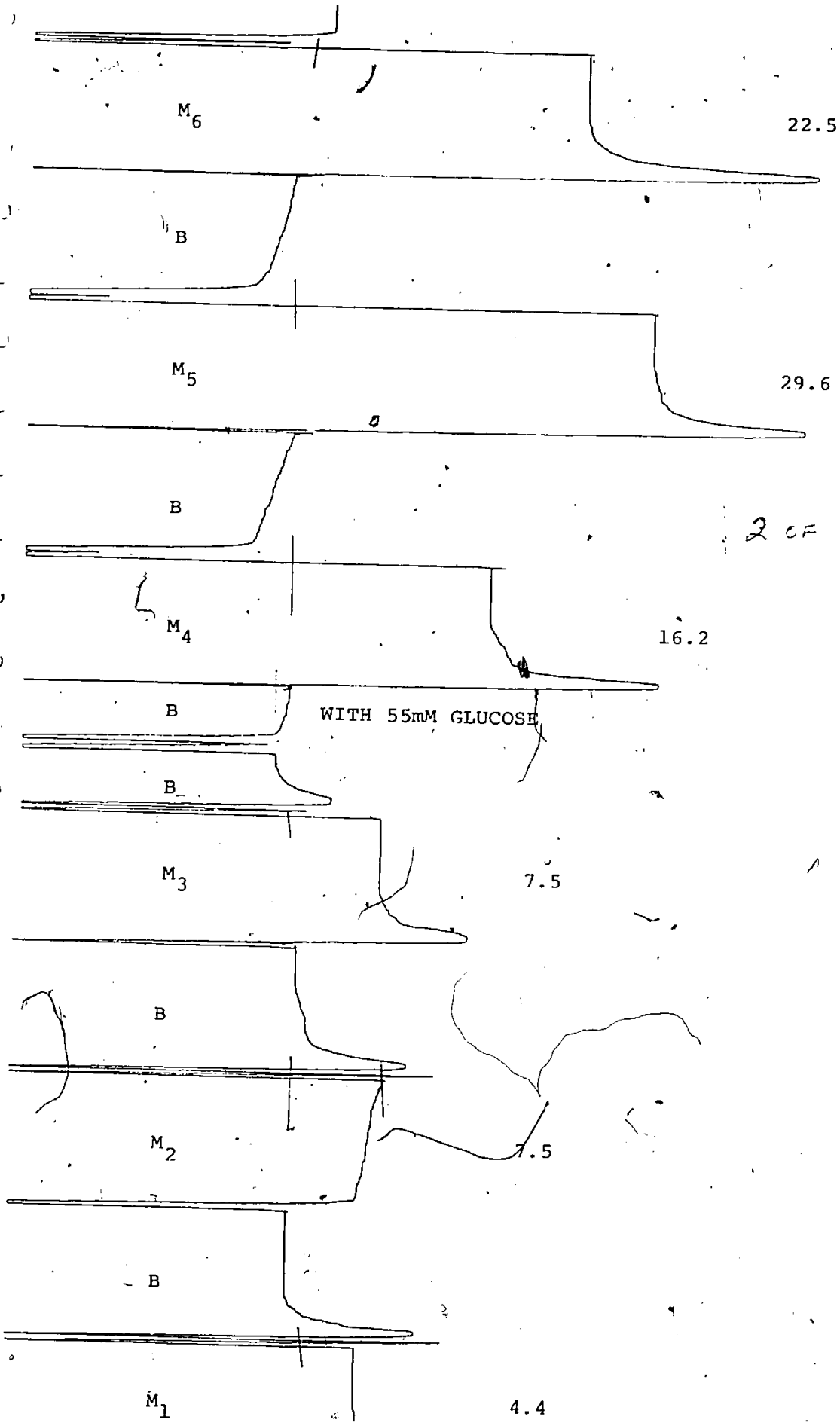
As mentioned above, samples were injected into the pH measurement chamber (figure 4) immediately following removal from the muscle bath. The 5% CO_2 in O_2 (figure 3A-E) bubbled through this chamber was equilibrated with a unit the same as that described in the previous section (figure 3A-A). Temperature of the pH measurement chamber was maintained at 37°C with a heating bath/circulating pump (Heto model 01 T 623; figure 3A-K). The glass pH measurement chamber was designed specifically to be used with a Radiometer GKS73041 KQ1 pH probe. This probe has a diameter of 4.5mm, and only requires 9mm immersion for reliable measurements. These characteristics allowed pH measurement of the .75ml sample collected from the muscle bath. pH was recorded with

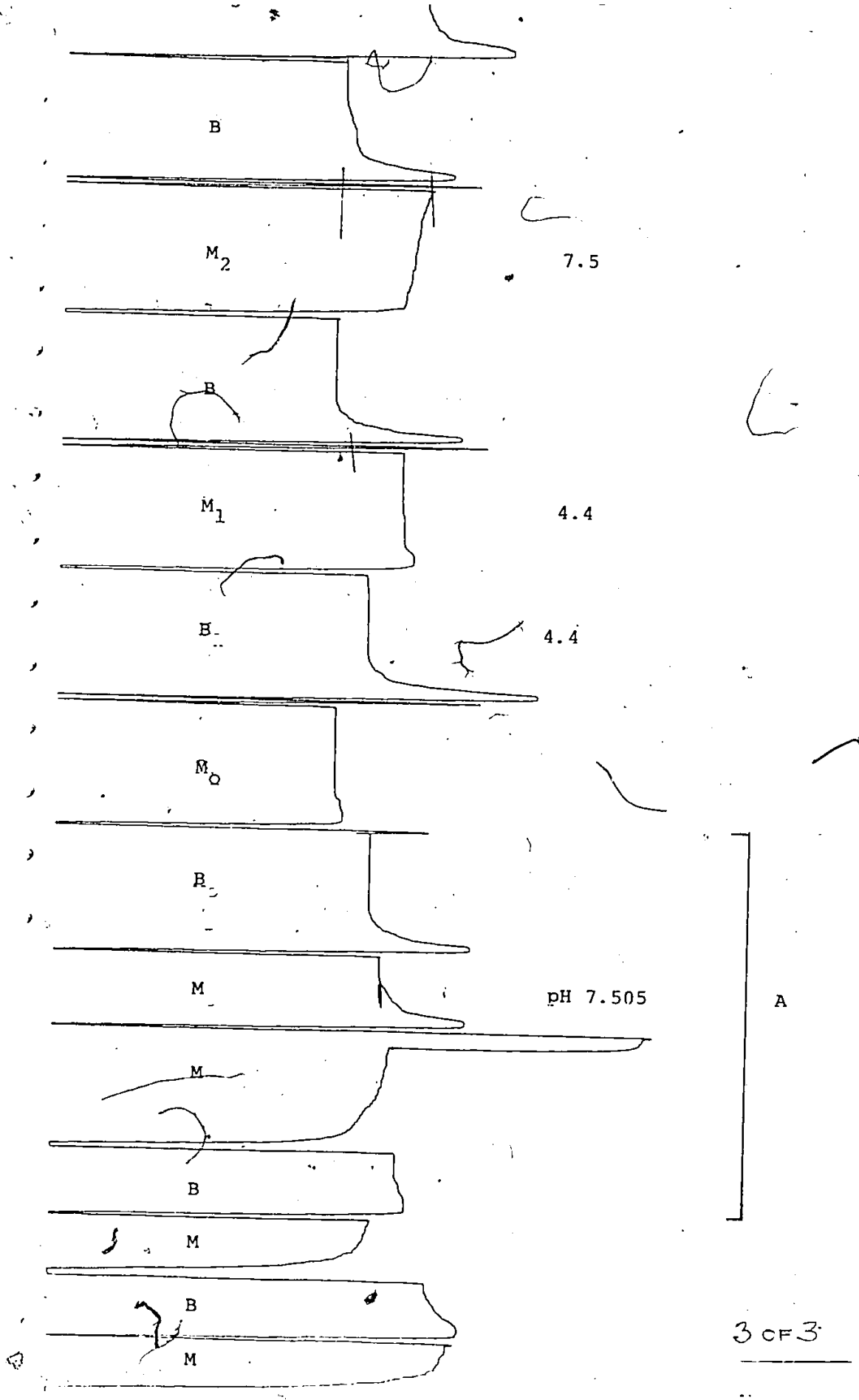
a Radiometer PHM64 research pH meter connected to a Sargent Welch model TRG chart recorder. The external recorder provided an expanded scale for measurement of H^+ concentration. Sample pH was recorded for a period of 10 minutes, after which the sample was removed from this chamber by a syringe with flexible polyethylene tubing and stored at $0^{\circ}C$ in glass culture tubes until used for biochemical assay, usually 1 hour. When the sample was removed from the pH chamber two individual .75ml aliquots of fresh Tyrode were injected. The first was simply used to rinse out the chamber from any muscle bath sample remaining. This was then removed and discarded and the second fresh Tyrode injected. The fresh Tyrode was used as the control pH of the buffer system alone, and the difference in H^+ concentration between the muscle bathing fluid and the control was taken to the H^+ which effluxed from the contracting diaphragm. Figure 5 is a duplication of an actual tracing from the pH recorder.

The muscle samples are labelled consecutively M1, M2, etc. in figure 5. On the tracing, each muscle sample is preceded and followed by a control bath sample, labelled "B" which was the fresh Tyrode solution sample. Before the muscle was positioned in the chamber, samples of Tyrode were tested from the muscle bath and the Tyrode stock to establish any difference in pH of the Tyrode which may be due to accumulation in the muscle chamber. The results of this test are illustrated in section "A" of figure 5. The muscle bath sample labelled "M" has the same pH as those labelled "B" which were samples of the Tyrode stock. A slight variation is evident, but is no more marked when samples from the muscle bath are compared to those from the stock bath, than comparison of

Figure 5. An actual tracing of the pH recordings on the Sargent-Welch recorder. pH was recorded using the apparatus illustrated in figure 4. Sample labelled "MO" was Tyrode solution from the muscle chamber before the muscle was put in place. "M1", "M2" etc are measurements of samples which bathed the muscle for 20 minutes. Sample "B" is fresh Tyrode solution. Numbers beside sample measurement represent difference in units between muscle sample and fresh tyrode solution. Unit scale: 25 units = 10 mpH units.


B
M10
19.4
B
M9
18.2
B
M8
19.4
B
M7
24.3
B
10mU/ml Insulin added


M6
22.5
B
M5
29.6
B
2 OF
M4
16.2
B
WITH 55mM GLUCOSE
B
M3
7.5
B
M2
7.5
B
M1
4.4

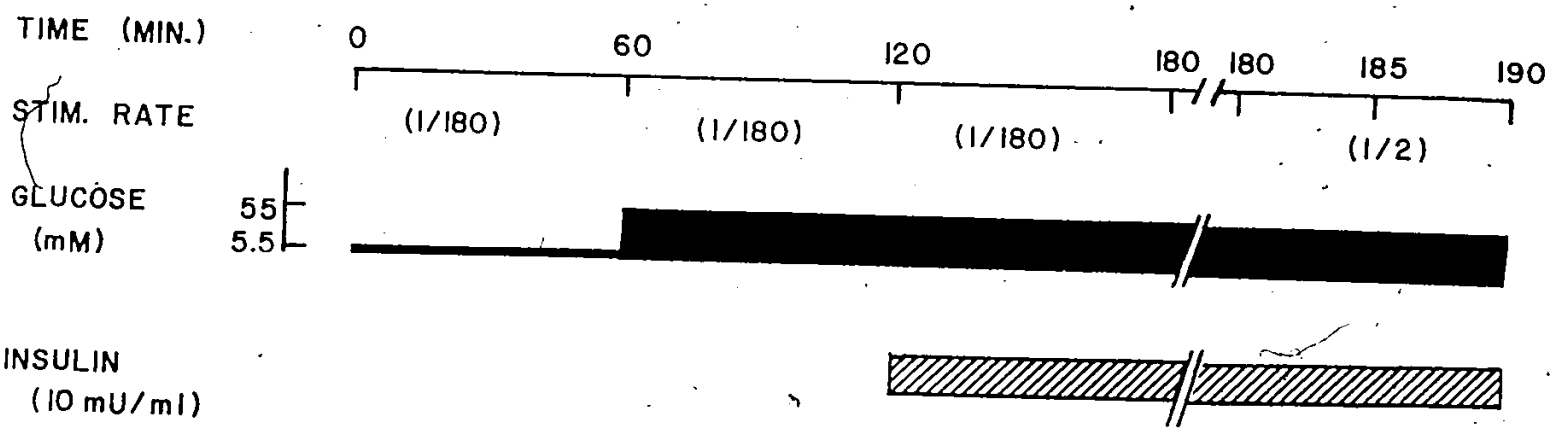

B
M2
7.5
B
M1
4.4
B
4.4
M0
B
M
pH 7.505
A
M
B
M
B
M

consecutive samples from the stock bath. Because of the indicated variation of Tyrode stock pH, a method of measurement to allow comparison to the samples from the muscle bath had to be standaridized. The final value of the Tyrode stock bath pH preceding and following any individual muscle sample determination were connected with a rule. A line transecting the line formed when the particular muscle sample was withdrawn, was drawn, and this was considered to be the mean pH measurement of fresh Tyrode for any particular muscle bath sample. The difference between this point and the tracing following 10 minutes pH determination of a muscle sample was used to calculate total H^+ efflux from the muscle over the 20 minute collection period of that sample. For a detailed analysis of H^+ concentration, see appendix A.

Experimental protocol

The protocol, as illustrated in figure 6, was the same throughout all experiments, once the equilibration period was over. For the first 60 minutes, Tyrode with 5.5mM glucose served as the muscle bathing medium. In the second 60 minute period, or the period from 60 minutes to 120 minutes, the concentration of glucose in the Tyrode solution was increased 10-fold to 55mM. One hundred and twenty minutes after the experiment was started, 10mU insulin was added to the high glucose-Tyrode solution. This Tyrode-high glucose-insulin solution was also used as the bathing medium for those muscles undergoing 10 minutes fatigue. For the first 180 minutes, the muscle received a tetanus inducing stimulus every 180

Figure 6. Diagram of experimental protocol. The muscle was bathed with 3 variations of tyrode solution of 60 minutes each. The first was a normal Tyrode solution with 5.5mM glucose. For the second 60 minutes, 55mM glucose-Tyrode solution was used as the bathing medium. Throughout the third 60 minute interval, 10mU/ml insulin was added to the 55mM glucose-Tyrode solution. The third solution was also the bathing medium when muscles were fatigued.

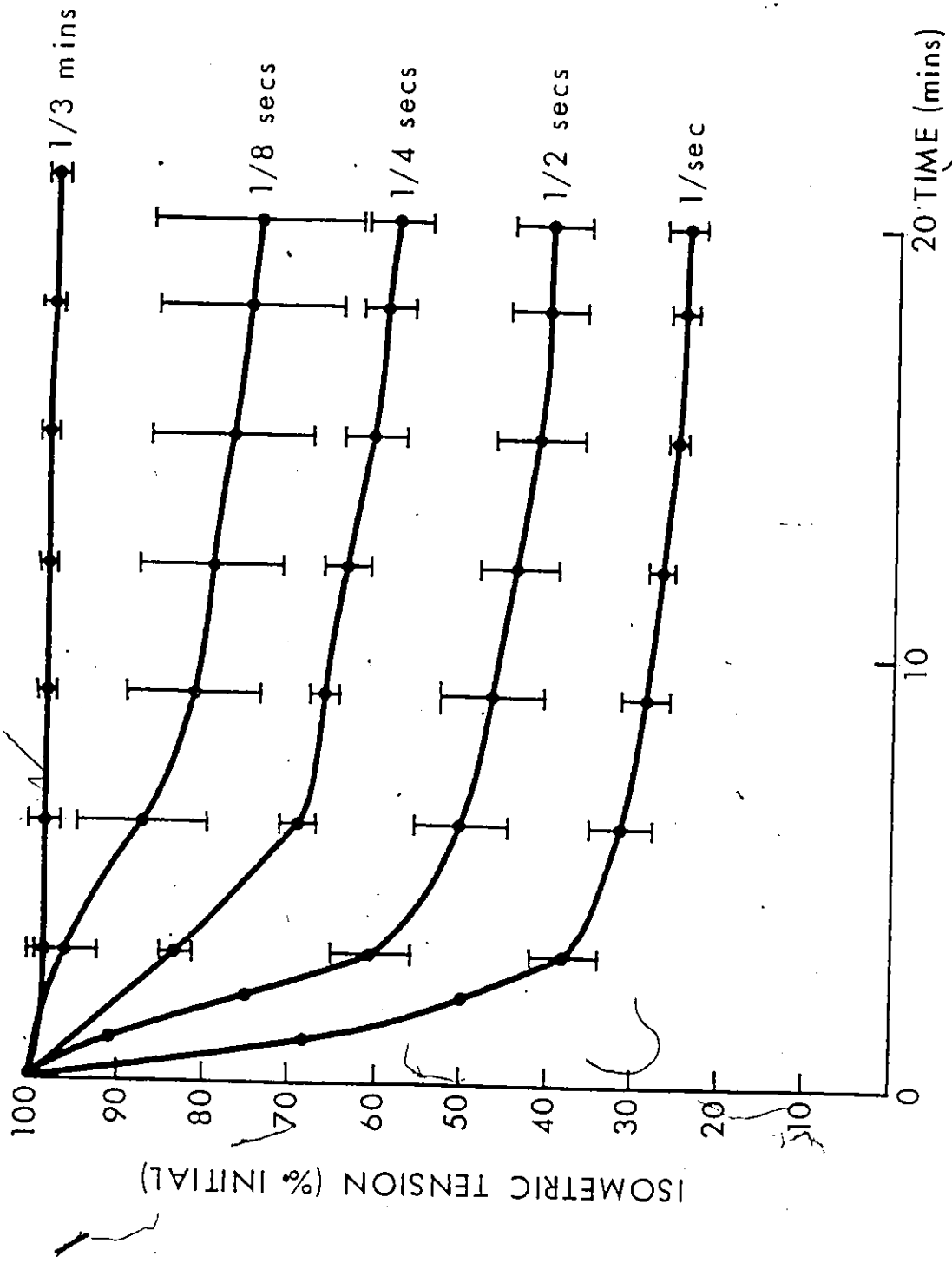

TIME (MIN.)
0
60
120
180
180
185
190
STIM. RATE
(1/180)
(1/180)
(1/180)
(1/2)
GLUCOSE
(mM)
55
5.5
INSULIN
(10 mU/ml)

seconds. Those muscles that were to be fatigued received a stimulus once every 2 seconds for a for a 5 or 10 min. period following the first 180 minutes. This frequency was chosen as a fatigue inducing stimulus from the results of some previous work in the laboratory. Figure 7 illustrates the effect of repetitive stimulation at different frequencies on isometric tension. Although a frequency of 1 every 2 seconds did not cause maximal fatigue it did result in a very noticeable and easily measurable fatigue to about 45% of pre-fatigue tension after 5 minutes. As well, it was felt that this level of fatigue would not result in relatively permanent damage to the tissue, leaving open the possibility for studies of recovery from fatigue.

Preparation of tissue for biochemical assay

Frozen diaphragm tissues were weighed and put in a tube containing 1ml of precooled 6% perchloric acid. The tissue was then homogenized with a Polytron PT 10/35 and model PCU1 power supply (Kinematica GmbH, Switzerland). The homogenizer probe and all homogenizing and centrifuge tubes were precooled to 0°C. Following homogenization, the mixture was placed on ice for 20 minutes and then spun at 7500 x g for 10 minutes in a model B-20A centrifuge (International Equipment Company). The supernatant was poured off into a second tube, and neutralized with a measured quantity of 2.5M K_2CO_3, usually 120-150µl. The solution was placed on ice for 10 minutes, and then centrifuged at 7500 x g for 5 minutes. The supernatant was immediately removed to a third tube, where it was stored, on ice, until assay.

Figure 7. The effect of repetitive stimulation on maximum isometric tension. Diaphragms from 170g rats were stimulated at various frequencies. Initial tension was that tension developed immediately preceding the increased stimulation frequency.


1/3 mins
1/8 secs
1/4 secs
1/2 secs
1/sec
TIME (mins)
0
10
20
ISOMETRIC TENSION (% INITIAL)
100
90
80
70
60
50
40
30
20
10

Spectrophotometric assay of lactate, pyruvate and creatine phosphate

Lactate was determined by an adaptation of the methods of Olson, 1961 and Bergmeyer, 1965. In this assay, lactate dehydrogenase catalyzes the conversion of lactate to pyruvate by reducing NAD^+ to NADH. Absorbance changes were measured with a Unicam SP800 UV spectrophotometer (England) at 340mμ. Reactions were carried out in a semicarbazide buffer, pH 9.8. A .100 ml sample was put into a total cuvette volume of 1.03ml.

In a similar method described by Bucher et al, 1965, pyruvate was quantitatively measured. A triethanolamine-EGTA buffer, pH 7.5 was used, and the change in absorbance due to oxidation of NADH to NAD^+ in the presence of lactate dehydrogenase measured. A sample volume of .100ml in a total cuvette volume of 1.02ml was used.

Determination of G-6-P, ATP, and CP was done in a combined assay as described by Lamprecht and Trautschald, 1965. In a triethanolamine buffer, pH 7.4, the conversion of $NADP^+$ to NADPH was spectrophotometrically measured at 340mμ. Successive addition of G-6-P dehydrogenase, hexokinase and creatine phosphokinase provided quantitative analysis for G-6-P, ATP and CP respectively. A .100 ml sample was added in a total cuvette volume of 1.125ml.

All biochemical determination were performed at 37°C. Enzymes and reagents were purchased from Boehringer Manheim (Montreal) preferentially, or Sigma Chemical Company, (St. Louis, Missouri).

Results

Due to limitations of physical facilities, only eight rats could be prepared for each of the thiamine deficient and pair fed groups at any one time. Because of this, and the 5 week special diet period, the results illustrated in graphs are occasionally from animals in groups prepared at different times. Table 1 provides identification of the number of animals in each group. The legend of each figure identifies the number of animals involved from each group undergoing any particular treatment. In this reference scheme, the number following the group type is the total number of animals used. The numbers in parentheses refer to the number from any specific group of animals. Thus, a reference like; TD 8(4(2)+4(3)) means a total of 8 thiamine deficient animals were used, 4 from group 2 and 4 from group 3.

Table 1. RAT GROUPS.

Group	Number of rats				Number of TD survived	Number of days on diet	Sacrifice date
	TD	TP	WC	AC			
1	6	6		6	6	24-32	January 1978
2	6	6	6	6	5	28-34	February 1978
3	8	8		8	7	34-39	April 1978
4	8	8	9	8	5	38-42	August 1978

Rats were divided into groups 3 to 4 days after arrival. All were housed in individual cages, in a controlled environment.

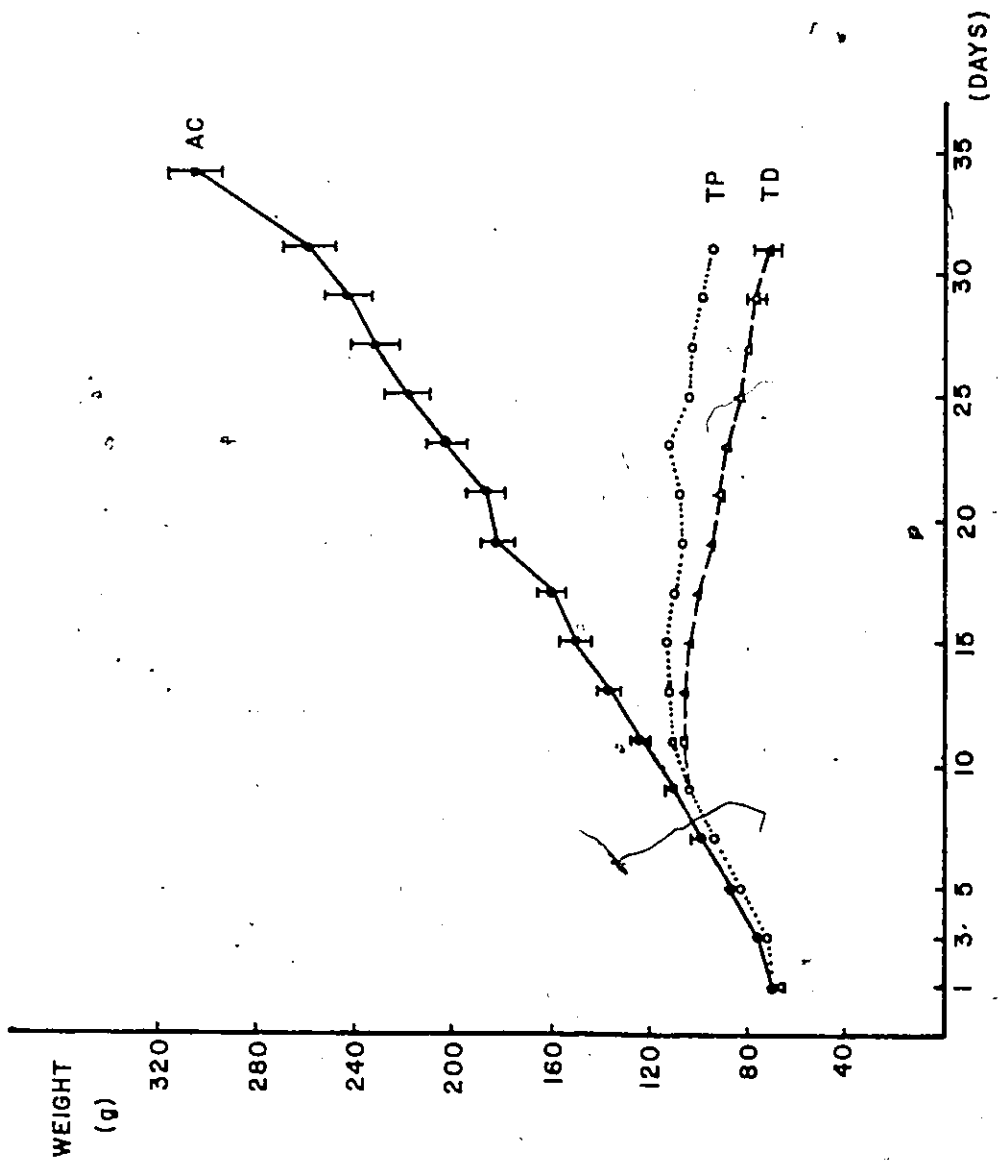
The effect of thiamine deficiency and restricted diet on rat growth

The growth of rats maintained on the three different diets used is illustrated in figure 8. Rats maintained on normal laboratory chow (Purina Rat Chow) grow with a relatively linear weight gain over the entire 35 day period. Rats maintained on the thiamine deficient diet or those that were pair fed, appeared to gain weight for the first 10-12 days, increasing in weight from a mean of 66g and 70g to 100g and 110g respectively. Whereas the thiamine deficient rats, following a plateau of 5-7 days, decreased in weight to a level non-significantly different from their starting weights, the weights of the pair fed rats also declined, but not to the same degree. When the animals were sacrificed, the thiamine deficient group and pair fed controls had mean weights of 72g and 94g respectively.

Certain qualitative changes were observed in the thiamine deficient and pair fed control groups, over the 31 day special diet period. The pair fed controls appeared to be extremely hungry. They remained relatively active in the cages, and would bite at anything within reach. These animals became difficult to handle simply because of the aggressive nature developed. In other respects the pair fed controls appeared normal, although growth stopped after about 12 days.

The thiamine deficient rats appeared much different. Again, no differences were obvious over the first 10-12 days. However, at this point gross changes began to appear. The most prevalent was anorexia,

Figure 8. The effect of thiamine deficiency on rat growth. The age control group (AC) was fed a standard Purina Rat Chow. Pair Fed (TP) and thiamine deficient (TD) groups were fed their respective special diets. Rats were weighed every second day for 31 days. All rats were from group 2, with 6 rats maintained on each diet.


WEIGHT (g)
320
280
240
200
160
120
80
40
AC
TP
TD
1
3
5
10
15
20
25
30
35
(DAYS)

which developed by day 25 into an apparent complete lack of food intake. The animals of the group became somewhat sluggish and apathetic. Between day 10 and day 20, very noticeable pilo-erection occurred. The eyes did not maintain their bright red brilliance. From day 20 on, the apathy became extreme, with the animals often remaining in a rear corner of the cage. After day 25, when movement did occur, problems of balance were obvious; the animals at this point were often not able to walk a straight line. This imbalance appeared to be of central origin, probably labyrinthine, as the animals also lost certain righting reflexes.. If they were hoisted by the tail, and slowly lowered toward a table top, no attempt was made to land on the fore-paws, with head upright. Rather the animals would most often tuck the head in. After day 25, the thiamine deficient group was also very weak, and indeed appeared to sleep most of the time. As the thiamine deficients were left on this diet, death would occur somewhere between day 31 and 35. The animals would become very disoriented, begin to convulse, and die within 1-2 hours. At sacrifice the thiamine deficient animals were very thin, the feet and paws being scaly, and having little flesh. At dissection, the muscular layers of the thorax and abdomen did not appear as robust as the pair fed or weight control groups, and of course the intestine appeared empty.

The concomitant weight loss of the thiamine deficient and pair fed groups suggests the weight loss during thiamine deficiency may be due to the anorexia, rather than being a direct effect of the induced avitaminosis.

Effect of thiamine deficiency on rat diaphragm thickness

Since, as discussed earlier, diaphragm thickness is of paramount importance to metabolic processes in the isolated diaphragm, this parameter was estimated. Figure 9 illustrates the diaphragm thickness for all four groups of rats employed. The age controls were thickest having a mean thickness of 0.69 ± 0.06mm. This was significantly larger than the thiamine deficient animals and the other two control groups, the mean diaphragm thickness being 0.29 ± 0.05mm (TD), 0.34 ± 0.05mm (TP) and 0.36 ± 0.04mm (WC). The diaphragm thickness of each of the latter three groups were non-significantly different from each other.

Effect of thiamine deficiency on plasma lactate and pyruvate levels

Figure 10 illustrates the effect that the various diets had on plasma lactate and pyruvate levels at sacrifice. The thiamine deficient animals had a significantly elevated plasma lactate level of 5.45 ± 0.45μmoles/ml as compared to 2.2 ± 0.2μmoles/ml, 3.2 ± 0.4μmoles/ml and 2.15 ± 0.3μmoles/ml observed in the age control, pair fed and weight control groups respectively. Whereas the lactate levels were significantly elevated in the thiamine deficient animals only, both the thiamine deficient and pair fed groups had pyruvate levels with mean values of 0.22 ± 0.03μmoles/ml and 0.19 ± 0.02μmoles/ml respectively. The age control and weight control mean values of 0.11 ± 0.03μmoles/ml and 0.08 ± 0.01μmoles/ml respectively were non-significantly different from the previous two groups and each other.

Figure 9. Effect of thiamine deficiency on rat diaphragm thickness. Diaphragm thickness was calculated by dividing the weight of the frozen diaphragm tissue by the tissue dimension multiplied by 1.05 to take into account tissue density. The number of animals used was: AC 6 (group 2), TD 9(5(2)+4(3)), TP 10(6(2)+4(3), WC 5(2). Asterisk denotes statistically significant difference ($p<.05$) relative to the other groups. Values represent mean ± S.E.M.

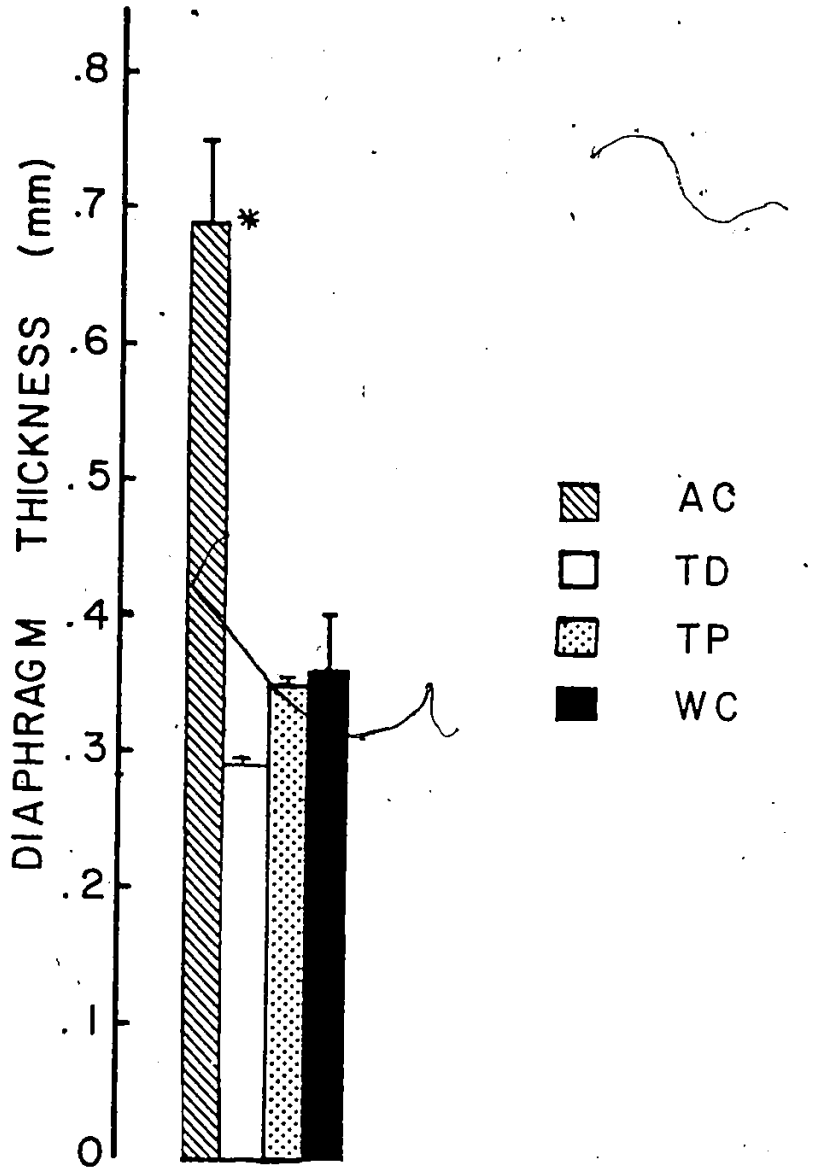
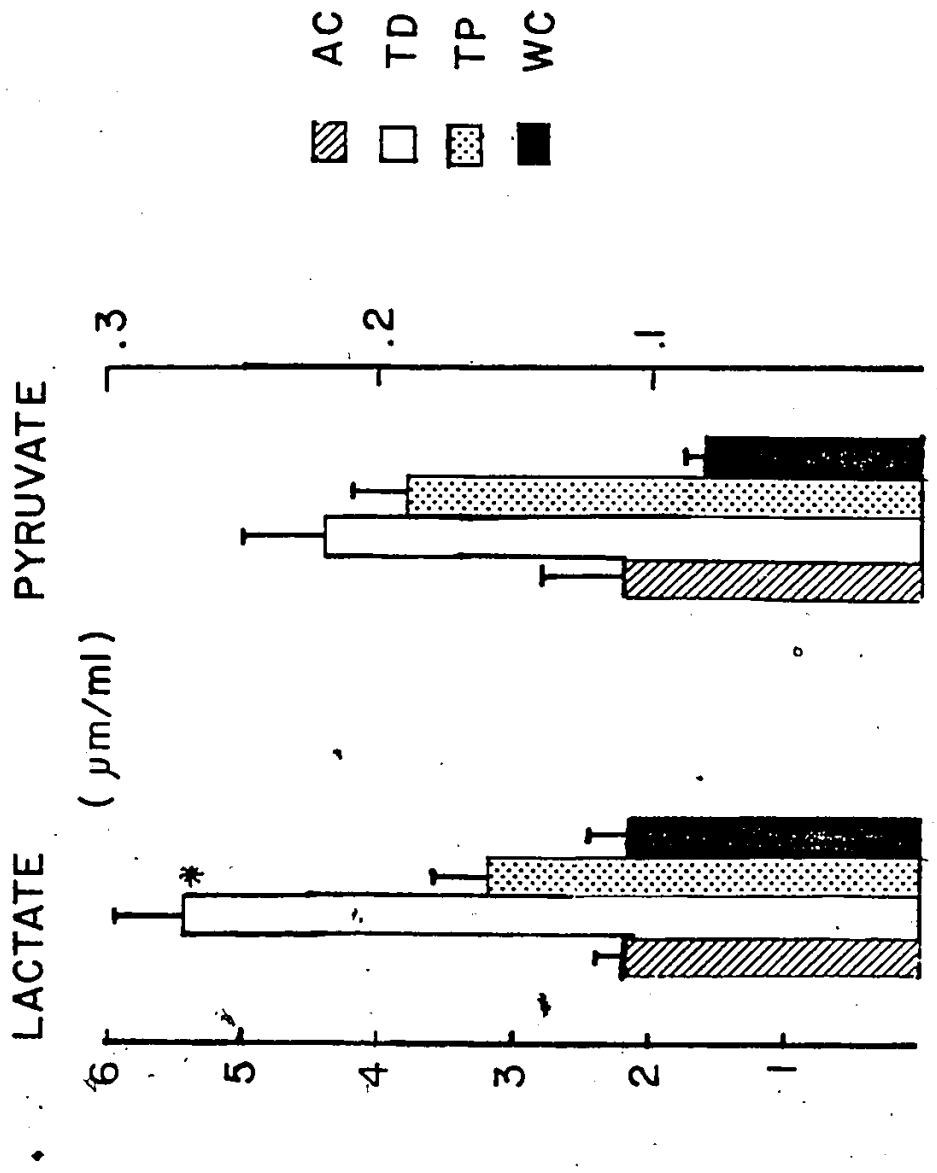

DIAPHRAGM THICKNESS (mm)
.8
.7
.6
.5
.4
.3
.2
.1
0
*
AC
TD
TP
WC

Figure 10. The effect of thiamine deficiency on plasma lactate and pyruvate concentration. Immediately before sacrifice, rats were anaesthetized with sodium pentabarbital. After allowing 25 minutes to take effect, blood samples were taken in a heparinized hematocrit tube by clipping the tail tip and milking. The number of animals in each group: AC 6(group 2), TD 9(5(2)+4(3)), TP 10(6(2)+4(3), WC 5(2). Concentrations were detected spectrophotometrically. Values represent mean ± S.E.M. Asterisk denotes significant difference $p<.05$.


AC
TD
TP
WC
PYRUVATE
.3
.2
.1
LACTATE
(µm/ml)
*
6
5
4
3
2
1

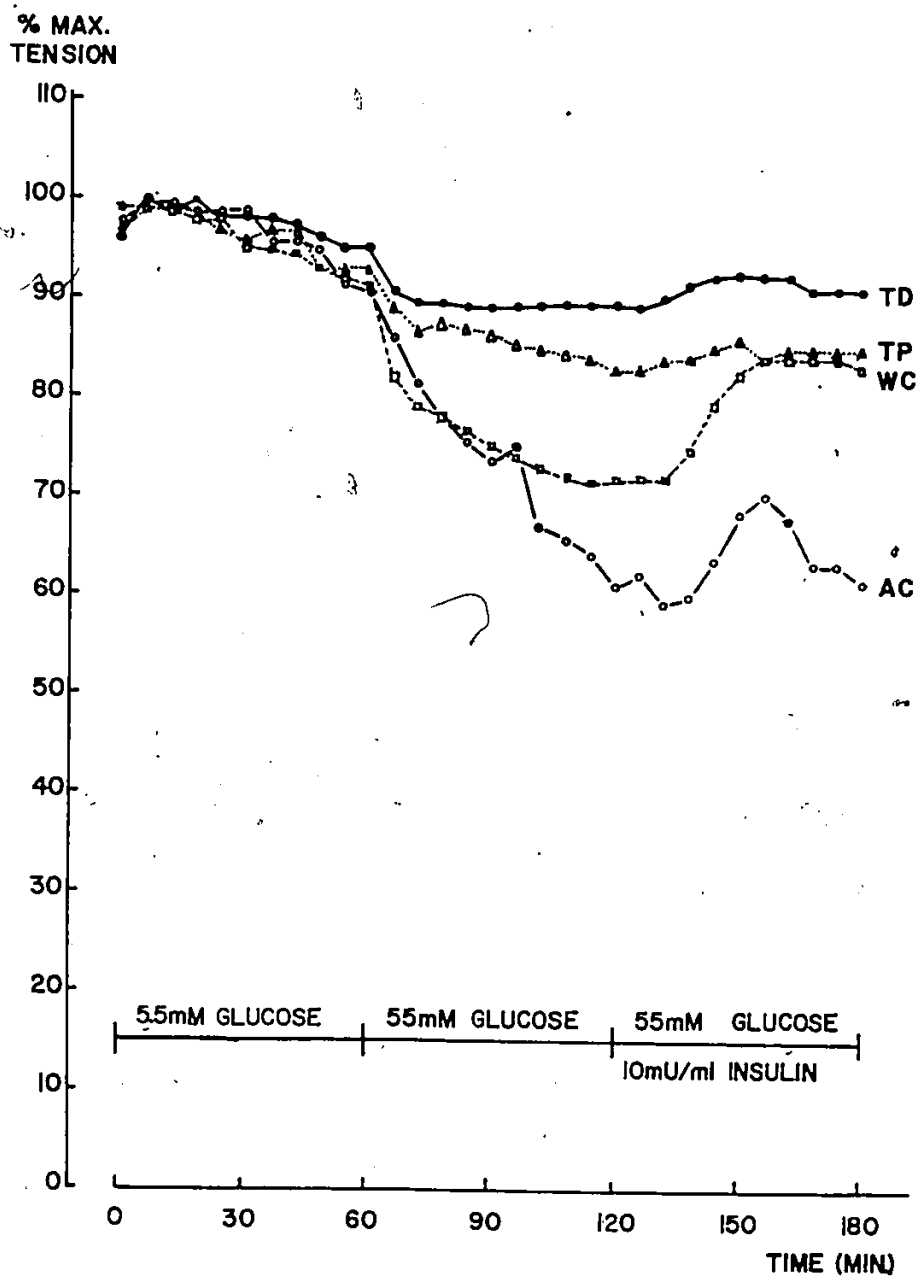
The elevated plasma lactate and possibly pyruvate levels lend support to the hypothesis that thiamine deficiency results in increased anaerobic energy production.

The effect of glucose and insulin on muscle contraction

Over the first hour, muscles were bathed in Tyrode solution containing 5.5mM glucose. Tissues from the 4 different groups all responded similarly, losing 5-10% of the maximum recorded isometric contraction tension (figure 11). The force of contraction usually increased over the first 18 minutes and then began to decrease gradually. For this reason, the strongest contraction in the first 18 minutes was considered maximum. When the bathing medium was replaced with a Tyrode-55mM glucose solution, the tissue from the weight control group underwent a characteristic response whereby tension dropped relatively rapidly from 91.5% at 60 minutes to 72% at 108 minutes, and plateaued there until insulin was added. Following the addition of insulin tension increased to 84% maximal tension observed. This increase of isometric contractile tension began 12 minutes after the addition of 10 mU/ml insulin to the bathing medium, and reached the 84% value 24 minutes later, at which point a new plateau was reached.

The thiamine deficient and pair fed groups had a tendency to react in the same manner to the bathing solution changes, but the changes were considerably less marked. Upon changing to the high (55mM) glucose-Tyrode solution, an accelerated rate of decline in isometric tension occurred for about 12 minutes. Following this

Figure 11. The effect of glucose and insulin on rat diaphragm isometric tension. Isolated rat diaphragm tissue was prepared in a 100 ml capacity glass bath for measurement of isometric tension developed upon electrical stimulation. Protocol was as explained in the Materials and Methods section, whereby the muscles were bathed for successive 60 minute periods with Tyrode solutions containing 5.5mM glucose, 55mM glucose, and 55mM glucose plus 10 mU/ml insulin. Tensions illustrated are mean values, indicated as a per cent of the maximum tension observed in the first 12 minutes. The largest contraction, in terms of tension, occurring in the first 18 minutes was considered the maximum tension. Number of animals used: TD 13(5(group 2) + 8(3)), WC 6(2), and AC 6(2).


% MAX.
TENSION
110
100
90
80
70
60
50
40
30
20
10
0
TD
TP
WC
AC
5.5mM GLUCOSE
55mM GLUCOSE
55mM GLUCOSE
10mU/ml INSULIN
0
30
60
90
120
150
180
TIME (MIN)

initial change of rate, the electrically induced isometric tension in the thiamine deficient tissues plateaued at a level of 89% maximum. Tetanic tension in the tissues isolated from the pair fed animals continued to decrease at approximately the same rate as during bathing with the 5.5mM glucose-Tyrode solution. The decline in tension of the thiamine deficient and pair fed groups was only 25% and 45% of that observed with the weight control tissues. Introduction of 10 mU/ml insulin to the 55mM glucose-Tyrode bathing medium at 120 minutes resulted in a tendency of the isometric tension to return towards that observed in the first 60 minutes of the experiment. Although all three groups recovered following the addition of insulin to levels approximating those observed immediately prior to the 55mM glucose-Tyrode solution bathing medium, the decline in isometric tension observed following the addition of this medium was significantly greater in the weight control tissues, compared to that observed in the thiamine deficient and pair fed tissues.

A progressive decrease in ability to develop isometric tension was observed with diaphragm tissue isolated from the age control animals when the tissues were bathed in 55mM glucose-Tyrode solution. Isometric tetanic tension decreased from 90.5% maximum immediately prior to changing the bathing medium, to 61% immediately before the addition of insulin. No plateauing of tension development occurred in this 60 minute interval. Addition of insulin to the 55mM glucose-Tyrode bathing medium resulted in an increased tetanic tension development, recovering to 70% maximum.

However, this recovery was not sustained, and by the end of the total 180 minutes, isometric tension had declined back to the 61% maximum level.

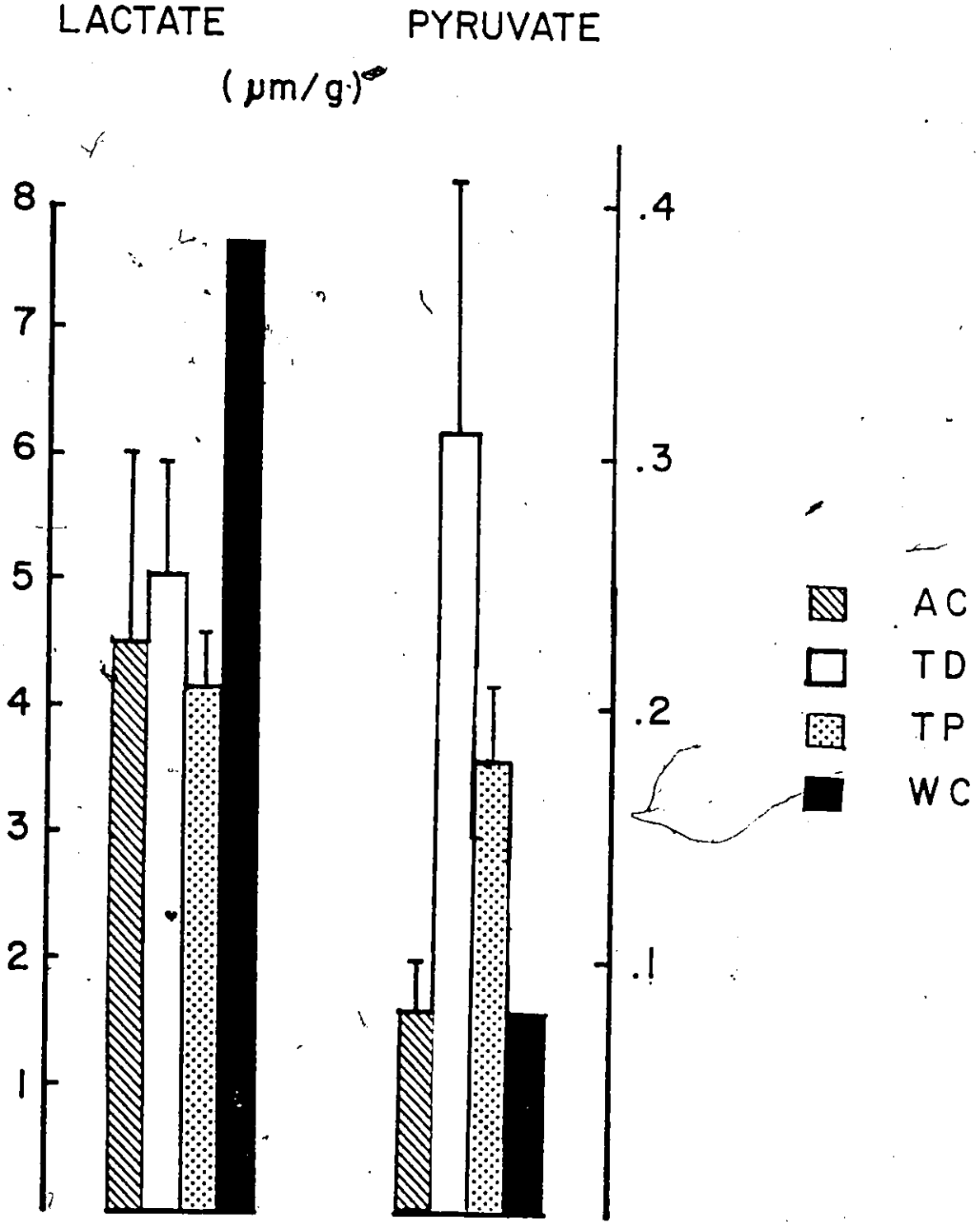
The inability of the tissues from the thiamine deficient and pair fed animals to respond to 55mM glucose-Tyrode solution, may reflect the dietary state of the muscle.

Effect of thiamine deficiency on tissue lactate and pyruvate levels

Whereas the plasma lactate concentration was elevated in animals maintained on a thiamine deficient diet, diaphragm tissue from these animals did not indicate the same (figure 12). The age controls, thiamine deficient and pair fed groups had lactate concentrations which were all non-significantly different from each other (4.5 ± 1.5μmoles/g, 5.05 ± 0.9μmoles/g, and 4.1 ± 0.5μmoles/g respectively). The weight control group was the only one to have an elevated tissue lactate, that being 7.7μmoles/g. However, the reliability of this difference is questionable, because samples from only two of these animals were available for biochemical analysis. Diaphragm tissues isolated from 3 other weight control animals, at a much later period of time, tend to support this doubt. Following the same 180 minute protocol, and freezing in liquid nitrogen, lactate levels were measured to be 2.02 ± 0.10μmoles/g.

Pyruvate concentrations were elevated in the thiamine deficient (0.31 ± 0.1μmoles/g) tissues, relative to the levels measured in the pair fed (0.18 ± 0.03μmoles/g), weight control (0.08 ± 0.02μmoles/g).

Figure 12. The effect of thiamine deficiency on tissue lactate and pyruvate concentration. Tissues were stimulated with a tetanus inducing stimulus once every 3 minutes for 180 minutes. The last 60 minutes the muscle was bathed in Tyrode solution containing 55mM glucose and 10 mU/ml insulin. Tissues were instantaneously frozen with Wallenberg clamps. Concentration of substrates was detected spectrophotometrically. Number of animals used in each diet: AC 3(group 2), TD 4(2(2) + 2(3)), TF 5(3(2) + 2(3)), WC 3(2). Values represent mean ± S.E.M.


LACTATE
PYRUVATE
(μm/g)
8
7
6
5
4
3
2
1
.4
.3
.2
.1
AC
TD
TP
WC

and age control (0.08 ± 0.04μmoles/g) tissues. There was no statistically significant difference between any two of the groups. Pyruvate concentration in the tissues of the animals sacrificed much later was 0.20 ± 0.07μmoles/g.

Effect of thiamine deficiency on normalized tension

Normalization of isometric tetanic tension on the basis of force generated per square unit area of muscle resulted in non-significant differences between animal groups. On this basis, the thiamine deficient and pair fed tissues developed tension of 20.63 ± 1.35 N/cm^2 and 18.69 ± 1.24 N/cm^2 respectively at minute 12 of the experiment. Tensions from minute 12 were normalized because the majority of tissues contracted maximally at this point in time. The tissues isolated from the weight control animals developed a mean normalized tension of 16.21 ± 1.58 N/cm^2 and the age controls 19.30 ± 2.02 N/cm^2. The lack of statistical significance between the various normalized tension suggests that the dietary restrictions imposed did not have significant effect on the contractile mechanisms of the muscle tissues.

Effect of thiamine deficiency on isolated rat diaphragm fatigue

Following the 5 minute period of fatiguing stimulation the mean tension produced by diaphragms from thiamine deficient rats was 64% of that produced in the immediate pre-fatigue period, (figure 13). Although tissues isolated from the weight control group had a similar pattern and degree of fatigue as did the thiamine deficient group (figure 13b), diaphragm tetanic tension of the pair fed group declined

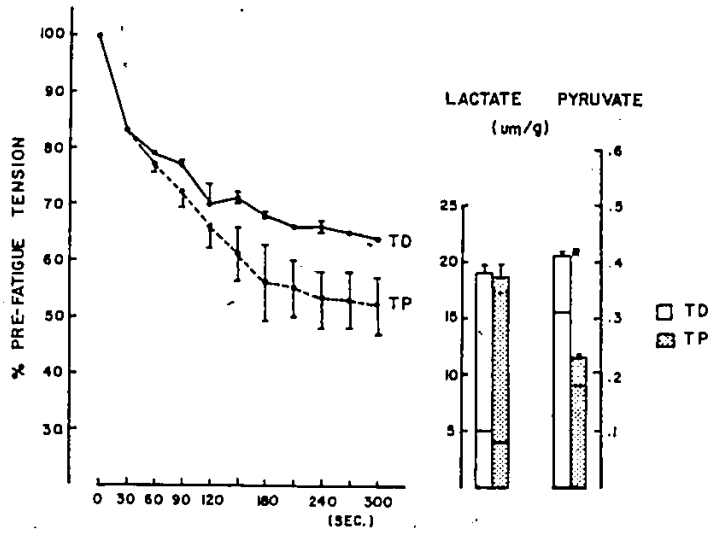
Figure 13a,b,c.

The effect of thiamine deficiency on isolated rat diaphragm fatigue.

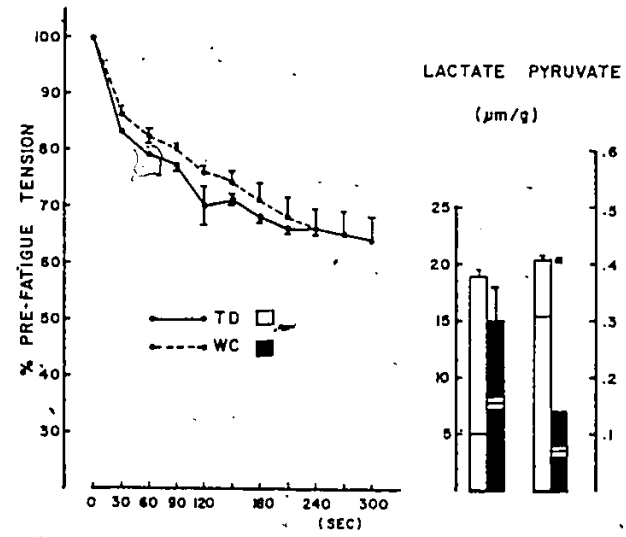
This is illustrated by the left portion of figures 13a, 13b and 13c. Isolated rat diaphragm was electrically stimulated every 2 seconds with a tetanus inducing DC train for a period of 5 minutes. The bathing medium was a Tyrode solution with 55mM glucose and 10 mU/ml insulin. The complete protocol is explained in the Material and Methods section. Each point represents mean ± S.E.M. for the following number of rats: TD 5(3(group 2) + 2(3)), TP 5(3(2) + 2(3)), WC 3(2), and AC 3(2).

Effect of thiamine deficiency on rat diaphragm lactate and pyruvate concentration following fatigue.

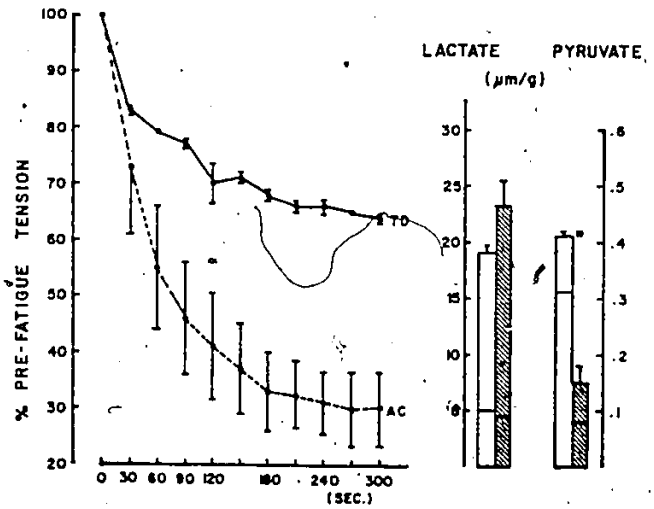
The muscles undergoing fatigue for 5 minutes were blotted and instantaneously frozen with Wollenberg clamps precooled in liquid nitrogen. Tissues were homogenized in 6% PCA and prepared for spectrophotometric assay. Values are μmoles/g tissue, represented as mean ± S.E.M. The transection of each bar represents the individual value of control tissues frozen without fatigue. Asterisk denotes a significant difference ($p<.05$). Number of animals used: Non-fatigued TD 4(2(group 2) + 2(3)1), TP 5(3(2) + 2(3)), WC 2(2), and AC 3(2); Fatigued TD 5(3(2) + 2(3)), TP 5(3(2) + 2(3)), WC 3(2), except pyruvate 2(2), and AC 3(2).


% PRE-FATIGUE TENSION
TD
TP
0 30 60 90 120 180 240 300
(SEC.)
LACTATE PYRUVATE
(um/g)
TD
TP

a


% PRE-FATIGUE TENSION
TD
WC
0 30 60 90 120 180 240 300
(SEC)
LACTATE PYRUVATE
(µm/g)

b


% PRE-FATIGUE TENSION
TD
AC
0 30 60 90 120 180 240 300
(SEC.)
LACTATE PYRUVATE
(µm/g)
TD
AC

c

to a mean of 52% of pre-fatigue levels (figure 13a). Not only were the final fatigue levels non-significantly different for these 3 groups, a similar statistical relationship was also found for each point of the curve throughout the 5 minute fatigue interval. The fatigued tissues from age control animals declined to a mean of 30% of pre-fatigue levels (figure 13c). There was a significant difference in degree of fatigue between the age control tissues and that of the other three groups. The difference was statistically significant after 60 seconds of the higher frequency stimulus, for the remainder of the 5 minutes.

Tissue lactate and pyruvate content following fatigue

Figure 13 also illustrates the tissue content of lactate and pyruvate following 5 minutes of fatigue. Lactate and pyruvate concentrations following fatigue were elevated in all tissues analyzed. The mean lactate concentration increased 3-fold in all groups with the exception of tissues from the weight control animals, where lactate concentration doubled. The levels of lactate, although varied in absolute concentration; 19.0 ± 0.68μmoles/g (TD), 18.71 ± 1.31μmoles/g (TP), 14.99 ± 3.39μmoles/g (WC), and 23.43 ± 2.54μmoles/g (AC), did not differ significantly.

Similar analysis for pyruvate proved interesting. Whereas once again, tissues of all 4 groups had an increase in pyruvate concentration to varying degrees, the mean absolute concentrations in the thiamine deficient tissues (0.41 ± 0.06μmoles/g) was significantly greater than that measured in the pair fed (0.23 ± 0.04μmoles/g), weight control (0.14μmoles/g) and the

age control (0.16 ± 0.03μmoles/g). The pyruvate concentrations of the latter 3 groups were non-significantly different. Although it appears that there should be a statistically significant difference between the pyruvate concentration following fatigue in the pair fed tissues and weight control tissues, the low number of tissues from the weight control group available for biochemical analysis (2) resulted in non-significance.

A significantly different degree of fatigue occurred only in tissues isolated from the age control animals. However, the observation that the elevation of lactate and pyruvate levels in the age control tissues was not significantly different from any of the other 3 groups suggests that increased glycolytic metabolite concentrations, of the order observed in these experiments, are not major factors in muscle fatigue.

Tissue concentration of high energy phosphates following fatigue

In all groups, except the thiamine deficient animals, there was a significant decline of ATP concentration (table 2) to 52%, 58%, and 73% for the pair fed, weight control and age control groups respectively. An even greater decline of creatine phosphate was seen, to 37%, 39%, and 29% for the respective groups. Although the observed mean glucose-6-phosphate level was different in all 4 groups, the difference was not of statistical significance.

The fact that the thiamine deficient, pair fed and weight control diaphragm tissues fatigued to about the same degree, but the ATP and CP levels declined in the latter 2 groups significantly more than in the

Table 2. THE EFFECT OF DIET ON RAT DIAPHRAGM ATP, CP AND G-6-P CONCENTRATION FOLLOWING FATIGUE.

	ATP (µmoles/g)			CP (µmoles/g)			G-6-P (µmoles/g)		
	Non-Fatigued	Fatigued	%	Non-Fatigued	Fatigued	%	Non-Fatigued	Fatigued	%
Thiamine deficient	3.93 ± .74	3.16 ± .66	80	18.76 ± 1.43	13.03 ± 3.25	69	.89 ± .11	.85 ± .09	96
Pair Fed	4.56 ± .78	2.36 ± .22*	52	19.34 ± 1.27	7.13 ± .90*	37	.46 ± .12	.69 ± .11	150
Weight Control	3.70	2.12 ± .29*	58	12.36	4.82 ± .69*	39	.51	.35 ± .10	68
Age Control	5.24 ± .25	3.82 ± .18*	73	17.14 ± 1.67	4.99 ± .37*	29	.68 ± .10	.91 ± .12	133

Tissues were frozen with Wollenberg clamps precooled in liquid nitrogen immediately following 10 minutes fatigue. Values given are mean ± S.E.M. Asterisk denotes statistically significant difference ($p<.05$) between non-fatigued and fatigued tissue values. The illustrated value, as a %, is calculated as the concentration following fatigue relative to that before fatigue. Number of animals used was: Non-fatigued TD (4(2(group 2) + 2(3)), TP 5(3(2) + 3(3), WC 2(2), and AC 3(2); Fatigued TD 5(3(2) + 2(3), TP 5(3(2) + 2(3)), WC 3(2), and AC 3(2).

first group, suggests depletion of high energy phosphate stores may not be a major contributor to muscle fatigue. Support of this idea is also found when one studies the data for the tissues of the age control group. In these tissues, the only group to fatigue to a significantly greater degree than any of the other 3 groups, ATP and CP levels also declined significantly. However no significant difference was found between the degree of decline of high energy phosphates in the age control tissues and the pair fed or weight controls.

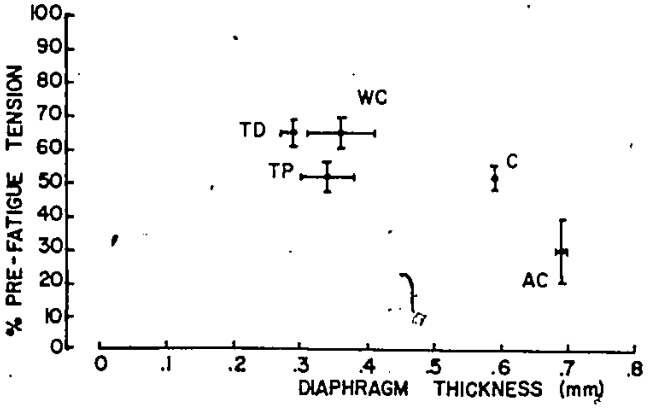
Relationship of rat diaphragm thickness to degree of fatigue, lactate and creatine phosphate levels

Plotting the degree of fatigue (from figure 13) as a function of diaphragm thickness illustrates a relationship which tends to suggest that fatigue of the isolated diaphragm preparation may be correlated with diaphragm thickness (figure 14a). Included in this figure is an extra group of rats, prepared and housed in the same manner as the age control and weight control groups, but sacrificed at about 170g. These rats, with a diaphragm thickness which was intermediate to the age and weight control diaphragm preparations, also fatigues to an intermediate degree, a mean of 50% for this group as opposed to 65% and 26% for the weight and age controls respectively. The illustration provided by this plot suggests the degree of fatigue increases as the isolated rat diaphragm muscle thickens.

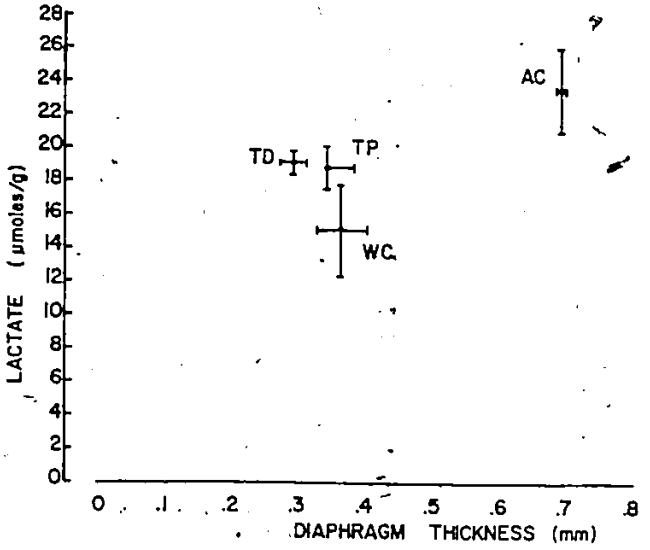
Figure 14. The effect of thickness on diaphragm fatigue, lactate, and creatine phosphate content following fatigue.

Figure 14a. Diaphragms were frozen between the surfaces of Wollenburg clamps, following 10 minutes of fatigue inducing stimulation. During this period, the Tyrode bathing medium contained 55mM glucose and 10mU/ml insulin. The complete protocol is described in the Materials and Methods section. Fatigue is expressed as % pre-fatigue tension. Values represent mean ± S.E.M.

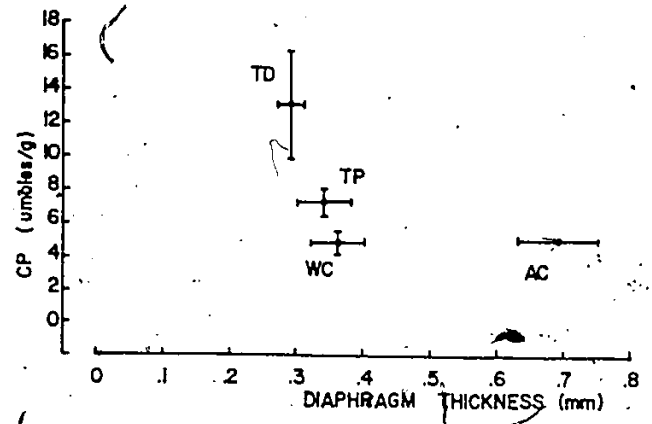
Figure 14b and 14c. The same muscles frozen in figure 12a were homogenized in 6% PCA and prepared for spectrophotometric enzymatic analysis. Values represent mean ± S.E.M. Diaphragm thickness was calculated by dividing the muscle weight by the product of the frozen muscle length, width and a factor of 1.05 to account for muscle density. The number of animals used: TD 5(3(group 2) + 2(3)), TP 5(3(2) + 2(3)), WC 3(2), and AC 3(2).


% PRE-FATIGUE TENSION
100
90
80
70
60
50
40
30
20
10
0
WC
TD
TP
C
AC
0
.1
.2
.3
.4
.5
.6
.7
.8
DIAPHRAGM THICKNESS (mm)

a


LACTATE (μmoles/g)
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
AC
TD
TP
WC
0
.1
.2
.3
.4
.5
.6
.7
.8
DIAPHRAGM THICKNESS (mm)

b


CP (umoles/g)
18
16
14
12
10
8
6
4
2
0
TD
TP
WC
AC
0
.1
.2
.3
.4
.5
.6
.7
.8
DIAPHRAGM THICKNESS (mm)

c

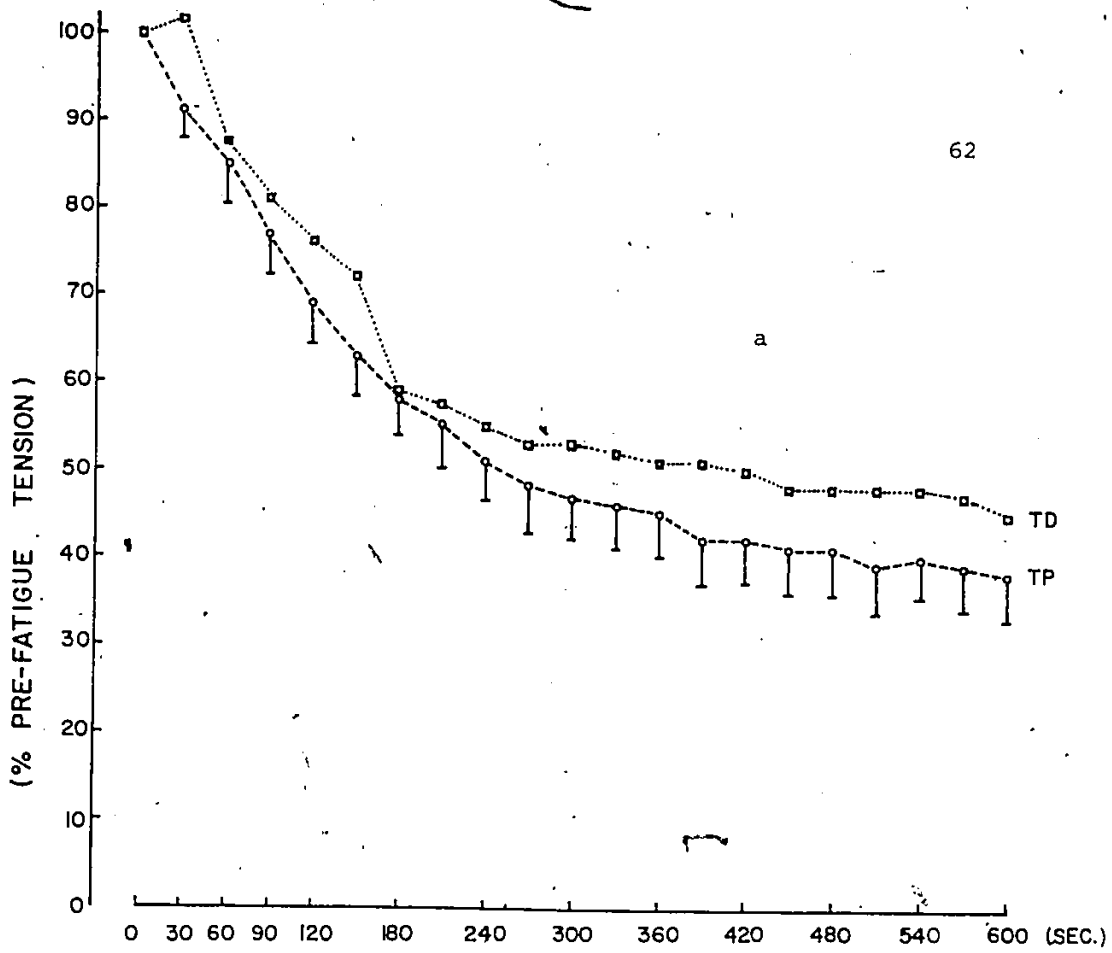
A similar pattern is suggested when lactate concentration (from figure 12) is plotted as a function of diaphragm thickness, figure 14b. Although muscles of the intermediate size were not available, the tendency is the same, indicating that muscle lactate concentration, following 5 minutes fatigue may also be correlated with diaphragm thickness.

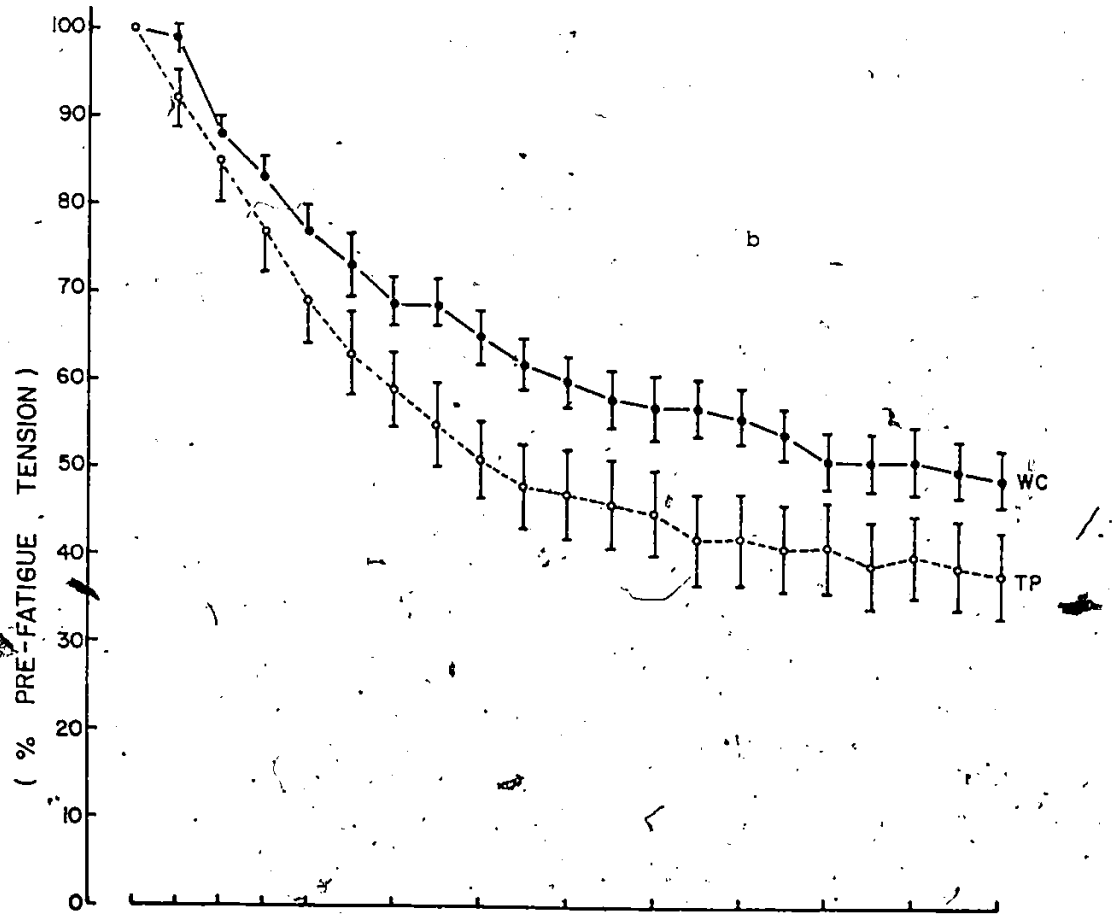
Correlation of isolated diaphragm creatine phosphate depletion, following fatigue (from table 1), with diaphragm thickness does not produce data which is easily interpretable (figure 14c). Although a general tendency of increased fatigue induced depletion of creatine phosphate stores with greater thickness of diaphragm tissues is suggested, the extreme decline of concentration from a mean of 13.0μmoles/g with diaphragm thickness of 0.29 mm (TD) to 7.1μmoles/g at 0.34mm thickness (TP) and 4.8μmoles/g with a thickness of 0.36mm (WC) makes one hesitant to draw conclusions. If such a relationship is so indicated in figure 14c, it also appears that a plateauing effect occurs whereby creatine phosphate levels in this preparation do not drop below a mean of approximately 4.8μmoles/g tissue even though diaphragm thickness of the groups may increase from 0.36mm (WC) to 0.69,, (AC).

Fatigue of rat diaphragm in small bath

When isolated rat diaphragm tissues were prepared in a specially designed small muscle bath, with a total bathing fluid volume of 0.75ml, the fatigue profile (figure 15) was statistically non-significantly different from that observed in the larger baths (figure 13). Following 10 minutes of fatigue, tissues isolated from the pair fed animals developed isometric tension upon stimulation which was a mean of 38% that observed

Figure 15a, b. The effect of thiamine deficiency on muscle contractility. Muscles were prepared and bathed in 0.75ml Tyrode solution with 55mM glucose and 10 mU/ml insulin. The complete experimental protocol used is described in the Materials and Methods section. Muscles were electrically stimulated with a tetanus inducing DC pulse at the rate of 1 every 2 seconds for 10 minutes. Values represent mean ± S.E.M. Number of animals used: TD 2(group 4), TP 8(4), and WC 9(4).


a
(% PRE-FATIGUE TENSION)
100
90
80
70
60
50
40
30
20
10
0
0 30 60 90 120 180 240 300 360 420 480 540 600 (SEC.)
TD
TP


b
(% PRE-FATIGUE TENSION)
100
90
80
70
60
50
40
30
20
10
0
WC
TP

before fatigue. Similarly, the thiamine deficient tissue tension declined to a mean of 45% and the weight control tissues to a mean of 49%.

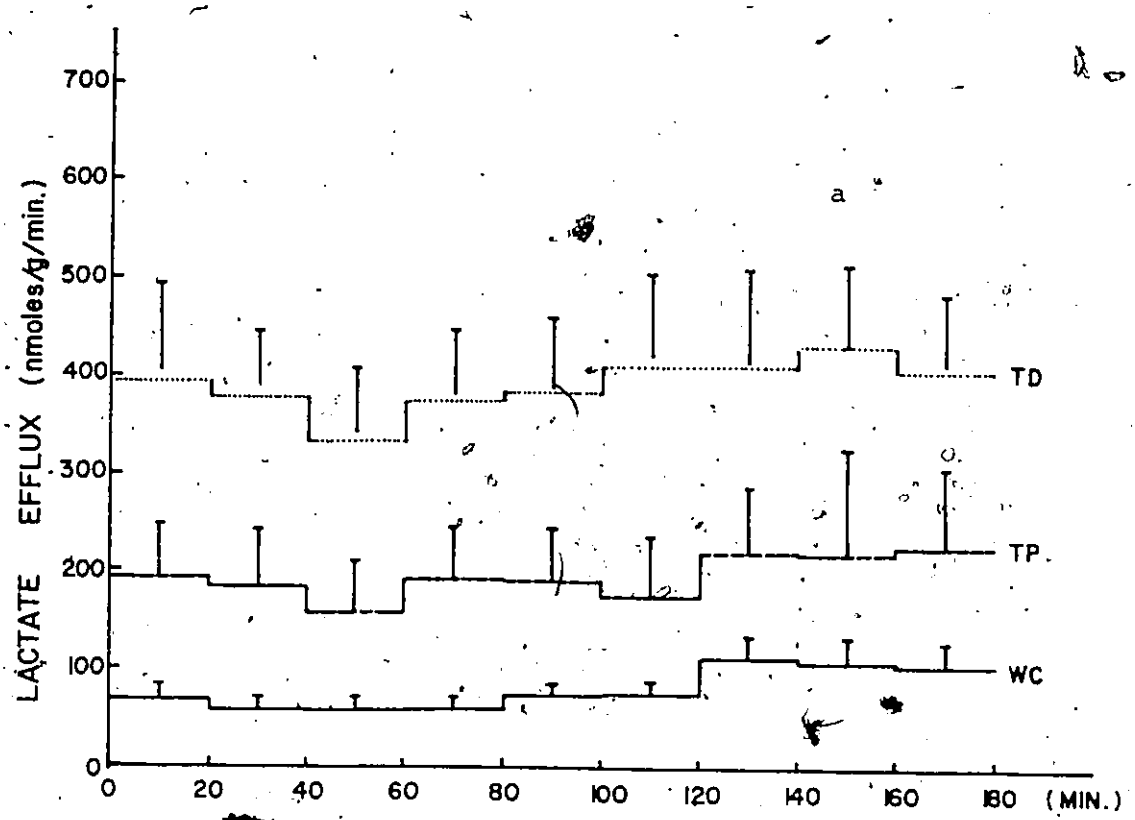
Following 5 minutes of fatiguing stimulus, the tensions had declined to means of 47%, 53% and 60% of pre-fatigue levels for the pair fed, thiamine deficient and weight control groups respectively. The fatigue observed in the small bath is similar to that observed with muscles prepared in the larger muscle bath (figure 12) where mean tension decline to 52%, 64% and 64% in the pair fed, thiamine deficient and weight control groups. These results suggest no significant change in diaphragm fatigue was measurable when the tissues were prepared in the smaller muscle bath, as compared to the larger bath.

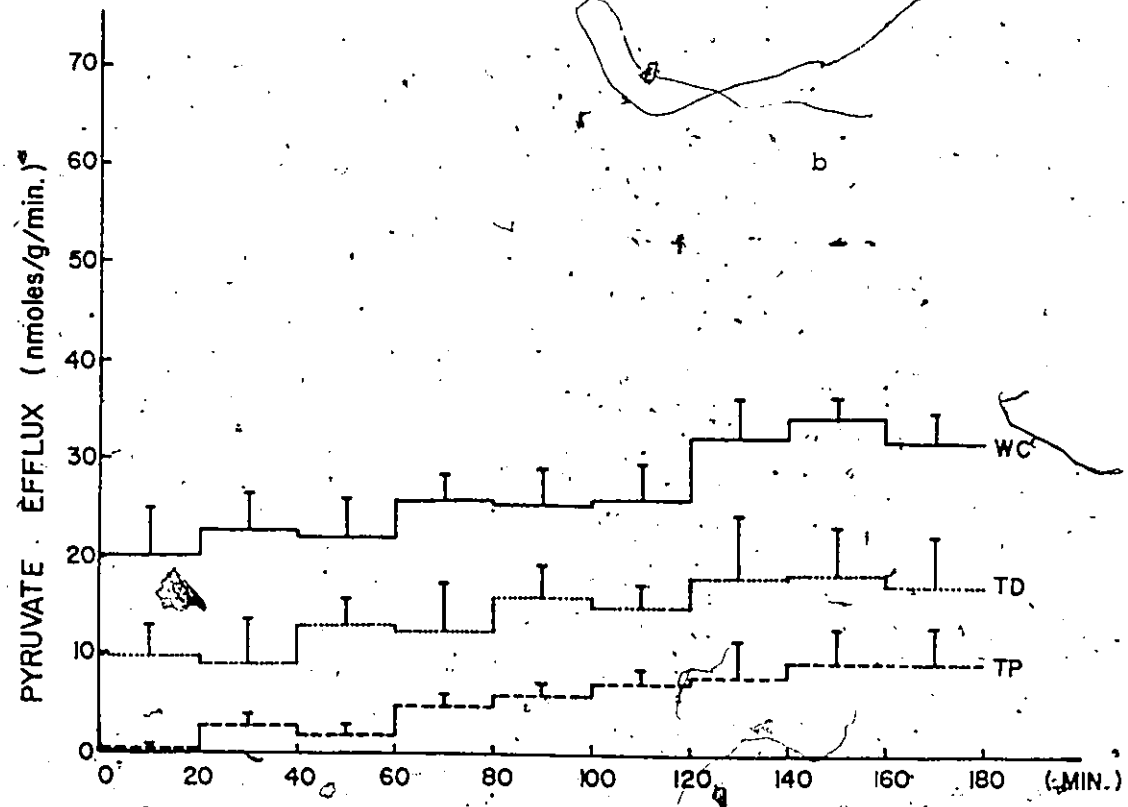
Effect of thiamine deficiency on lactate and pyruvate efflux from rat diaphragm

Lactate efflux from isolated rat diaphragm remained at a relatively constant level for all groups throughout the 60 minute period of perfusion with the 5.5mM glucose-Tyrode solution (figure 16a). The efflux rate was consistently greater from the thiamine deficient group than the pair fed, as was the pair fed relative to the weight control group. When the efflux rates for the first 3 individual 20 minute periods are pooled for each group of animals, a significant difference can be seen between any 2 groups. When pooling is performed, lactate efflux was calculated to be 367.0 ± 19.1nmoles/g/minute (TD), 181.0 ± 11.8nmoles/g/minute (TP), and 65.0 ± 4.0nmoles/g/minute (WC).

Figure 16a, b. The effect of thiamine deficiency on the efflux of lactate and pyruvate from isolated rat diaphragm.

Samples of bathing medium were collected at 20 minute intervals as explained in the Materials and Methods section. The muscle was bathed for successive 60 minute periods in Tyrode solution containing 5.5mM glucose, 55mM glucose, and 55mM glucose plus 10 mU/ml insulin, as explained in the experimental protocol. Samples were retained on ice until used for spectrophotometric enzymatic analysis. Values illustrate mean ± S.E.M. efflux over a 20 minute period. Number of animals used: TD 5(group 4), TP 8(4), and WC 9(4).


a
LACTATE EFFLUX (nmoles/g/min.)
700
600
500
400
300
200
100
0
TD
TP
WC
0
20
40
60
80
100
120
140
160
180
(MIN.)


b
PYRUVATE EFFLUX (nmoles/g/min.)
70
60
50
40
30
20
10
0
WC
TD
TP
0
20
40
60
80
100
120
140
160
180
(MIN.)

Dietary status also appeared to affect the rate of pyruvate efflux from diaphragm tissue, but not in the same way as lactate. Pyruvate efflux, in the first 60 minutes bathing period, was consistently significantly greater from tissues of the weight controls relative to the thiamine deficient and pair fed groups (figure 16b). Pooling of the 3 individual 20 minute collection periods for each group of animals results in pyruvate efflux rates of 10.7 ± 1.2nmoles/g/minute (TD), 1.8 ± 0.7nmoles/g/minute (TP), and 21.7 ± 0.9nmoles/g/minute (WC) (table 3). Statistical analysis of the pyruvate efflux rates illustrated that pyruvate efflux from the thiamine deficient group was significantly greater than from the pair fed tissues.

The fact that lactate efflux from the pair fed tissues was significantly greater than from the weight control tissues suggests that restricted diet does have an effect on the production of glycolytic metabolites. A similar, more pronounced, effect is indicated in thiamine deficiency. This latter point may indicate that thiamine deficiency does reduce the oxidative metabolism of pyruvate.

The effect of glucose and insulin on lactate and pyruvate efflux from isolated rat diaphragm

Increasing the Tyrode solution concentration of glucose from 5.5mM to 55mM resulted in no statistically significant effect on lactate efflux rate from any of the 3 groups of tissues (table 3). The addition of insulin to the Tyrode-55mM glucose solution increased the rate of lactate efflux from the thiamine deficient group but the change was non-significant.

Table 3. THE EFFECT OF GLUCOSE AND INSULIN ON LACTATE AND PYRUVATE EFFLUX FROM ISOLATED RAT DIAPHRAGM.

	TD		TP		WC	
TYRODE SOLUTION	LACTATE	PYRUVATE	LACTATE	PYRUVATE	LACTATE	PYRUVATE
5.5 mM glucose	367.0 ± 19.1	10.7 ± 1.2	181.0 ± 11.8	1.8 ± 0.7	65.0 ± 4.0	21.7 ± 0.9
55 mM glucose	388.3 ± 11.3	14.3 ± 1.2	187.0 ± 6.5	6.0 ± 0.6*	73.0 ± 2.6	26.0 ± 0.0*
55mM glucose + 10 mU/ml insulin	415.3 ± 7.4	17.7 ± 0.3*	231.7 ± 7.1*	8.7 ± 0.3	113.0 ± 2.1*	33.0 ± 0.6

Tissues were prepared for mounting in a specialized muscle bath, as discussed in Materials and Methods. After a 40 minute equilibration period, the tissues were bathed for successive 60 minute periods in Tyrode solution containing 5.5mM glucose, 55mM glucose, and 55mM glucose with 10 mU/ml insulin. The bathing medium was changed every 20 minutes, and the extracted medium was retained at 0°C for spectrophotometric enzymatic analysis. Values represent mean ± S.E.M. of the efflux rate, illustrated as μmoles/g/minute. Number of animals used: TD 5(group 4), TP 8(4), and WC 9(4).

Similar treatment of the pair fed and weight control groups resulted in statistically significant increases from 187.0 ± 6.5nmoles/g/minute and 73.0 ± 2.6nmoles/g/minute to 231.7 ± 7.1nmoles/g/minute and 113.0 ± 2.1nmoles/g/minute respectively. Lactate efflux rate was consistently significantly greater from the tissue of the thiamine deficient group relative to either of the other 2 groups during similar treatment. The same relationship existed between lactate efflux rates from the pair fed and weight control tissues.

The rate of pyruvate efflux from the various tissues differed somewhat. Changing the glucose concentration to 55mM (from 5.5mM) in the Tyrode bathing solution resulted in a significantly increased rate of pyruvate efflux from the pair fed and weight control tissues (Table 3). These rates increased from 1.8 ± 0.7nmoles/g/minute and 21.7 ± 0.9nmoles/g/minute to 6.0 ± 0.6nmoles/g/minute and 26.0 ± 0.0nmoles/g/minute respectively in the pair fed and weight control tissues. Pyruvate efflux rate was increased non-significantly following similar treatment of the thiamine deficient tissues. Addition of 10 mU/ml insulin to the bath significantly increased the rate of pyruvate efflux from all 3 groups of tissues, when compared to the efflux rate in the absence of insulin. Pyruvate efflux rate increased from 14.3 ± 1.2nmoles/g/minute to 17.7 ± 0.3nmoles/g/minute in the thiamine deficient tissues, from 6.0 ± 0.6nmoles/g/minute to 8.7 ± 0.3nmoles/g/minute in the pair fed group and from 26.0 ± 0.0nmoles/g/minute to 33.0 ± 0.6nmoles/g/minute in the weight control group. Unlike the rate of efflux observed for lactate, pyruvate efflux was consistently

significantly greater from tissues of the weight control group, relative to the thiamine deficient and pair fed groups, during similar treatments.

These results suggest that membrane permeability changes may occur in thiamine deficiency because throughout the entire 180 minute period lactate efflux was significantly greater from the thiamine deficient than the control tissues.

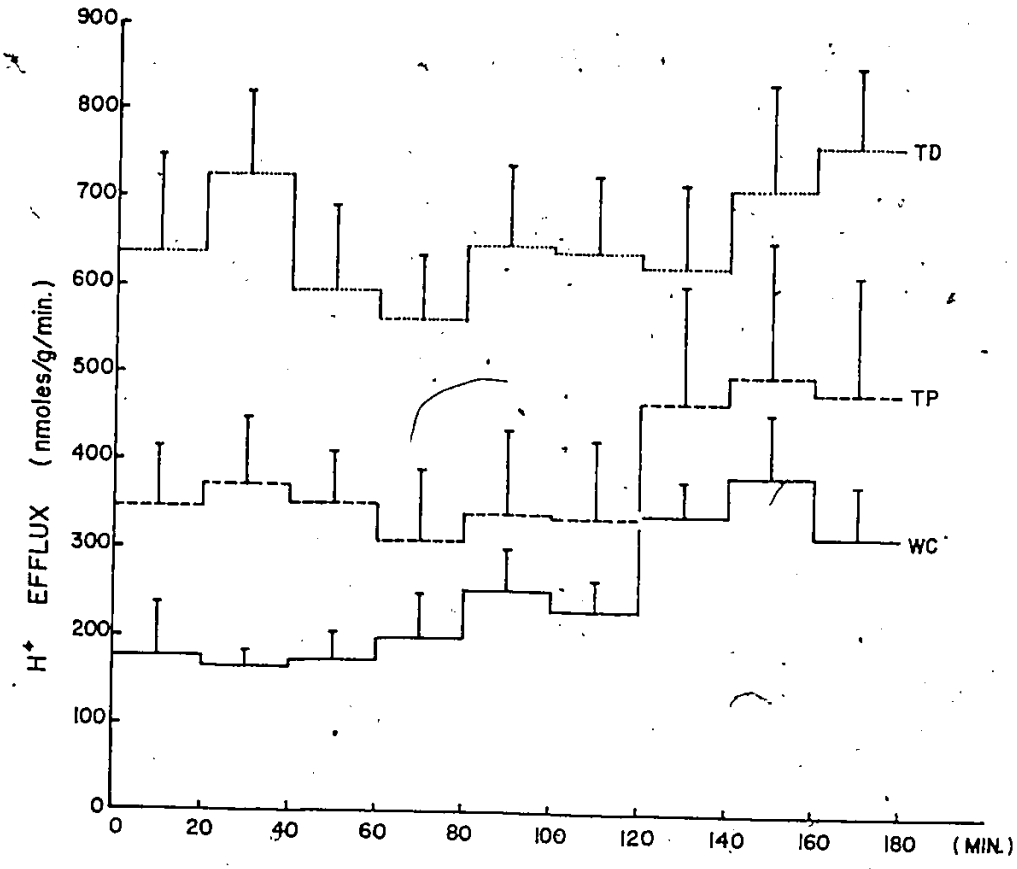
H^+ efflux from isolated rat diaphragm

Throughout the first 60 minutes, when the bathing medium contained 5.5mM glucose-Tyrode bathing medium, H^+ efflux was consistently significantly greater from the thiamine deficient tissues than the pair fed tissues, (figure 17). A similar relationship was observed with H^+ efflux from the pair fed control tissues in relation to the weight control tissues.

Increasing the glucose concentration of the Tyrode solution to 55mM appeared to increase slightly the rate of H^+ efflux from the thiamine deficient and weight control tissues. Analysis of the efflux rates from these tissues individually in the 5.5mM glucose-Tyrode bathing medium compared to the 55mM glucose-Tyrode medium did not illustrate a significant difference. H^+ efflux from the pair fed tissues did not appear to be significantly altered with the change of glucose concentration.

Whereas the mean H^+ efflux from the pair fed tissues was elevated significantly following the addition of insulin, this was not the case in the thiamine deficient and weight control groups. A significant elevation of mean H^+ efflux did occur over the first 20 minutes collection

Figure 17. The effect of thiamine deficiency on H^+ efflux from isolated rat diaphragm. Rat diaphragm muscle was isolated and prepared as discussed in the materials and methods section. Muscle bathing samples were collected for a period of 20 minutes and then H^+ concentration measured. Total H^+ concentration of the sample was calculated by the method in Appendix A. Experimental protocol was as discussed in Materials and Methods. Values represent mean ± S.E.M. efflux over any individual 20 minute period. Number of animals used: TD 5(group 4), TP 8(4), and WC 9(4).


900
800
700
600
500
400
300
200
100
0
H+ EFFLUX (nmoles/g/min.)
TD
TP
WC
0
20
40
60
80
100
120
140
160
180
(MIN.)

period following the addition of insulin in the latter 2 groups, but the significance was not sustained over the whole 60 minute period when insulin was present.

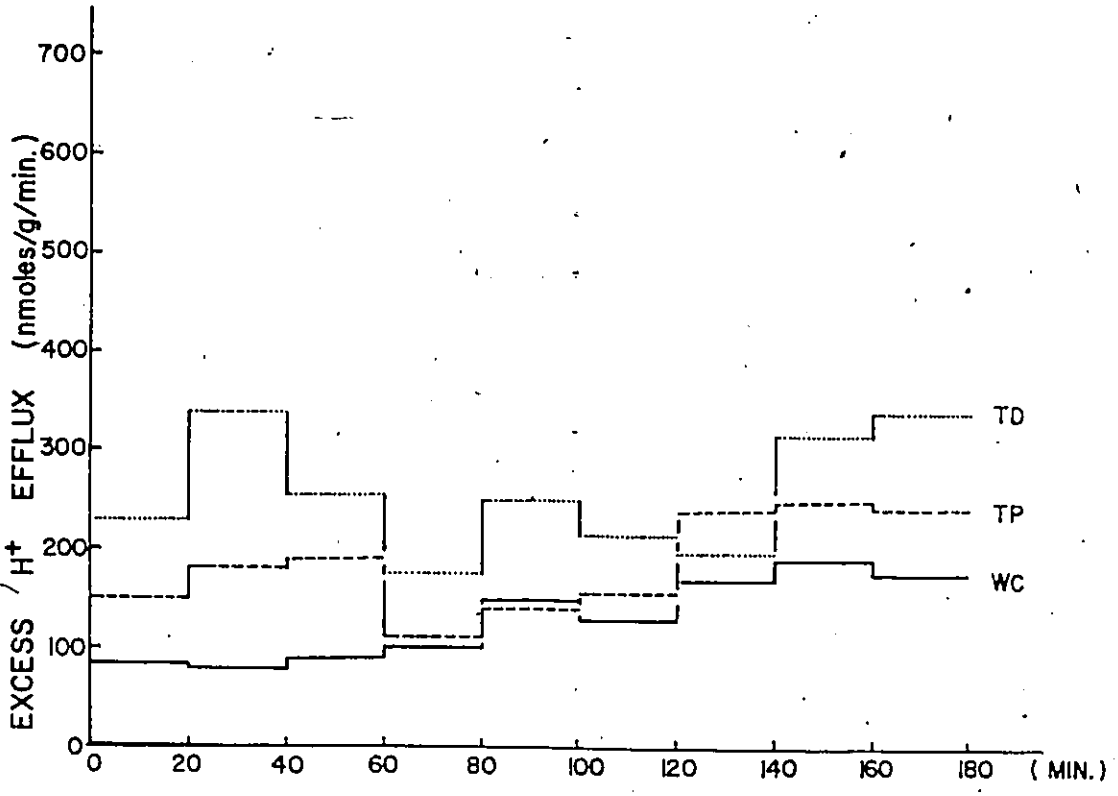
Pooling of the mean H^+ efflux rates, in any individual group, over the first 120 minutes and last 60 minutes separately, results in a statistically significant difference between any 2 of the 3 groups throughout the whole 180 minutes. The actual mean H^+ efflux rates over the first 120 minutes are 635.00 ± 3.53nmoles/g/minute, 334.17 ± 2.39nmoles/g/minute, and 200.33 ± 7.34nmoles/g/minute for the thiamine deficient, pair fed and weight control groups respectively. In the presence of insulin the efflux rates increased to means of 485.33 ± 9.96nmoles/g/minute (TP), 718.00 ± 46.00nmoles/g/minute (TD), and 325.67 ± 5.67nmoles/g/minute (WC).

That lactate and H^+ efflux rates are elevated significantly in tissues from the pair fed animals relative to the weight controls, and that tissues from similarly treated animals do not contain significantly higher levels of lactate following fatigue (figure 12), suggests an increased permeability of the membrane to lactate during starvation. A further increase of permeability may be indicated in thiamine deficiency, as the mean lactate and H^+ efflux from these tissues was also significantly greater than the tissues of the pair fed group.

Difference between H^+ and lactate plus pyruvate efflux

Figure 18 illustrates the excess H^+ efflux, relative to the sum of lactate and pyruvate (where measurable) efflux. In the first 60

Figure 18. Difference between the efflux rate of H^+ and lactate plus pyruvate. The arithmetic difference between the means of figures 14 and 15 has been plotted. Number of animals used: TD 5(group 4), TP 8(4), and WC 9(4).


EXCESS H+ EFFLUX (nmoles/g/min.)
700
600
500
400
300
200
100
0
0
20
40
60
80
100
120
140
160
180
(MIN.)
TD
TP
WC

minutes (5.5mM glucose-Tyrode bathing medium) the excess efflux of H^+ appeared to be greater from the thiamine deficient tissues (mean of 275.33nmoles appeared to be greater from the thiamine deficient tissues (mean of 275.33nmoles/g/minute) than the pair fed group (mean of 174.99nmoles/g/minute) which was in turn greater than that observed with the weight control tissues (mean of 85.33nmoles/g/minute). The excess H^+ efflux from the thiamine deficient tissues was significantly greater than that observed with the weight control tissues.

Increasing the bathing medium glucose concentration to 55mM did not result in major changes of excess H^+ efflux. Excess H^+ efflux, relative to the total of lactate plus pyruvate efflux, from the thiamine deficient tissues decreased to a mean of 215.00nmoles/g/minute over this 60 minute period, and this rate was significantly greater than that observed from the other 3 tissues. During the same period excess H^+ efflux from the pair fed tissue decreased to a mean of 137.33nmoles/g/minute. The efflux from the weight control tissues increased significantly to a mean of 129.00nmoles/g/minute.

Addition of 10mU/ml insulin to the bathing medium did not have a statistically significant effect on the excess H^+ efflux from the thiamine deficient tissues. The efflux rate from this tissue increased to a mean of 285.00nmoles/g/minute over the 60 minute period. Insulin did increase H^+ efflux from the pair fed and weight control tissues, to 245.00nmoles/g/minute and 179.67nmoles/g/minute respectively. These efflux rates, in both cases, were not only significantly greater when compared to that observed with 55mM glucose-Tyrode in the absence of insulin, but were also greater than the efflux from these tissues into 5.5mM glucose-Tyrode solution.

Close observation of figure 18 illustrates a general tendency for the excess H^+ efflux to increase over the whole 180 minute experimental period from the pair fed and weight control tissues.

The fact that insulin significantly increase excess H^+ efflux form the pair fed and weight control tissues, but not the thiamine deficient tissues further supports the suggestion that thiamine avitaminosis may affect the muscle fibre membrane.

Discussion

Effect of thiamine deficiency on rat growth

In the present study, rats maintained on a thiamine deficient diet gained weight for about 12 days and then began to lose weight. The weight loss continued for the duration of the experiment, with the rats attaining the weight at which the experiment started by approximately day 30. Similar effects of thiamine on rat growth have been reported (Davies and Jennings, 1970; Meghal et al, 1977; Bitter et al, 1968). Gubler in 1961, observed that rats began to lose weight after 5 or 6 days of thiamine deficient diet, but these were rats with a starting weight twice that used in this study. The fact that a very similar pattern, with regard to weight gain and loss was observed in the pair fed control animals suggests that this is a function of food intake, and not due primarily to an effect of thiamine deficiency. The first tendency is to suggest that the anorexia results from thiamine deficiency in the central nervous system. Gubler (1976), reported that after 25 days of thiamine deprivation the brain total thiamine levels decreased to approximately 35% of control, and the sum of brain TPP and thiamine triphosphate (TTP) declined to 45% of control values. The effect of thiamine deficiency, in this case, may however, be through a mechanism other than inhibition of pyruvate decarboxylation as Meghal et al (1977) reported no gross changes in rat brain pyruvate dehydrogenase activity in thiamine

deficiency. Notwithstanding the mechanism by which thiamine deficiency results in anorexia, the observed weight loss suggests that the animals used in this study were reacting to thiamine deprivation as those in other reports.

It is also interesting to note that only tissues from the age control group had a diaphragm thickness significantly greater than that of the thiamine deficient group, the pair fed and weight control groups being non-significantly different. Gubler (1976) observed that the rat brain to body weight ratio was similar for rats on experimental thiamine deficiency which had attained a higher weight and then subsequently lost weight, and for those which had attained the same body weight by uninterrupted growth. Although first impression suggests that once again anorexia may account for the lack of changes in the thiamine deficient animals, the similarity of diaphragm thickness amongst the 3 groups may have implications.

It is probable that the number of muscle fibres is maximally fixed at birth. The fact that the thiamine deficient and pair fed animals first gained and then lost weight indicates atrophy of the existing fibres and not a decrease in the number of cells.

Effect of thiamine deficiency on plasma lactate and pyruvate concentration

Plasma lactate levels of the thiamine deficient animals were significantly greater than the other 3 groups studied in these experiments.

Similarly high levels of blood lactate have been reported in the thiamine deficient calf, 40mg/100 ml, (Blaxter and Rook, 1955) and dog, 60 mg/100 ml, (Chesler et al, 1944). Values from the present study converted to these units, were 45 mg/100 ml blood. Park and Gubler (1969) reported a somewhat lower blood lactate concentration of 400 µmoles/100 ml in thiamine deficient rats. Whereas the plasma pyruvate levels were elevated in both the thiamine deficient and pair fed groups, the difference between these and the other 2 groups was not significant. Other authors have recorded blood pyruvate levels during thiamine deficiency to be somewhat higher than those of this study; 2.38mg/100ml in rats (Gubler, 1961), and 5mg/100ml in calves (Benevenga et al, 1966). Conversion of the results of the present study to the same units, give mean pyruvate levels of 1.6mg/100ml. In a later study Park and Gubler (1969) reported blood pyruvate values in thiamine deficient rats to be 175µmoles/100ml, a value very similar to that recorded in the present study.

The fact that plasma lactate levels were significantly greater in the thiamine deficient animals relative to the 3 control groups suggests that pyruvate metabolism was indeed being affected by the induced thiamine deficiency.

The diaphragm as a model of skeletal muscle

In order to investigate the role which glycolytic metabolites and/or high energy phosphate depletion may play in fatigue, the skeletal

muscle model chosen had to conform to several requirements. It had to be a tissue which could be easily removed from the animal without the fibres being damaged, and was thin enough that diffusion of oxygen into the fibres and metabolites out of the fibres would not be a restrictive property. As well, the tissue sample had to have enough mass to permit biochemical analysis. The tissue dimensions also had to permit quick freezing. The tissue used in these studies was the rat diaphragm, because it fulfilled all of these requirements, as discussed in the introduction. However, Beatty et al (1960) have cautioned against interpreting data collected from diaphragm preparations as being representative of skeletal muscle.

In a series of papers, Engel (1962, 1965, 1970) developed a classification system for muscle fibres; type I, which were considered oxidative fibres in that the enzymes of aerobic metabolism found within these fibres have high activity and also show low phosphorylase and myosin ATPase, and type II, which had low activity levels of the aerobic enzymes, and high activity in the phosphorylase and myosin ATPase enzymes. This system of muscle fibre typing was expanded by Edgerton and Simpson (1969) so that at least three different muscle fibre types are studied in recent literature: a) type I, which is still classed by the definition of Engel, b) Type IIa, which are those which have high activity for myosin ATPase and both aerobic and anaerobic enzymes; and c) Type IIb, which have high myosin ATPase activity, high activity of anaerobic enzymes and low activity of the aerobic

enzymes. This fibre classification system has for the most part, replaced the earlier "red and white" fibre system which related the speed of muscular contraction to the red or white colour of the muscle. It has now become evident that the red colouration of muscle fibres is due to the myoglobin content.

Although this latter classification system appears to be quite functional, some hazy areas still exist. In trying to relate the earlier and later fibre typing systems, Essen and Haggmark (1975), noted both red and white fibres contained high levels of glycogen. Glycolytic activity was more pronounced in type II fibres, but they suggested the type I glycolytic potential may be enough to maintain a high rate of glycolysis. They exposed subjects to maximal bicycle exercise, taking samples 10, 20, and 40 seconds after exercise started, and after exhaustion (3 to 4 minutes). Lactate levels in both fibre types, at rest, were 1.8mmoles/kg wet weight. These increased to 2.8mmoles/kg wet weight at 10 seconds and continued to increase to 20mmoles/kg wet weight at exhaustion.

Davies and Gunn (1972) observed some further discrepancies during histochemical studies of fibre types from diaphragms of several species. They reported that the rat diaphragm consisted of at least the 3 fibre types described earlier. Their analysis revealed that rat diaphragm consisted of about 60% fast fibres, and as well, 60% of the fibres were high in activity of oxidative enzymes. A most interesting inconsistency however, is their observation that whereas the rat

diaphragm contains 61% "fast" fibres, and the dog diaphragm 64%, the contraction time of the rat diaphragm is 18msec., as compared to 65msec. for the dog diaphragm.

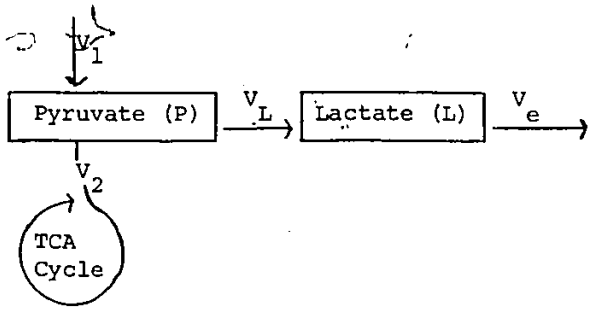
These studies only serve to emphasize that although a particular muscle may be classified from histochemical data as a type I or type II fibre this representation may not entirely correlate with function. Whereas it would be preferable to study a muscle with homogeneity of fibre type, these are practically non-existent, and certainly do not fulfill the particular qualifications required for this study. The rat diaphragm can be regarded as consisting of predominantly oxidative and fast fibres, as well as meeting the other restrictions of tissue thickness etc.

Effect of thiamine deficiency on tissue lactate and pyruvate levels

With respect to the observed elevation of plasma lactate levels in the thiamine deficient animals, it was a surprise to find that tissue lactate concentrations did not follow the same pattern. The thiamine deficient group tissues did not have an elevated lactate or pyruvate content relative to the 3 control groups, following 180 minutes perfusion in the muscle bath (see experimental protocol in Materials and Methods section for details). The pyruvate levels in the plasma suggested that tissue pyruvate metabolism was being affected by thiamine deficiency. Increasing the rate of glucose metabolism in muscle was then expected to result in an increased lactate content,

based on the fact that the lactate in the blood must be produced by the tissues. In order to drive glycolysis at an elevated rate, greater amounts of glucose were made available to the tissue by bathing it with 55mM glucose-Tyrode in the presence of 10mU/ml insulin. Parks et al (1955) were the first to demonstrate glucose uptake in the rat diaphragm was a function of the external glucose and insulin concentration. More recently, Mainwood et al (1977), observed that with an external glucose concentration of 55mM in the presence of 10 mU/ml insulin, the mean intracellular glucose concentration increased to 19.0 μmoles/ml fibre from 1.2 μmoles/ml fibre in the absence of insulin.

The overall sequence of events being investigated may be represented schematically as:



where V_e = lactate efflux

V_1 = pyruvate production rate

V_L = rate of conversion of pyruvate to lactate

V_2 = pyruvate oxidation rate

Then the change in concentration of pyruvate in the tissue to increase with time is $\frac{dP}{dt}$ and the change of lactate concentration to increase with time is $\frac{dL}{dt}$. Then;

$$\frac{dP}{dt} = V_1 - (V_L + V_2) \text{ and } \frac{dL}{dt} = V_L - V_e$$

Thus, for the pyruvate concentration to increase with time ($\frac{dP}{dt} > 0$), $V_1 > (V_L + V_2)$ and similarly for $\frac{dL}{dt}$ to be positive $V_L > V_e$. Therefore for pyruvate and lactate concentrations to increase, $V_1 > V_2 + V_e$. Thus the lack of lactate and pyruvate buildup in the thiamine deficient tissues could be due to any one, or combination of 4 reasons; a) the glycolytic pathway was being inhibited at a rate limiting step, such as PFK, by increased metabolite concentrations, b) pyruvate metabolism via the TCA cycle was not, in this case, being inhibited by thiamine deficiency, c) that even glucose loading does not maximally activate the glycolytic enzymes, and although the maximum potential activity of pyruvate dehydrogenase may be reduced by a lack of TPP, the rate of pyruvate production still does not approach the rate of oxidation, or d) lactate and pyruvate was being produced, but the rate of efflux from the muscle tissue had also increased, and thus the increased production could not be detected at this point.

The first solution can be discounted with evidence from the results of the present study. Although Henderson et al (1978) reported that there is a decreased rate of protein synthesis in rat brain and viscera during thiamine deficiency, the production of ATP did not appear to be affected in the present study (table 3). As well, if the ability of the thiamine deficient tissues to produce ATP had declined, one would expect that the thiamine deficient tissues would fatigue at a greater rate than the control tissues, but figures 14a, b and c, suggest

this is not the case.

The suggestion that the oxidative metabolism of pyruvate in diaphragm muscle may not be inhibited by thiamine deficiency brings to light an even more obvious question: does thiamine deficiency result in TPP depletion from this particular tissue? A second review of the literature did not help resolve this issue, because although data of TPP depletion was available for several tissues including human (Ferrebee et al, 1942; Taylor et al, 1942), and rat (Schultz et al, 1939; Mitchell et al, 1942) data in skeletal muscle was conspicuously absent, or discredited due to variability. Lowry, 1952, did report however, total thiamine levels decreased in skeletal muscle in rats at the rate of about 10% per day of deficiency (20 µg/100g/day), with a beginning mean concentration of 2 µg/g. Thus he observed that skeletal muscle thiamine content declined from 1.8 µg/g to 0.14 µg/g after 20 days. No data was available for longer periods of time.

The reason for this lack of data in the literature with regard to TPP content of skeletal muscle, became obvious when the attempt was made to measure TPP spectrophotometrically. Several modifications of the method of Ullrich (1974) were attempted, to no avail. In this method the apo-decarboxylase (pyruvate dehydrogenase minus TPP) enzyme is combined with the tissue homogenate and incubated for 30 minutes, allowing any TPP present to combine with the apo-enzyme to reform holoenzyme pyruvate decarboxylase. A base slope is then obtained on the spectrophotometer (set at 340mµ). A fixed amount of pyruvate is then

added to the cuvette and the rate of change in absorption due to the oxidation of NADH to NAD^+ measured. The difference in slopes observed after and before the addition of pyruvate can then be related to the amount of holo-enzyme reformed in the first step, providing for the calculation of the TPP concentration in the tissue homogenate. Whereas accurate standard curves were easily attained, addition of the centrifuged tissue homogenate interfered with measurement. Indeed, addition of a specific concentration of TPP standard to the diaphragm homogenate, still gave an indicated level of 0 TPP.

In light of the fact that tissue TPP levels could not be measured, it was assumed, on the basis of reports from other tissues, that diaphragm TPP levels were depleted by the induced athiaminosis.

The possibility that the efflux rates, and thus membrane permeability to lactate and/or pyruvate may have been altered was explored, and will be discussed later. It may be sufficient to say at this point that lactate efflux may be increased significantly in thiamine deficiency.

The effect of thiamine deficiency on normalized muscle tension

It has generally been assumed for many years that thiamine deficiency results in a decreased muscle strength, ie a decline of muscle contractility. This is so well accepted, that very general statements can be found in many medical textbooks, as examplified by the following quotation from Follis and Mann, 1970.

> "Over the course of days or weeks, the patient comes to be easily fatigued and experiences heavy feelings in the legs, together with stiffness and aching in the muscles. In time, the muscles become weaker, acutely painful and then atrophic."

The results of the present study suggest the muscle fatigue observed clinically in thiamine deficiency, is not a result of decline in muscle contractility because the normalized tensions from the 4 groups of tissues were not statistically significantly different from each other, nor was the fatigue profile. Overall strength decreases may be indicated in this condition because tissue atrophy does occur. Thus, even though the contractile tension developed per unit area of muscle does not decline, total tension developed may decrease because the amount of tissue decreases.

The effect of glucose and insulin on muscle contraction

Mainwood et al (1977), using the same preparation as in the present study, observed that maximal isometric tension would decline if the external concentration of glucose was increased. In their report, altering the external glucose concentration from 5.5mM to 55mM resulted in maximum isometric tension dropping by about 30%, and this was almost completely reversible by restoring the glucose concentration of the bathing medium to 5.5mM. This change in contractility was attributed to the increase in osmolarity of the external medium associated with the increased glucose concentration. The decline in contractile tension was also reversible by adding insulin to the bathing medium. The

increased glucose uptake apparently assisted in causing isotonicity between the intracellular and extracellular medium. In the present study, the weight control group underwent the characteristic response to high glucose and insulin, observed by Mainwood et al. The thiamine deficient and pair fed groups tended to react in the same fashion, but the decline of tension occuring when the bathing medium was changed to one with 55mM glucose, was much less. Similarly, the insulin induced recovery was not as dramatic. With regard to the work of Mainwood et al (1977), the results of this study may also be explained in terms of osmolarity, and lead one to suggest that thiamine deficiency may cause membrane permeability changes. Although this has not been documented quantitatively, one can extrapolate from the work of Mainwood et al (1972) that the osmolarity induced decline in maximal isometric tension will reach a plateau when the intracellular and extracellular fluids are isosmolar. Since the isometric tension developed in the thiamine deficient and pair fed tissues plateaued at a much greater level relative to maximum than did the weight and age controls one can assume they reached isosmolarity with the external medium quicker. The rapidity of this equilibration may be explained by either an increased intracellular osmolarity in these tissues, or an increased permeability of the membrane to facilitate equilibration.

The response of the age control tissues was unique in that maximum isometric tension generated continued to decline throughout the entire 60 minute exposure to 55mM glucose, and while an increase in this

parameter was observed when insulin was added, the increase was transient. This response is probably a result of diffusion problems due to the thickness of the muscle tissue. Creese et al (1957) suggested a muscle thickness greater than .5mm results in delayed diffusion into and out of the inner fibres. Tissues of this group had a mean thickness of .69mm, which would account for the slow equilibration during the bathing in high concentration glucose. The transient response to insulin may be due to its effect on the outer diaphragm fibres where it may have been metabolized before ever reaching the inner fibres.

Effect of thiamine deficiency on muscle fatigue

Over a 5 minute stimulation period, at a frequency of 1 every 2 seconds, maximal isometric contraction of the thiamine deficient tissue decreased to 64% of the level observed immediately before the fatiguing stimulus was begun. The pair fed tissues underwent a somewhat greater degree of fatigue, but the difference in degree of fatigue between these 2 groups was statistically non-significant. In contrast, the age control tissues did fatigue to a much greater degree, and the difference between these tissues and those of the thiamine deficient and pair fed groups was significant. At this point, the only difference found between any of these groups has been the diaphragm thickness, that being significantly greater for the age control tissues, the tissues which fatigue to a significantly greater

degree. If the degree of fatigue can be correlated with diaphragm thickness, then one would anticipate, in light of the non-significant difference between diaphragm thickness of the thiamine deficient and pair fed groups, that the fatigue characteristics of these 2 groups would be similar, and this was indeed the observation.

Tissue lactate and pyruvate concentrations following fatigue

The increase of the tissue lactate and pyruvate concentrations following 5 minutes fatigue may be relevant to several ideas discussed in this thesis. It is interesting to note that the lactate concentration in all tissues increased 3-fold following fatigue, excepting the weight control group, where the concentration doubled. The differences between groups were non-significant when comparing non-fatigue tissue levels with post-fatigue levels. Perhaps of greater importance however, are the pyruvate levels observed following fatigue. Whereas the levels of pyruvate were elevated in all tissues, the thiamine deficient samples were significantly greater, in this case, than any of the other 3 groups. The implications of this are important. In particular, the fact that lactate and pyruvate concentrations were elevated suggests that the muscles were producing energy anaerobically, at least to some degree (Karlsson, 1971a,b; Knuttgen and Saltin, 1972).

The hypothesis that thiamine deficiency would have an effect on pyruvate metabolism is supported by the fact that the elevated

pyruvate levels following fatigue become significantly greater in the thiamine deficient tissues, as compared to any of the 3 control groups.

Effect of fatigue on muscle ATP, CP, and G-6-P concentration

The levels of ATP, CP, and G-6-P were measured in an attempt to answer the question of the involvement of high energy phosphates in muscle fatigue. In the present study, it was observed that not only did CP concentration decline significantly following fatigue in the pair fed, weight control and age control tissues, but ATP levels did as well. The ATP concentration did not decline to levels as dramatic as the CP, but one might predict this, since the function of the phosphate stored with creatine is to replenish ATP concentrations as it gets used up. These results contradict other studies in rats (Hohorst et al, 1962), mice (Spande and Schottelius, 1970), and frogs (Mainwood, et al, 1972) where only a significant decline of CP concentration was reported. Hohorst et al, 1962, did report a drop in ATP concentration, but this was only 15%, whereas the decline observed in the present study was to between 52 and 73% of non-fatigued tissue levels. A further twist enters into the picture when the TD tissue ATP and CP concentrations are compared to the other tissues. The concentration of both these compounds dropped, but not significantly. This may suggest that the capability of the glycolytic enzymes to convert glucose to pyruvate has increased in thiamine deficiency. Inherent to this idea would be an increased capacity of the glycolytic pathway due to an

increased reaction rate or decreased inhibition of certain glycolytic enzymes, resulting in an increased ability to maintain energy supplies.

The G-6-P concentration of the various tissues appeared not to correlate to fatigue at all as they remained non-significantly different from the levels observed in non-fatigued tissues in all cases. The present results indicated that the intracellular levels of high energy phosphates following fatigue do not correlate to the degree of fatigue.

Correlation of degree of fatigue and post-fatigue lactate and CP concentration to diaphragm thickness

At this point, the data of the present study suggest that fatigue of isolated diaphragm tissue does not correlate with lactate concentration and dietary status. Another possibility however, is that another parameter may be involved. Since the only major difference between groups appeared to be body size (weight), a related parameter, diaphragm thickness may provide an integral contribution. After plotting, fatigue and post-fatigue lactate concentration appeared to correlate with diaphragm thickness. Creatine phosphate, which Spande and Schottelius (1970) found related linearly to fatigue, did not appear to correlate with diaphragm thickness. These results stimulate some interesting thoughts. Muscle fatigue is a state which we know to exist. Rearrangement of the data suggests that the degree of fatigue of the isolated diaphragm correlates in a curvilinear fashion to

diaphragm thickness. As well, a similar rearranging of data illustrates the diaphragm lactate concentration following 5 minutes fatigue increases with increasing diaphragm thickness. However, data discussed earlier illustrated that fatigue did not correlate with lactate concentration and thus in total, the present data tend to suggest in the isolated diaphragm preparation, that even though the correlation of fatigue with diaphragm thickness may provide some insight into the muscle fatiguing process, the fact that lactate also correlates with this parameter is probably a result of decreased diffusion rates and does not have a causative role in muscle contractile response.

Diaphragm fatigue in small muscle bath

The custom made muscle bath (for details see Materials and Methods section) was designed for studies of H^+, lactate and pyruvate efflux from isolated diaphragm tissues. In order to analyze these compounds accurately, a small volume of bathing fluid had to be used, because the quantity of these substances leaving the muscle was very small and greater dilution would render them undetectable by the analytical methods used. Thus the bath was designed such that the entire muscle sample could be bathed with volumes of 0.75m. to 1.0ml. In these particular studies, 0.75ml fluid was used.

What effect, if any, the increasing concentrations of substances from the muscle tissue on muscle contractility had, was of great concern. However, muscle fatigue in this bath was non-significantly different than in the larger 100ml bath used in the earlier fatigue studies.

Effect of diet on lactate and pyruvate efflux from isolated rat diaphragm

The mean lactate efflux rate from the weight control group over the 180 minute period was 83.67 nmoles/g/min. This rate is relatively low when compared to other values reported in the literature. Using various other types of diaphragm preparation, steady state lactate efflux rates of about 330 nmoles/g/min. have been reported (Hollanders, 1968; Rowlands, 1969ab). However, much earlier, Wallaas and Wallaas (1950) and Beatty et al (1960) reported control lactate efflux rates of 104 to 124 nmoles/g/min and 80 nmoles/g/min respectively using preparations of manometrically incubated diaphragms. Cechetto and Mainwood (1978) using the same preparation as this study found a control lactate efflux rate of 120 nmoles/g/min.

The fact that the steady state lactate efflux in the present study is somewhat lower than other reported values is somewhat of a puzzle, but may be explained by several ideas. Firstly, the rats used in this study were much smaller, weighing between 70 and 90 grams, whereas other studies utilized rats of 200 to 300 grams. Krebs (1950) and Zeuthen (1955) have both published reports in which metabolic activity has been related to body size, the smaller the animal, the more increased the capacity of oxidative processes. As well, Gauthier and Padykula (1966) suggested that in general, muscle fibre type changes as the animal species get larger; the mouse diaphragm consisting entirely of red fibres, the elephant of white. Although

the adult (350g) rat diaphragm was reported to consist of a mixture of red and white fibres, the majority being white, at this smaller weight, the composition of fibre type may be closer to that of the mouse. Further support to this idea is suggested by the fact that although, according to the authors previously mentioned, these smaller animals would have a higher metabolic rate, the non-fatigued diaphragms of the thiamine deficient, pair fed, and weight control animals did not contain greater amounts of lactate than did the age controls.

The smaller diaphragm thickness of these animals may also be an aid to a greater metabolic rate by providing greater oxygen availability. Although Creese et al (1958) observed that diaphragms from rats weighing 150 to 250 grams maintained fibre potentials to a depth of 250 μ, even if the bubbling gas contained only 60% 0_2, he did not demonstrate that this could maintain optimum metabolism. With the smaller diaphragms used in the present study, bubbling with 95% 0_2 on both sides of the muscle, may facilitate better 0_2 availability if the oxidative processes should require it for increased activity.

It was of great interest to note that lactate efflux rate was significantly greater from the thiamine deficient tissues (390nmoles/g/min) relative to the pair fed group, as was the pair fed group relative to the weight control tissues. This leads to speculation that the nutritive state of the tissue can affect membrane permeability,

in that even the restricted diet of the pair fed group resulted in an elevated lactate efflux rate of 189 nmoles/g/min.

The reports of several authors have inferred that lactate efflux is more complicated than a simple diffusion process. Karpatkin et al (1964) suggested that lactate efflux from frog sartorius muscle may be a saturable process, and measured the efflux V_{max} to be 0.8 µmoles/ml intracellular water per minute. Others have tended to support the hypothesis that maximal lactate efflux may not depend on a simple diffusion process (Hirche et al, 1970; Karlsson et al, 1971; Mainwood and Worsley-Brown, 1975). It has been suggested by one author that lactate efflux is an active process (Kubler et al. 1966).

If lactate efflux is carrier mediated, the maximal rates of efflux from different tissues vary greatly. Maximal lactate efflux from the dog gastrocnemius was observed to be 0.6 µmoles/g/min (di Prampero et al, 1970) and for frog muscle 0.3 to 0.4 µmoles/g/min. In the isolated rat diaphragm, Hollanders (1958) reported maximum lactate efflux values of between 1 and 8 µmoles/g/min. However, even the maximal rate observed with the thiamine deficient tissues was only 66% of the lowest value reported in these publications, and thus would not be a factor. A carrier mediated mechanism for lactate efflux from muscle may not be merely academic speculation, even though no specific function of such a process has been suggested, as we do know of the existence of a pyruvate specific carrier mediated mechanism for mitochondrial entry (Pande and Parvin, 1978; Halestrap 1975; Halestrap and Denton, 1975), and carrier mediated entry of

pyruvate and lactate into erythrocytes (Halestrap, 1976). In what respect thiamine deficiency may further affect membrane permeability of lactate can not be answered by the data of the present study.

The pyruvate efflux rates observed in the present study were very low, and difficult to interpret. It is indeed doubtful that they represent the true pyruvate efflux, particularly from the thiamine deficient and pair fed tissues. Two inherent problems became obvious when performing the enzymatic assay for pyruvate. The first was that the concentration of pyruvate being measured was at the minimum level detectable with the assay, and thus open to large error. Secondly, some other substance, probably enzyme, had also undergone efflux from the thiamine deficient and pair fed (to a lesser degree) tissue samples and remained in the bathing fluid samples. This became evident when performing the assay, because some time was required to obtain a steady base line, even before the enzyme was added, a phenomenon which never occurred with the standard curves or the bathing fluid of the weight control group. In fact, often a completely steady base line was never observed before the exogenous enzyme was added. In the spectrophotometric analysis for pyruvate used, the enzyme is added last, after a steady base line is obtained, and thus it must be assumed that either lactate dehydrogenase, or another enzyme capable of metabolizing pyruvate to lactate, was present in the bathing medium of the thiamine deficient and pair fed tissues.

Effect of glucose and insulin on lactate and pyruvate efflux

Increasing the glucose concentration from 5.5mM to 55mM in the bathing medium did not alter significantly the lactate efflux rate for any of the 3 groups of tissues. The addition of insulin in the presence of 55mM glucose did increase significantly lactate efflux from both the pair fed and weight control groups. This result is most likely a reflection of the increased glucose uptake induced by insulin. The purpose of bathing the muscles in a high glucose-insulin medium was to drive the glycolytic pathway at a maximum rate, the result being an increased production of pyruvate, which, if the concentration was greater than the oxidative processes could handle, would be converted to lactate. The fact that lactate efflux was stimulated by insulin and high glucose, in the pair fed and weight control tissues, suggests that the hypothesis of this thesis was valid.

The fact that insulin and high glucose concentration in the bathing medium did not increase lactate efflux from thiamine deficient tissues suggests that either glycolysis was operating at a maximal rate before the addition of insulin, or that the insulin-sensitive glucose uptake system had lost the ability to respond to insulin. Since in all experiments where the tissues were fatigued, tissue lactate values increased, it is obvious that the capacity of the glycolytic pathway was not a limiting factor. The evidence tends to suggest that thiamine avitaminosis may render diaphragm tissue glucose uptake insulin-insensitive.

Efflux of H^+ from isolated rat diaphragm

H^+ efflux from diaphragm tissue followed basically the same pattern as lactate. H^+ efflux was consistently greater from the thiamine deficient tissue than either the pair fed or weight control tissues, but was significantly greater than the efflux from the weight control tissues only. Efflux of H^+ was also consistently greater from the pair fed tissues than the weight control tissues, but the difference was not statistically significant. However, pooling of the efflux values from any similarly treated group for the first 120 minutes and last 60 minutes produced a significant difference when efflux rates from any 2 of the 3 groups are compared.

Of interest was the observation that insulin did not significantly increase H^+ efflux from the thiamine deficient diaphragm tissue, but did from the pair fed and weight control tissues, when efflux is considered over the whole 60 minute period. A similar pattern was observed in the lactate efflux from the pair fed and weight control tissues relative to that from the thiamine deficient tissues. These results indicate that lactate and H^+ efflux rates tend to respond concomitantly to similar stimuli.

Difference between H^+ and lactate plus pyruvate efflux

The recalculation of data of this study was presented in this fashion in an attempt to add some knowledge to ideas already published on the form that the lactate molecule takes during efflux; as lactic

acid or the lactate ion. Ample evidence is available in the literature to support either opinion.

Hill et al, in 1924, and Bock et al, in 1927, reported that the CO_2 dissociation curve in human blood shifted following heavy exercise. This shift, which was in the direction as if pH had fallen, was attributed to the accumulation of lactic acid in the blood by other authors (Turrell and Robinson, 1942). Roos (1975) also reported, based on his observations of the ratio of intracellular and extracellular lactate in the equilibrated state, and the ratio of H^+ between extracellular and intracellular compartments, that lactate permeated the membrane in the undissociated form.

On the other hand, as lactic acid can be considered to be almost completely ionized at physiological pH, with a pK of about 3.8 (Benade and Heisler, 1978; Lockwood, Yoder and Zienty, 1965), can the small amount of undissociated lactic acid present in the intracellular compartment account for the large rates of efflux discussed earlier.

Several authors have provided evidence which tends to support lactate ion permeation. Mainwood and Worsley-Brown (1975), using a low buffer concentration (10mM) bathing medium reported a higher rate of lactate efflux than H^+ from frog sartorius muscle. Benade and Heisler (1978), reported the efflux of H^+ exceeded that of lactate by a factor of 14 from diaphragm and 50 from sartorius. As well, Woodbury and Miles (1973), calculated from experiments with frog sartorius muscles that the membrane

had not only a low permeability to lactate, but also that the efflux rate was limited to a level approximating 0.07 the permeability of chloride.

Hirche et al (1975) observed efflux rates which support both sides of the argument, depending on the experimental conditions. In alkalosis, H^+ and lactate permeated into the blood at approximately equal rates from the stimulated dog gastrocnemius perfused with blood. However, during acidosis, H^+ ions were found to permeate even more slowly than lactate during the first 3 to 4 minutes of exercise.

The results of the present study indicate H^+ efflux occurs at a rate 1.5 to 2 times that of lactate in all tissues tested. These values concur closely with efflux rates obtained in other similar experiments carried out in the same laboratory. The relatively small difference in efflux rates may lend some support to those that hypothesize that H^+ and lactate efflux are independent or occur by separate mechanisms, but this evidence is not very convincing alone.

Concluding Remarks

The hypothesis of this thesis was based on the theory that if a thiamine deficient diet was fed to rats, thiamine levels in the body would be depleted, and thus TPP levels would decline. The lack of TPP to act as a cofactor for the pyruvate dehydrogenase enzyme complex theoretically would result in an accumulation of pyruvate and lactate. By observing the fatigue profile of the various groups of

rats, and determining the tissue concentrations of lactate, pyruvate, ATP, and CP following fatigue, it was anticipated that a determination of which, if any, of these substances have a role in muscle fatigue, could be made.

The evidence that tissue concentration of lactate and pyruvate did not increase more in the thiamine deficient tissues than the pair fed, weight and age control tissues, following glucose loading, suggested perhaps that either deprivation of thiamine in the diet did not result in the depletion of TPP from muscle tissue, or that skeletal muscle pyruvate dehydrogenase does not require TPP as a co-factor. The observations appeared to support the idea that the oxidative metabolism of pyruvate was not decreased. The observation that pyruvate levels increased in all tissue following fatigue, but the concentration in the thiamine deficient tissues was significantly greater than in the other tissues supported the original hypothesis of this thesis. These results also suggest that whereas the capacity of the thiamine deficient tissues to oxidatively metabolize pyruvate may remain adequate for the maintenance of ATP levels at rest, the decrease from maximum capacity becomes evident when energy demands increase.

The efflux studies produced some more interesting evidence to support the hypothesis. Lactate and H^+ efflux from the thiamine deficient tissues was significantly greater than that observed from any of the 3 control tissues. More to the point, the efflux of these substances remained significantly greater from the thiamine deficient

tissues even in the presence of insulin. This is important because only the pair fed and weight control tissue lactate and H^+ efflux rates were significantly increased when insulin was added to the medium of high glucose, suggesting that the efflux of these substances from the thiamine deficient tissues was occurring at a maximal rate throughout the study. Thus, the efflux rate was significantly greater. Inherently implied in this argument is that whether or not the activity of the pyruvate oxidizing enzymes is completely or partially decreased by thiamine deficiency can not be determined, but glucose loading was successful in increasing the rate of pyruvate production beyond the capacity of the oxidizing enzymes.

The evidence presented may also suggest that thiamine deficiency causes changes in the membrane. This is supported by the fact that insulin did not have a significant effect on lactate or H^+ efflux from the thiamine deficient tissues. This implies that the glucose uptake mechanism of the membrane of the thiamine deficient tissues was not sensitive to insulin or that glucose influx into the tissue occurred independent of this mechanism. This latter point is more probable because lactate and H^+ efflux was not significantly altered by increasing glucose concentration in the bathing medium or by the further addition of insulin, and thus the lactate production, even in the presence of 5.5mM glucose was greater than that observed in the pair fed and weight control tissues in the presence of the 55 mM glucose and insulin.

It was apparent in this study that thiamine deficiency does not cause a greater depletion of high energy phosphates in muscle tissue, and may actually enhance the ability of the muscle to produce ATP and CP. However, this may be a consequence of the increased H^+ efflux resulting in less pH induced inhibition of certain glycolytic enzymes, particularly PFK. The role of ATP and CP in muscle fatigue is questionable because the levels of these phosphates did not decline significantly more in the age control tissues which fatigued to a significantly greater degree than the thiamine deficient, pair fed, weight or age control tissues.

The role of increased lactate concentration in muscle tissue fatigue could not be answered by the results of this study. It was observed that a 2 to 3 fold increase of lactate concentration occurred in fatigue, but a relationship between these variables was not apparent. It is conceivable that further increases of muscle lactate concentration may affect this parameter.

APPENDIX A

Calculation of H^+ Efflux

To calculate total change of H^+ content of the buffer system used, 4 quantities of H^+ must be summed: a) ΔH^+, b) $-\Delta OH^-$, c) $-\Delta HCO_3^-$ and d) $-\Delta B^-$ (non-bicarbonate buffer).

These four quantities of H^+ can be represented mathematically as:

$$a = V[10^{-pH_2} - 10^{-pH_1}]$$

$$b = -V[10^{-(14-pH_2)} - 10^{-(14-pH_1)}]$$

$$c = V \cdot B[1 - 10^{pH_2-pH_1}]$$

$$d = V \cdot A\left[\frac{1}{1 + 10^{pH_2-pk_A}} - \frac{1}{1 + 10^{pH_1-pk_A}}\right]$$

Where B = the concentration of bicarbonate buffer

A = the concentration of non-bicarbonate buffer in the acid form

V = the volume

pH_2 = the measured pH following the 20 minute muscle bathing period

pH is the pH of the tyrode solution

NOTE: The concentration of bicarbonate buffer must be corrected from the original value. The presence of the non-bicarbonate buffer will cause a change in concentration of B; the working concentration of B may be represented mathematically as:

$$B' = B - A\,\frac{10^{pH_1} - pk_A}{1 + 10^{pH_1-pk_A}}$$

Where B' is the working concentration of B.

A more complete derivation of the formulae used can be found in the works of Cechetto (1979).

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