EFFECT OF CARBONIC ANHYDRASE INHIBITION ON MUSCLE METABOLISM DURING EXERCISE STUDIED BY ^{31}P-MRS

by

Shelly Ann Smith

Graduate Program in Kinesiology

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ABSTRACT

Carbonic anhydrase (CA) inhibition with acetazolamide (ACZ) has been shown to lower the plasma [La^-] for a given power output during exercise. The mechanism for this response is unknown. Nine males performed dynamic forearm flexion to volitional fatigue on a wrist flexor dynamometer. Exercise consisted of raising and lowering (1 s contraction, 1 s relaxation) a cylinder the volume of which increased in a ramp-like fashion at a rate of 200 ml/min. The protocol was performed during control (CON) and following 45 min CA inhibition with ACZ (10 mg/kg BW i.v.). ^{31}Phosphorus magnetic resonance spectroscopy (^{31}P-MRS) was used to measure intramuscular pH (pHi), PCr, ATP, and the intramuscular thresholds for pHi (T_{pHi}) and log Pi/PCr ($T_{logPi/PCr}$). Plasma [La^-] was measured using venous blood sampling techniques in a separate exercise session. Venous plasma [La^-] at fatigue was lower ($p<0.05$) in ACZ (3.7 ± 1.7 mmol/l) than CON (5.0 ± 1.7 mmol/l), and in early recovery (Fig 5). The T_{pHi}, which reflects the onset of glycolytic metabolism and La^- production in muscle, occurred at a similar power output in ACZ (722 ± 50mW) and CON (855 ± 211mW). The pHi was similar in ACZ and CON during exercise but with ACZ recovery of pHi was slower during the first 7 minutes after exercise. The lactate threshold (T_{La^-}) occurred at a higher power output in ACZ (1433 ± 243 mW) than CON (1041 ± 414 mW). Also, during ACZ treatment the T_{La^-} power output (1433 ± 243 mW) was greater than the T_{pHi} and $T_{logPi/PCr}$ power output (T_{pHi}, 722 ± 50 mW; $T_{logPi/PCr}$, 796 ± 75 mW) but not the CON condition (T_{La^-}, 1041 ± 414 mmol/l; T_{pHi}, 855 ± 211; $T_{logPi/PCr}$, 835 ± 235)($p<0.05$). These data suggest that the lower plasma [La^-] observed during ACZ-induced CA inhibition may be related to a slower rate of La^- efflux from muscle as muscle glycolysis (as determined by the T_{pHi} and slope of the pHi - power output relationship) was not affected by ACZ.

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CHAPTER 1
INTRODUCTION

1.1 Carbonic Anhydrase and Metabolism

Carbonic anhydrase (CA) catalyzes the hydration and dehydration of CO_2 to form carbonic acid (H_2CO_3), according to the reaction: $CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$. While the role of CA in the transport of CO_2 from exercising muscle and its elimination from the lungs has been known for some time, a less well understood function is related to the metabolic response to exercise. Côté and coworkers (1997) observed that inhibition of the intracellular CA isozyme (CA III) was associated with an increased rate of glycogen breakdown. Kowalchuk et al., (1992; 1994) have observed a lower plasma lactate concentration (plasma [La^-]) during recovery from 30 s high intensity cycle ergometer exercise during both acute (Kowalchuk et al., 1992) and chronic (Kowalchuk et al., 1994) acetazolamide (ACZ)-induced CA inhibition. Scheuermann et al. (1998c, unpublished observations) used a progressive leg cycling exercise model to demonstrate a rightward shift in the La^--power output curve during ACZ-induced CA inhibition. In addition, Scheuermann et al. (1998a, unpublished observations) demonstrated a lower plasma [La^-] during constant-load moderate and heavy intensity exercise following CA inhibition.

The mechanism for a reduced plasma [La^-] following acute ACZ administration is unknown. The plasma [La^-] represents a balance between La^- appearance into, and its disappearance out of, the blood compartment. Therefore, the lower plasma [La^-] with ACZ may be associated: with an increased uptake of La^- from blood into inactive skeletal muscle or other tissues; a decreased La^- efflux from active muscle; a decrease in La^- production as a consequence of an increase in pyruvate oxidation; or a decrease in pyruvate production

within muscle.

A greater uptake of La^- from blood into inactive skeletal muscle or other tissue is probably not responsible for a lower plasma [La^-]. Kowalchuk et al. (1994) demonstrated a lower arterial-venous [La^-] difference across the inactive forearm muscle during recovery from short term heavy cycling exercise during CA inhibition, suggesting that La^- uptake by inactive muscle (and perhaps other tissues) was lower, not higher, during ACZ.

Impaired translocation of La^- across the sarcolemma may also contribute to a lower plasma [La^-] during ACZ. McLellan and co-workers (1988) found a lower plasma [La^-] during exercise and an increased muscle-to-plasma [La^-] ratio during ACZ-induced CA inhibition suggesting that decreased La^- efflux across the muscle membrane may contribute, in part, to the lower plasma [La^-]. In that study however, ACZ was administered chronically for 2 days and exercise was performed under conditions of hypobaric hypoxia. Chronic ACZ administration is associated with a metabolic acidosis which has been shown to impair La^- efflux from muscle independent of effects of CA activity (Sutton et al., 1981). De Hemptinne et al. (1987) demonstrated the inhibition of the sarcolemmal-bound CA (CA IV) leading to a greater acidification of the extracellular surface membrane during recovery from an intracellular acid load which may lead to a decreased efflux of H^+ or lactic acid (Juel, 1997). Also observed in our laboratory, Scheuermann et al. (1998d, unpublished observations) demonstrated with muscle biopsy data a higher muscle-to-plasma [La^-] ratio after exercise following acute ACZ treatment suggesting that there was a slowing in the efflux of La^- from the muscle.

A third mechanism for a lower plasma [La^-] may be a decrease in La^- production, either because of a decrease in the rate of glycolysis and pyruvate production, or an increase

in pyruvate oxidation. La^- appearance in blood is sometimes associated with a slower adaptation of VO_2 during the on-transient phase of exercise. This implies a relationship between La^- appearance, pyruvate oxidation and mitochondrial respiration (Roston et al., 1987; Cerretelli et al., 1979). However, in spite of a lower plasma [La^-] during ACZ administration, Scheuermann et al. (1998a) found that the rate of adaptation of VO_2 at the onset of moderate and heavy, constant-load exercise, was similar to control conditions implying that pyruvate oxidation was not affected by CA inhibition. Rose et al. (1990) demonstrated a decrease in glycogen breakdown during heavy exercise following 3 days of ACZ-induced CA inhibition in horses suggesting that the rate of glycolysis was reduced due to ACZ-induced CA inhibition. However, in that study ACZ was administered chronically for 3 days and was associated with a metabolic acidosis prior to the start of exercise. In addition, muscle glycogen levels were reduced prior to the start of exercise which may have contributed to the lower rate of glycolysis as previous studies have shown that the initial rate of glycolysis may be related to the initial glycogen level in muscle (Pernow & Saltin, 1971). Inhibition of CA is associated with an increase in plasma and tissue PCO_2 (Cardenas et al., 1998; Taki et al., 1993). According to physicochemical principles, the PCO_2 is a major determinant of [H^+] in biological fluids (Stewart, 1981). Therefore, an increase in plasma and tissue PCO_2 following CA inhibition would be expected to contribute to a rise in plasma and tissue [H^+]. It has been suggested that a decrease in pHi may inhibit phosphofructokinase activity thereby reducing the rate of glycolysis and pyruvate production during exercise (Sutton et al., 1981; Jones et al., 1977). Geers & Gros (1988a, 1988, 1990) observed a lower pHi in resting muscle following CA inhibition. However, studies have demonstrated a higher rate of glycogen depletion. Consequently, resistance to fatigue has been shown following

inhibition of the intracellular CA isozyme (Frémont et al., 1991a; Frémont et al., 1991b; Côté et al., 1993; Côté et al., 1997). Côté et al. (1997) have also demonstrated a lack of a decrease in pHi and have suggested that an increased breakdown of PCr during CA inhibition acts to consume a greater amount of H^+ thus attenuating a fall in pHi of the muscle during exercise. Further investigation is required to determine the effect of CA-inhibition on the rate of glycolysis.

1.2 ^{31}P-MRS and Intracellular Thresholds

^{31}Phosphorus magnetic resonance spectroscopy (^{31}P-MRS) has been used to study skeletal muscle metabolism noninvasively, by examining changes in the intramuscular phosphate pool (PCr; ATP; Pi) and intracellular pH (pHi) during exercise and in various disease states (Kent-Braun et al., 1995). ^{31}P-MRS can also be used to determine whether intramuscular thresholds of high-energy-phosphate compounds (logPi/PCr) and pH occur during progressive exercise. Marsh et al. (1991) and Kent-Braun et al. (1993) administered progressive exercise to fatigue and used ^{31}P-MRS to demonstrate a threshold for skeletal muscle metabolism. The threshold can be identified from the point at which there is a rapid decrease in the Pi/PCr-, pHi-power output curve ($T_{logPi/PCr}$; T_{pHi}). Marsh et al. (1991) have suggested that stable values of Pi/PCr indicate an adequate energy status, while rapid changes in this ratio show that energy homeostasis cannot be maintained. Pi/PCr is used to estimate the intramuscular phosphorylation potential (ATP/ADP•Pi) or cytosolic [ADP], both of which are metabolic regulators for mitochondrial respiration and glycolysis (Erecinska and Wilson, 1982; Connett and Sahlin, 1996). An increase in Pi/PCr at power outputs above $T_{logPi/PCr}$ has been interpreted as an inability to provide ATP through oxidative processes alone, thus requiring the activation of glycolysis (Marsh et al., 1991; Kent-Braun et al., 1993). In

support, Marsh et al. (1991) and Kent-Braun et al. (1993) found a close approximation between the $T_{logPi/PCr}$ and T_{pHi} indicating the possibility that both intracellular thresholds reflect an increase in the rate of glycolysis and lactic acid production. Also, Systrom et al. (1990) measured ventilation, venous [La^-] and pHi and demonstrated that the T_{pHi} coincided with both the ventilatory and plasma La^- thresholds suggesting a common cause for all three variables (Systrom et al. 1990). Systrom et al. (1990) also implied that the T_{pHi} was a threshold for increased glycolytic metabolism. ^{31}P-MRS can be used to study the rate of glycolysis by examining the intramuscular threshold and high-energy-phosphate compounds providing further insight into the mechanism responsible for a CA inhibition-induced decreased plasma [La^-].

1.3 Purpose

In the present study, ^{31}P-MRS was used to examine the effects of CA inhibition with ACZ on the metabolic response to progressive exercise to volitional fatigue using a model of human forearm flexion. Specifically, this study attempted to identify a mechanism responsible for the lower plasma [La^-] which is commonly seen during exercise following CA inhibition.

1.4 Hypotheses

The following hypotheses were tested:

1. The plasma [La^-] would be lower at any given power output during progressive exercise (to fatigue) and the "plasma La^- threshold" would shift to the right and occur at a higher power output following CA-inhibition.
2. During progressive exercise, a greater intracellular acidosis associated with CA inhibition would inhibit glycolysis and cause the intracellular threshold for pHi (T_{pHi}) and log Pi/PCr ($T_{logPi/PCr}$) to shift to the right and occur at a higher power output

following CA-inhibition.

3. The intramuscular pH (pHi) would be more acidic at rest, and throughout exercise, and recovery following CA-inhibition, as a consequence of CO_2 and/or lactate retention within the muscle.

CHAPTER 2
LITERATURE REVIEW

2.1 The Transport of Carbon Dioxide (CO_2)

CO_2 formed in the cell it diffuses into the venous blood and is transported to the lungs. CO_2 is transported in three forms: as a gas dissolved in plasma (5%); as carbonate, chemically bound to haemoglobin in red blood cells (20%), and as a bicarbonate ion (HCO_3^-) in plasma (70%). Hydration of CO_2 to form carbonic acid (H_2CO_3) in followed by dissociation into hydrogen ions (H^+) and HCO_3^-. CO_2 conversion to HCO_3^- is a slow reaction requiring an enzyme, Carbonic Anhydrase, to speed up the hydration/dehydration reaction. The reaction is summarized as follows: $CO_2 + H_2O \overset{CA}{\rightleftharpoons} H_2CO_3 \rightleftharpoons HCO_3^- + H^+$.

2.2 Carbonic Anhydrase

Carbonic anhydrase (CA) is the enzyme responsible for catalyzing the hydration of CO_2 and the dehydration of HCO_3^-. In striated muscle, at least four isozymes of CA have been demonstrated: 1) CA III, a sulfonamide resistant isozyme, found in the cytosol of type I and type IIa skeletal muscles (Geers & Gros, 1991; Frémont et al., 1991a; Bruns et al., 1986; Jeffery et al., 1986; Geers et al., 1985; Gros et al., 1984; Geers & Gros, 1991; Frémont et al., 1988; Frémont et al., 1989), 2) CA II, a sulfonamide sensitive cytosolic isozyme, found in the cytosol of type I, Iia, IIb muscle fibers (similar to that found in the cytoplasm of red blood cells) (Siffert & Gros, 1982), 3) CA IV, a membrane-bound form present in the sarcolemma (Geers et al., 1985; Wetzel & Gros, 1988), the sarcoplasmic reticulum (SR) (Bruns et al., 1986; Geers & Gros 1991; Geers et al., 1985; Geers & Gros, 1988; Côté et al., 1989; Frémont et al., 1988;), and endothelial cells of capillaries of all fibre types (Sender et al., 1994), and 4) CA V, a mitochondrial isozyme (Dodgson et al., 1980). A summary of the skeletal muscle CA isozymes is presented in Table 1 and Figure 1.

2.2.1 CA III

The predominant CA isozyme in skeletal muscle is CA III. CA III has a very low-activity level and is resistant to CA inhibitors (Geers & Gros, 1991; Frémont et al., 1991a). CA III is found in high concentrations in the cytosol of Type I fibers (Bruns et al., 1986; Jeffery et al., 1986; Geers et al., 1985; Gros et al., 1984; Geers & Gros, 1991; Frémont et al., 1988; Frémont et al., 1989) and, to a lesser degree, in type IIa fibers of rat skeletal muscle (Frémont et al., 1989; Frémont et al., 1988). It is generally assumed that CA III functions to facilitate CO_2 diffusion through the cytosol and across the sarcolemma (Geers and Gros, 1991), although additional roles have been proposed. Côté et al. (1997) demonstrated that inhibition of CA III was associated with increased resistance to fatigue which may have been related to an increase in utilization of carbohydrates, implying that CA III may be a key modulator of energy metabolism.

2.2.2 CA IV

A membrane-bound CA isozyme, associated with, the sarcolemma (Geers et al., 1985; Wetzel & Gros, 1988) of all fiber types (Geers & Gros, 1991; Geers & Gros, 1988; Côté et al., 1989) and is believed to be part of the CA IV family of isozymes. CA IV has its catalytic activity directed towards the extracellular surface of the sarcolemma and may function to maintain H^+- HCO_3^-- CO_2 equilibrium and promote CO_2 transport into the vasculature (Geer et al., 1985; Wetzel & Gros, 1988). CA IV activity may prevent drastic acidification of the interstitium when lactic acid moves out of the muscle fibers (Wetzel & Gros, 1988). There is a higher specific CA IV activity is found in type II muscle fibres and may be related to the greater potential for lactic acid (Wetzel & Gros, 1988). The sarcolemmal-bound CA IV of type I muscle fibres may be important in the release of CO_2 from muscle under conditions of

exercise when blood flow and CO_2 production increases (Geers & Gros, 1985).

Studies have revealed intracellular staining for CA IV on the sarcoplasmic reticulum (SR) membrane of type I and type II muscle fibers (Bruns et al., 1986; Frémont et al., 1989). This SR-bound CA IV isozyme may be necessary for optimal Ca^{2+}-ATPase activity of intact skeletal muscle providing H^+ in exchange for Ca^{2+} during Ca^{2+} uptake into the SR. (Bruns et al., 1986).

CA IV has also been localized in the liver of capillaries of muscle, independent of fibre type (Sender et al., 1994). CA IV in the muscle capillaries may act to promote CO_2 uptake into plasma and transport away from muscle (Sender et al., 1994). The catalytic activity of CA IV has been found to be similar to that of CA II (Maren et al., 1993).

The heart is devoid of cytosolic CA isozymes but the CA IV has been identified on the sarcolemma and the capillary endothelial layer (Vandenberg et al., 1996). The CA activity associated with cardiac muscle probably plays a role, similar to the CA IV found on the sarcolemma and vascular membrane of skeletal muscle.

2.2.3 Summary

The CA isozymes found in striated muscle have a wide range of activity levels and resistance to or sensitivity to CA inhibitors. While many of these isozymes function to facilitate transport of CO_2 out of muscle and into blood, they may also, play a role in acid-base balance and metabolism.

2.3 The Effects of CA Inhibition on CO_2 Transport

The inhibition of CA leads to an increase in the PCO_2 of blood and muscle tissue (Cardenas et al., 1998). A dosage of 5 mg/kg of ACZ administered in dogs inhibited the endothethial capillary CA and partially inhibited CA in RBC as demonstrated by an increase

in the PCO_2 in venous blood (P_vCO2) and a lower pH (Cardenas et al., 1998). Also, the arterial PCO_2 ($PaCO_2$) increased and a lowered pH in the control group (Cardenas et al., 1998; Maren & Swenson, 1980). At a dose of 100 mgACZ/kg, to achieve complete CA inhibition, an even greater increase in P_vCO_2 and P_aCO_2 and lower pH in both arterial and venous blood was observed.

Maren & Swenson (1980) demonstrated that pulmonary CO_2 output was only slightly reduced when CA was completely inhibited suggesting that the elimination of CO_2 was subserved by residual enzyme activity or due to compensatory adjustments in CO_2 transport with dissolved CO_2 and carbonate formation playing a greater role. Steady-state, CO_2 output decreases according to the amount of CA inhibited. Cardenas et al. (1998) observed a decrement of 10% in VCO_2 with 5 mg/kg ACZ and a 26% decrement in VCO_2 with 100 mg/kg ACZ. Cain & Otis (1961) demonstrated in anaesthetized dogs during complete inhibition of CA, that less CO_2 was taken up from tissues per unit of blood, and caused an increase in CO_2 stores within tissues, thereby forming an increase gradient for PCO_2. The higher PCO_2 within the tissues would then promote greater diffusion of CO_2 into the blood. An increase in ventilation has also been demonstrated with CA inhibition, and results in a decrease in alveolar PCO_2 (P_ACO_2) (Cardenas et al., 1998; Maren & Swenson, 1980; Cain & Otis, 1961), and widening of the Δ (v-A) PCO_2 gradient, thus further promoting CO_2 elimination at the lung.

In summary, inhibition of CA leads to an increase in tissue and blood PCO_2; an increase in ventilation and decrease in P_ACO_2 and a widening of Δ(v-A)PCO_2. These factors, along with compensatory adjustments to CO_2 transport, help maintaain pulmonary CO2 output in steady-state conditions at near normal levels.

2.4 Effects of CA Inhibition on Contractile Parameters

CA may play a role in muscle contraction. Changes observed in contractile parameters during CA inhibition include decrease in isometric force, prolonged relaxation times, and increased time to peak tension (Dawson et al., 1980).

2.4.1 Isometric Force

Barclay (1987) studied the importance of CA on isometric tetanic force of mouse soleus muscle by stimulating the muscle in the absence and presence of ACZ. Isometric tension was not affected when the mouse soleus muscle was exposed to 10^{-5} M of ACZ for 25 min (Barclay, 1987). Barclay (1987) concluded that ACZ had no affect on isometric tension which may have been due to a failure of ACZ to inhibit CA III possible because of its low membrane permeability (Barclay, 1987).

2.4.2 Relaxation Time & Time to Peak Tension

The relaxation time is prolonged with CA is inhibition. The EDL and soleus muscle were incubated in bathing solution and CA was inhibited by adding chlorzolamide ($5 \cdot 10^{-4}$M). The half-relaxation times of twitches and tetani for both muscles were markedly prolonged by incubation with chlorzolamide (Geers & Gros 1988a, 1988, 1990).

The time to peak tension increased were CA inhibition. Geers & Gros (1988a, 1988, 1990) studied the time to peak tension in EDL and soleus muscle after incubation in CLZ ($5 \cdot 10^{-4}$M). Results demonstrated the time to peak tension increased in EDL and soleus muscle after incubation in CLZ (Geers & Gros 1988a, 1988, 1990).

2.4.3 Summary

CA inhibition may impair CO_2 elimination from muscle, loading to a respiratory acidosis which may then have an affect on the contractile parameters and high-energy

phosphates. These decreases in isometric force, prolonged relaxation time, increased time to peak tension, greater breakdown of PCr and increase in Pi are similar to the changes seen in muscle fatigue.

2.4.4 CA Inhibition and Resistance to Fatigue

CA inhibition leads to a greater breakdown in PCr and a resistance to fatigue during muscle stimulation (Côté, et al., 1997). The hydrolysis of PCr is a proton-consuming reaction, leading to an increase in pHi if no other metabolic changes occur. Côté et al. (1997) found no visible decrease in pHi of the soleus muscle of rats deprived of CA activity (1 mM methazolamide) and have suggested that the greater breakdown of PCr observed with CA inhibition may explain the lack of decrease in pHi.

Frémont et al. (1991a , 1991b) also demonstrated a resistance to fatigue in the rat soleus muscle when CA III was inhibited using a membrane permeable CA inhibitor, methazolamide. It was suggested that this was attributable to a higher rate of glycogen utilization induced by CA inhibition (Côté et al., 1993; 1997). The mechanism responsible for the lower rate of fatigue in the presence of a CA inhibitor was unexplained. Côté et al. (1989) also incubated rat soleus muscle in 10^{-5} M of methazolamide, a concentration expected to inhibit the extracellular sarcolemmal CA isozyme but not the cytosolic CA III, to determine whether the sarcolemmal CA isozyme had an effect on fatiguability. The fatigue profile in this condition was similar to that found in the control group suggesting that the sarcolemmal CA isozyme did not contribute to the fatiguability of the muscle. Fatigue resistance of the EDL, a muscle devoid of CA III, was not affected by incubation with the CA inhibitor (Côté et al., 1989). Côté et al. (1989) concluded that the observed resistance to fatigue during a long duration, low intensity stimulation protocol was due to the inhibition of cytosolic CA III,

since resistance to fatigue was demonstrated only with the inhibition of CA III in the rat soleus but not when the EDL muscle was incubated with a CA inhibitor or when only the CA IV of the sarcolemma was inhibited.

Studies have suggested that CA III can influence the rate of utilization of carbohydrates (CHO) and fatiguability of type I muscles during prolonged contractile activity (Côté et al., 1993, 1997). The resistance to fatigue of the soleus during CA inhibition was linked to an increased rate of CHO utilization and a greater breakdown of PCr (Côté et al., 1993, 1997).

2.5 The Effects of Acetazolamide (ACZ) on the Metabolic Response to Exercise

A decrease in plasma [La^-] has been demonstrated during exercise following ACZ-induced CA inhibition (Kowalchuk et al., 1992; 1994; Scheuermann et al., 1998a,b,c,d, unpublished observations). Kowalchuk et al. (1992, 1994) observed a lower arterial [La^-] during recovery from short-term heavy exercise after acute (1000mg ACZ) and chronic ACZ (500 mg/day for 3 days) administration compared to control (CON) conditions. Recently, Scheuermann et al. (1998a,b, unpublished observations) observed a lower plasma [La^-] during constant-load cycling exercise performed at exercise intensities below and above the ventilatory threshold following the acute admnistration ACZ (acACZ; 10 mg/kg of body weight). The mechanism responsible for the decrease plasma [La^-] following ACZ-induced CA inhibition has not been established.

The plasma [La^-] represents a balance between muscle La^- production and appearance in blood, and La^- removal. One mechanism for a lower plasma [La^-] may be a consequence of a reduced translocation of La^- across the sarcolemma.. McLellan and co-workers (1988) found a lower plasma [La^-] after chronic ACZ administration and a higher muscle-to-plasma

[La^-] ratio implying that the lower plasma [La^-] may be due to a reduced translocation across the sarcolemma. However in that study, ACZ was administered chronically for two days and exercise was performed under conditions of hypobaric hypoxia. Scheuermann et al. (1998d, unpublished observations) have also demonstrated a similar glycogen breakdown and lower plasma [La^-], and a tendency for a higher muscle- to-plasma [La^-] ratio during acute ACZ administration suggesting a reduced translocation across the sarcolemma. It is known that the release of La^- from the muscle to the blood is influenced by the extracellular acid-base status. An acidic plasma will slow La^- release from muscle (Jones et al., 1977; Sutton et al., 1981). Therefore the metabolic acidosis associated with chronic CA may contribute to an impairment of La^- translocation across the sarcolemma (Jones et al. 1977, Sutton et al. 1981; Juel, 1997).

A second possible mechanism for a lower plasma [La^-] after the administration of ACZ may be a greater removal of La^- from blood into inactive muscle and other tissues. Kowalchuk et al. (1992; 1994) examined arterial-venous [La^-] differences to determine the effect of CA inhibition on La^- uptake by inactive forearm muscle during recovery from heavy exercise. These studies showed that La^- uptake by inactive muscle was reduced following CA inhibition and appeared to be related to the lower arterial [La^-].

A third possible mechanism for a lower plasma [La^-] may be a decrease in production, either because of a decrease in the rate of glycolysis and pyruvate production, or because of an increase in pyruvate oxidation. Conditions associated with a lower plasma [La^-] are sometimes accompanied by a faster adaptation of VO_2 suggesting an increase in pyruvate oxidation (Roston et al., 1987; Cerretelli et al., 1979). However, Scheuermann et al. (1998a, unpublished observations) found that in spite of a lower plasma [La^-] during steady-state

exercise below and above the ventilatory threshold following CA inhibition the rate of adaptation of VO_2 at the onset of exercise was similar to control conditions implying that pyruvate oxidation was not affected by CA inhibition. Conditions associated with a decrease in plasma [La^-] may be related to a decrease in the intracellular activity of the rate limiting enzyme systems in the glycolytic pathway (Gevers et al., 1963). Rose et al. (1990) demonstrated a lower rate of glycogen depletion and a reduced accumulation of La^- in muscle following ACZ-induced CA inhibition in horses (Rose et al., 1990). However, in that study the ACZ was administered chronically for three days and the muscle glycogen levels were reduced by approximately 50% prior to the start of exercise in ACZ-treated horses, which may contribute to the decrease in glycolysis. It was suggested that the reductions in La^- production were attributed to a pH-induced depression in glycolysis (Rose et al., 1990).

2.6 ^{31}P-MRS

^{31}P-MRS is a noninvasive tool allowing continuous monitoring of muscle metabolic parameters. Wilkie et al. (1984) compared muscle metabolite concentrations using muscle biopsy technique and the ^{31}P-MRS. They observed a higher [Pi] and lower [PCr] in the biopsy sample relative to ^{31}P-MRS measurements. They attributed the difference to hydrolysis of PCr during the time taken to freeze the biopsy sample. The ^{31}P-MRS spectra collected in muscle provides information on the relative concentration of metabolite including α-adenosine triphosphate (ATP), β-ATP, γ-ATP, inorganic phosphate (Pi), and creatine phosphate (PCr) (Inch et al,. 1986). In addition, the intracellular pH (pHi) can be determined by calculating the chemical shift of the Pi peak relative to the PCr peak (Chance et al., 1981).

2.6.1 Muscle Energetics

The energy state of the muscle is often represented by the ratio Pi/PCr. Similarly,

Pi/PCr can be used to express as log(Pi/PCr). Studies have used progressive exercise during which a metabolic steady state is achieved at each work level to observe the relationship between work rate and energy cost and to determine its relationship to mitochondrial function of the muscle (Chance et al., 1985). As workload increases, there is an increase in Pi/PCr (Chance et al., 1985). The oxidative potential of the muscle can be estimated by studying the initial linear portion of the work - Pi/PCr relationship (Chance et al., 1985). An increase in Pi/PCr relative to low work level indicates a poor capacity for oxidative metabolism. However if the slope is steep this would indicate less of an increase in Pi/PCr at any given relative workload, thus indicating an improved ability to keep pace with energy needs by means of oxidative phosphorylation (see Fig. 2, Chance et al., 1985).

The pathway of ATP production during muscular work can be studied using the ^{31}P-MRS. The rate of PCr decrease at the onset of exercise is a good indicator of the rate of ATP production from the creatine kinase reaction while the initial rate of recovery of PCr after exercise can estimate the rate of ATP production from oxidative phosphorylation because PCr resynthesis after exercise is primarily through oxidative phosphorylation (Boska, 1991). In addition, during exercise, the rate of change in pHi has been used to estimate the rate of ATP production (Boska, 1991). Studying the kinetics of PCr and pHi at the onset of exercise and during recovery with the use of ^{31}P-MRS will help determine the relative roles for each pathway of ATP production during muscular work.

^{31}P-MRS is a useful tool to investigate noninvasively the relationship between muscle performance and the supply of energy, by measuring the alterations of metabolic parameters.

2.6.2 Rest-Exercise-Rest Transitions

Using ^{31}P-MRS to study the on- and off- transients of rest-exercise-rest may provide

better insight about the muscle's energy capacity. Marsh et al. (1993) studied the on-off transients for rest to steady-state exercise and back to rest and demonstrated that the time constant for the decrease in PCr at the onset of exercise was the same as that for the recovery of PCr after exercise (approximately 30 secs) and similar to that reported for O_2 consumption measured during cycle ergometry (Whipp et al., 1982). These results suggested that the on- and off-transients for PCr kinetics may be useful for measuring muscle oxidative capacity (Marsh et al, 1993). Studies have demonstrated that both the PCr and Pi time constants at the onset of exercise are independent of exercise intensity (Yoshida & Watari, 1993). However, the time constant for Pi recovery is slowed by the lower pH at the end of exercise generated by the high-intensity exercise (Yoshida & Watari, 1993). In summary, measuring the on- and off-transients of PCr provides insight into the energy system providing ATP.

2.6.3 Thresholds During Exercise

With the use of the ^{31}P-MRS the intramuscular threshold can be monitored noninvasively throughout exercise. Systrom et al. (1990) studied the thresholds of ventilation, venous lactate, and pHi. Subjects performed a plantar flexion exercise of progressively increasing intensity while ventilation, venous lactate, and pHi were measured. The results showed a pHi threshold that coincided with ventilatory and lactate thresholds. It was concluded that the developing acidity in the muscle during the progressive exercise protocol may explain the lactate threshold, and the increase in ventilation at this point (Systrom et al., 1990). Systrom et al. (1990) also suggested that the pHi threshold was a threshold for glycolytic metabolism. Marsh et al. (1991) and Kent-Braun et al. (1993) investigated intracellular metabolic thresholds during a progressive exercise protocol involving wrist flexor muscles and found that at approximately 60 % of maximum force, there

was a rapid increase in the rate of change in the log of Pi/PCr and pHi.

2.6.4 Metabolic Recovery From Exercise

The resynthesis of PCr after muscular contraction is accomplished primarily by means of oxidative phosphorylation. The half-time of recovery is used as an index of oxidative metabolism. The resynthesis rates of PCr are influenced by the degree of acidosis that develops during exercise (Arnold et al., 1984). Fig. 3 demonstrates the very fast recovery of PCr with trained subjects who would have a larger oxidative capacity compared to normal and subjects with a disease that affects oxidative capacity (Bendahan et al., 1990).

2.6.5 The Effects of ACZ on Intracellular pH

Intracellular pH decreased when isolated rat skeletal muscle was incubated with a CA inhibitor. The pHi values in EDL and soleus were found to be about 7.10 and 7.16 (Geers & Gros, 1988a, 1988, 1990) and pHi decreased when incubated in CLZ ($5 \cdot 10^{-4}$M) to about 6.97 for EDL and 7.05 for soleus. The effect of CLZ on pHi was related to the suppression of facilitated CO_2 diffusion.

2.6.6 The Effects of ACZ on Energy-Rich Phosphates

The metabolites involved in energy production (ADP + PCr + H^- → ATP + creatine) and utilization (ATP → ADP + Pi + energy) were shown to be affected by CA inhibition during exercise. With moderate intensity exercise under control conditions without CA inhibition, there is a progressive increase in the Pi concentration and decline in the intramuscular PCr concentration (Marsh et al., 1993b). Administration of a CA inhibitor resulted in a greater increase in Pi and breakdown in PCr during muscle stimulation (Geers & Gros 1988, 1990).

The increase in Pi and decrease in pHi following CA inhibition may affect muscle

contractile properties. A decrease of pHi and increase in Pi following CA inhibition may contribute to the increase in time to peak tension, decrease in force and a prolonged relaxation time observed by (Geers & Gros (1990; 1995). The observed changes in phosphate metabolites and pHi may result from a respiratory acidosis that develops in skeletal muscle following CA inhibition (Geers & Gros, 1990).

2.6.7 Summary

The ^{31}P-MRS is a noninvasive tool to study human skeletal muscle metabolism. By understanding the changes that occur with high energy phosphates during exercise and in recovery the oxidative and glycolytic metabolism can be studied..

TABLE 1 SUMMARY OF THE CARBONIC ANHYDRASE ASSOCIATED WITH MUSCLE

Carbonic Anhydrase	Activity Level	Resistance Level	Location	Role
CA II	highest specific activity	sensitive	cytosol type II muscle fibres cyosol type I muscle fibres cytoplasm of RBC	facilitating CO_2
CA III	lowest specific activity	resistant	type I muscle fibres type IIa muscle fibres	facilitating CO_2 regulating pHi metabolic modulator
Membrane Bound (Family of CA IV)	between CA II and I	sensitive	all fiber types	provides a pool of protons to compensate for the Ca^{2+} facilitates CO_2 diffusion, accelerating uptake of CO_2
CA IV	same as CA II	sensitive	endothelial cells of capillaries of all fiber types	accelerates the amount of CO_2 taken up by the blood during capillary transit

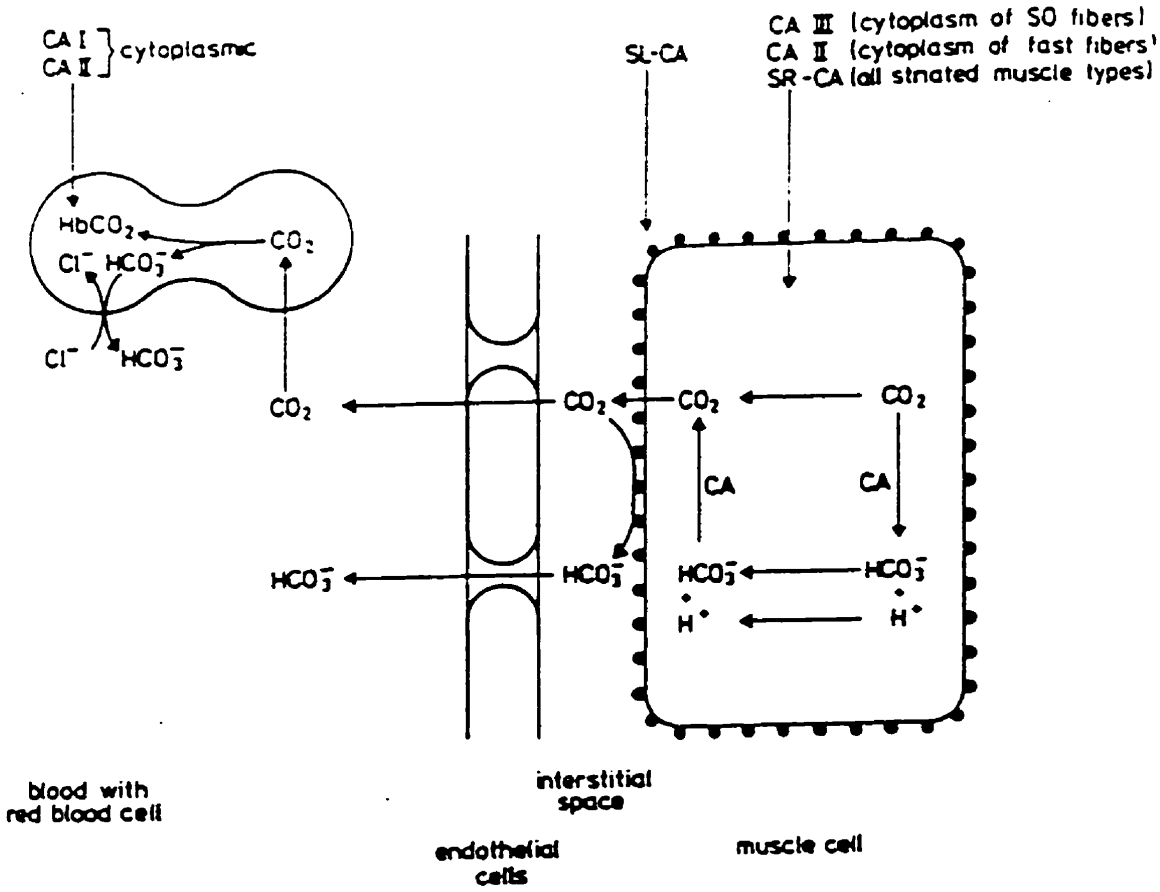

Fig 1. Schematic diagram of the localization of CAs inside striated muscles. Their functions for the transfer of CO_2 from the site of production within muscle cells to the blood are 1. facilitation of CO_2 diffusion within the muscle cell and 2. extracellular catalysis of CO_2 transport in muscle capillary. SL-CA. extracellular sarcolemmal CA; SR-CA. sarcoplasmic reticulum CA.
(Geers & Gros, 1991)

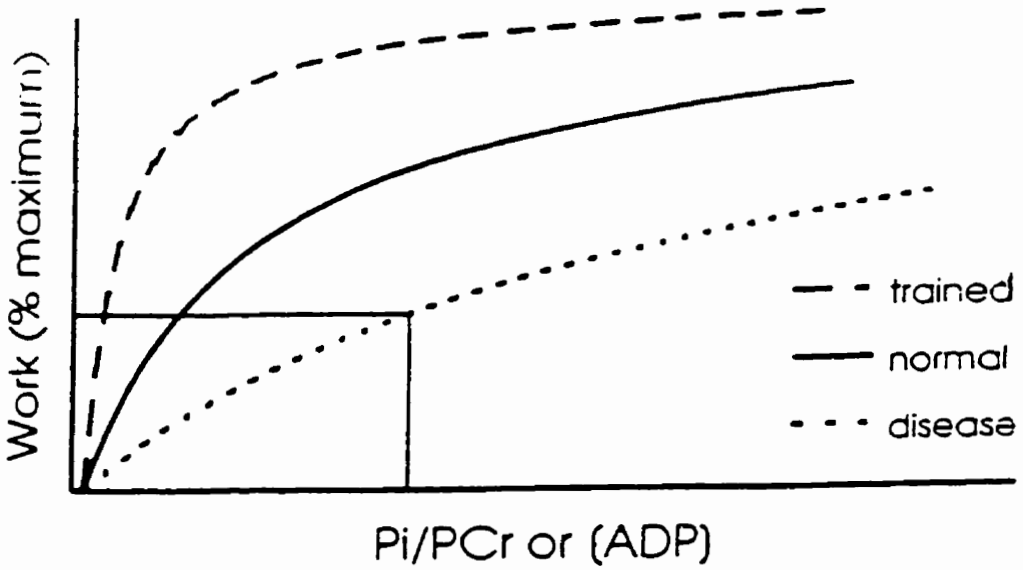

Fig 2 Schematic of the general relationship between power or force and Pi/PCr during progressive exercise. As the workload increases, there is a concomitant increase in Pi/PCr as the muscle responds to the increased energy demand. The initial, linear portion of this curve (workload <40% maximum) can be used to estimate the oxidative potential of the muscle (box). In trained compared with untrained subjects, there is less of an increase in Pi/PCr at any given relative workload, thus indicating an improved ability to keep pace with energy needs by means of oxidative phosphorylation. In contrast, in persons with disease that impairs muscle metabolism (either directly or indirectly), the initial slope of work versus Pi/PCr is decreased, indicating a poor capacity for oxidative metabolism.
(Kent-Braun et al., 1995)

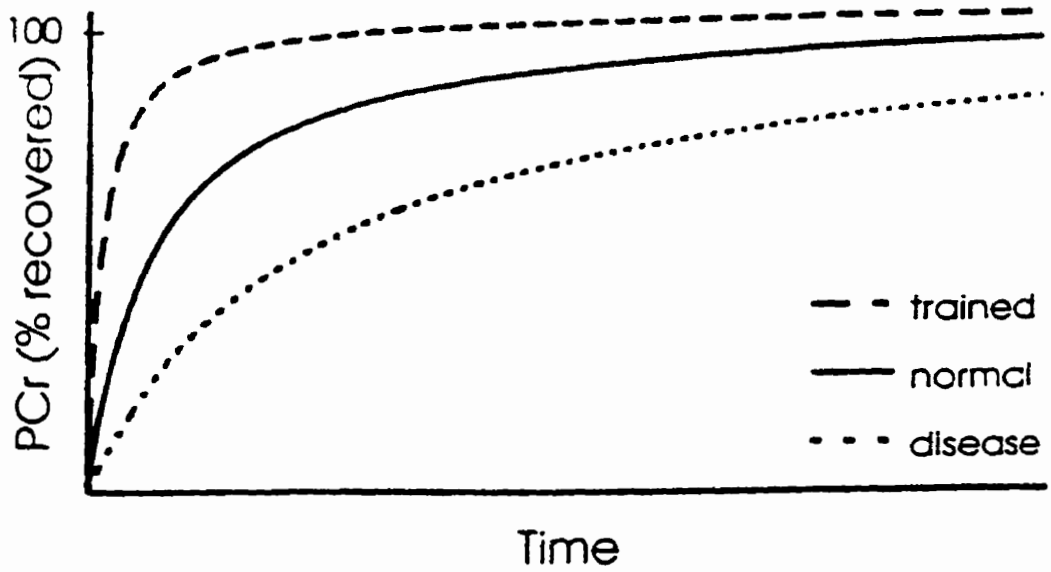

Fig 3 Schematic of PCr recovery after exercise. Phosphocreatine resynthesis after muscular contraction is accomplished primarily by means of oxidative phosphorylation. Individual data are fit to a single exponential and the half-time of recovery is used as an index of oxidative metabolism. This analysis requires exercise conditions in which PCr declines to approximately 50% of initial with no significant decline in intramuscular pH. In trained subjects, PCr recovery is very fast compared with normal subjects. In subjects with disease that affects oxidative capacity, the rate of PCr recovery is slowed.
(Kent-Braun et al., 1995)

CHAPTER 3
METHODS

3.1 Subjects

Adult male subjects (n=9; age, 20-25 yrs; body mass, 80 ± 9.1 kg) participated in this study. The subjects were healthy, active and relatively fit third year undergraduate students enrolled in the Kinesiology programme at The University of Western Ontario. The procedures and any potential risks were explained to each subject and an informed consent was signed prior to participating in the experiment. The study was approved by The University of Western Ontario Review Board for Health Sciences Research involving Human Subjects.

3.2 General Protocol

Subjects were studied twice for each condition, control (CON) and following acetazolamide (ACZ) administration. Two exercise tests were performed under these conditions to evaluate i) muscle metabolism and acid-base status using ^{31}P-MRS and ii) plasma [La^-] and acid-base status in the venous blood draining the active forearm muscles using standard venous sampling techniques.

The exercise tests were performed at the same time of day for each subject. Subjects reported to the laboratory at least 4 hours after a light meal and were instructed not to consume any caffeine-containing foods or beverages prior to testing. On arriving at the laboratory subjects rested in a supine position while a percutaneous Teflon catheter (Angiocath, 20 g) was placed retrograde into the median cephalic vein of the dominant arm (towards the perforating vein) at the cubital fossa to facilitate blood sampling and ACZ administration. After 15 min of rest a blood sample was drawn (pre-infusion), followed by

ACZ infusion (10 mg/kg iv. over a 2-min period). The administration of ACZ was randomly assigned for each of the 2 types of exercise tests (^{31}P-MRS; blood sampling, (BLD)). A placebo was not administered in this study because in our experience the side effects of ACZ administration, although minor in discomfort, are recognized by the subjects. The subjects rested for an additional 45-min, and then blood samples were drawn (post-infusion).

The exercise protocol was identical for each of the four tests and consisted of progressive wrist flexion exercise to fatigue, performed on a specially built wrist ergometer (Marsh et al., 1991). The subjects were required to repeatedly depress a lever at a frequency of 0.5 Hz (1 s contraction/1 s relaxation) through a range of motion of 70°. This action raised and lowered a variable resistance (a water reservoir) suspended outside via a cable-and-pulley system. The resistance on the wrist dynamometer was increased in a ramp-like fashion by pumping water into the reservoir at a constant rate (200 ml/min) by means of a roller pump (Cole-Parmer Instruments, Chicago, IL). The subject's dominant arm was positioned into the wrist flexor dynamometer with the arm extended, abducted to 90°, and the forearm pronated with the hand grasping the contraction lever of the ergometer. The arm was aligned such that the pivot of the lever was centered on the wrist joint. With the arm in this position the contracting forearm musculature was positioned at heart level thus ensuring adequate perfusion during the relaxation phase of each contraction/relaxation cycle. The subjects remained supine throughout the protocol.

Exercise began with 4 min of forearm flexion against a zero load so subjects could accommodate to the exercise protocol. The load was then increased by attaching the empty collection cylinder (mass = 1.07 kg) to the apparatus and contractions continued for another 1 min. After this initial accommodation period the resistance began to increase in a ramp-like

fashion by starting the roller pump and adding water to the reservoir. The forearm contractions continued to volitional fatigue. Power output was calculated as the product of the mass of the reservoir plus water (1.07 kg + (flow rate • time)), the contraction frequency (0.5 Hz), and the vertical distance traveled by the reservoir per contraction (0.09 m). Work was initiated at 0.47 W (representing the weight of the empty reservoir) and increased at approximately 0.09 W/min until fatigue. Before each experiment the roller pump was calibrated and adjusted to ensure a constant flow rate of 200 ml/min. In addition, the actual rate of water flow into the reservoir was calculated as the total water volume added to the reservoir during the exercise test divided by the time to fatigue. The average rate of flow for each of the exercise tests was similar in CON (204 ± 2 ml/min) and ACZ (201 ± 3 ml/min).

3.3 ^{31}P-MR Spectroscopy

Forearm muscle metabolism was studied with ^{31}P-MRS using a replica of the wrist ergometer positioned within the bore of a 20-cm bore 1.89-T (Tesla) superconducting magnet. This magnet was interfaced with a SMIS console (Surrey Medical Imaging Systems, Guilford, U.K.). Before starting the exercise protocol, each subject's arm was imaged using a multislice gradient echo sequence to ensure proper positioning of the flexor digitorum profundus (FDP) and flexor digitorum superficialis (FDS) above the phosphorus surface coil. The proton signal was then used to shim the magnet homogeneity and improve spectral resolution. Homogeneity was adjusted until the full width, half maximum of the water peak was less than 0.4 PPM and the peak was Lorentzian in shape.

Subjects rested in the supine position with their arm inserted into the bore of the magnet and positioned as described previously. A 4-cm dual tuned coil was positioned under the belly of the forearm approximately 7-9 cm distal to the medial epicondyle of the humerus.

In this position, the ^{31}P-MRS signal obtained during exercise was primarily from the flexor digitorum superficialis muscle.

Spectra were collected sequentially throughout rest (15 min), the accommodation period, ramp exercise, and recovery (16 min). All spectra were acquired using a 3ms adiabatic 90 degree RF pulse, 12μs delay time, 3.33 kHz receiver bandwidth, and 2048 complex data points. The initial nine excitations were used to establish steady state T_1 saturation then four resting spectra were collected in total to establish a baseline just prior to starting exercise. All spectra collected during baseline, exercise, and recovery were acquired with eight averages to give a sampling time of 24 seconds.

3.4 Blood Samples

During the BLD studies, blood was drawn from the deep arm vein at the following times: pre- and post-infusion of ACZ or pre-accommodation in CON; at 4 min (after zero load contractions) and 1 min (after reservoir-only contractions) of the accommodation period; at 30 s intervals during the ramp protocol; at the point of fatigue (0 min recovery); at 1 min recovery and at 2 min intervals to 15 min recovery. Blood was drawn into heparnized syringes, mixed, and stored in ice water and analyzed after a short delay. The whole blood samples (200 μl) were analyzed (at 37° C) for plasma pH, PCO_2, and plasma concentration of La^- using selective electrodes (StatProfile 9 Plus Blood Gas-Electrolyte Analyzer, Nova Biomedical Canada Ltd., Mississauga, Ontario). The electrodes were calibrated prior to each test and at regular intervals during analysis. Plasma concentration of H^+ was calculated from the measured pH.

3.5 Data Analysis

Metabolite quantification was performed in the time (acquisition) domain by fitting the

free induction decay (fid) data to a sum of damped sinusoids. These exponentially damped sinusoids, could be varied in amplitude, phase, delay time, damping constant, and frequency. The quantification software (Potwarka, 1995; Bartha, 1995) used a-priori knowledge and the Levenberg- Marquardt algorithm (Marquardt, 1963) to iteratively reduce the difference between the data and exponential model. The software required little operator intervention and all spectra were fit to the same template after application of a 2 Hz Lorentzian filter. The first 1.5 ms of data were not used in order to eliminate the very broad (FWHM>100Hz) phosphorus components originating from regions with large in homogeneities or bone. The relative concentration of each metabolite was taken as the amplitude of the exponential model function at time equal zero. The amplitudes at t=0 were used to calculate the relative contributions of the phosphate metabolites, α-ATP, β-ATP, γ-ATP, PCr, and Pi; the ratio Pi/PCr. Intracellular pH (pHi) was determined from the chemical shift of Pi with respect to PCr (Taylor et al., 1983).

The logarithm of the ratio Pi/PCr (logPi/PCr) and pHi were plotted against power output and the log Pi/PCr ($T_{logPi:PCr}$) and pHi (T_{pHi}) thresholds were determined. This was done in two ways: i) by visual inspection of the data plotted against power output, and ii) by computer analysis. In the first instance, 3 investigators were unaware of the subject and condition, and a threshold or breakpoint was identified by visual inspection of the logPi/PCr and pHi versus power plots. The threshold was considered to be the power output corresponding to the point of intersection of two straight lines fit through the data (Fig 4). In the second instance, a piecewise linear regression analysis was applied to individual data plots (Vieth, 1989). The program estimated the parameters of two regression functions and determined a breakpoint where the two lines diverged. This point of intersection represented

the respective intramuscular threshold ($T_{logPi/PCr}$; T_{pHi}). The computer- and investigator-derived thresholds were compared statistically and found to be statistically the same (see Fig. 4). The actual threshold values reported are the means of the investigator-derived thresholds. In a previous study a high test-retest correlation of 0.92 and 0.98 was reported for identifying the $T_{logPi/PCr}$ and T_{pHi}, respectively, demonstrating the reliability and reproducibility of these threshold measurements (Marsh et al., 1991).

The plasma lactate threshold (T_{La^-}) was determined by visual inspection of the plasma [La^-]-power output curve. The investigators were unaware of the subject and condition, and the T_{La^-} was identified as the power output corresponding to a 1.0 mmol/l increase in plasma [La^-] above resting levels.

3.6 Statistical Analyses

Statistical analyses were performed using a computerized statistical program for the PC (SigmaStat, Jandal Scientific, San Rafael, CA.). Muscle and plasma metabolic data were analyzed for condition (CON; ACZ) and time main effects using a two-way repeated measures analysis of variance. During studies involving blood sampling, we were unable to collect samples from subject one due to excessive blood clotting within the catheter. As a consequence, statistical comparison involving blood data were performed in the remaining 8 subjects only. Intracellular and plasma thresholds were analyzed for condition (CON; ACZ) and test type (^{31}P-MRS; BLD) main effects using a two-way repeated measures analysis of variance. In both instances, a significant F-ratio was further analyzed using Student-Neuman-Keuls post-hoc test. Statistical significance was accepted at $p<0.05$. Data were reported as the mean ± SD.

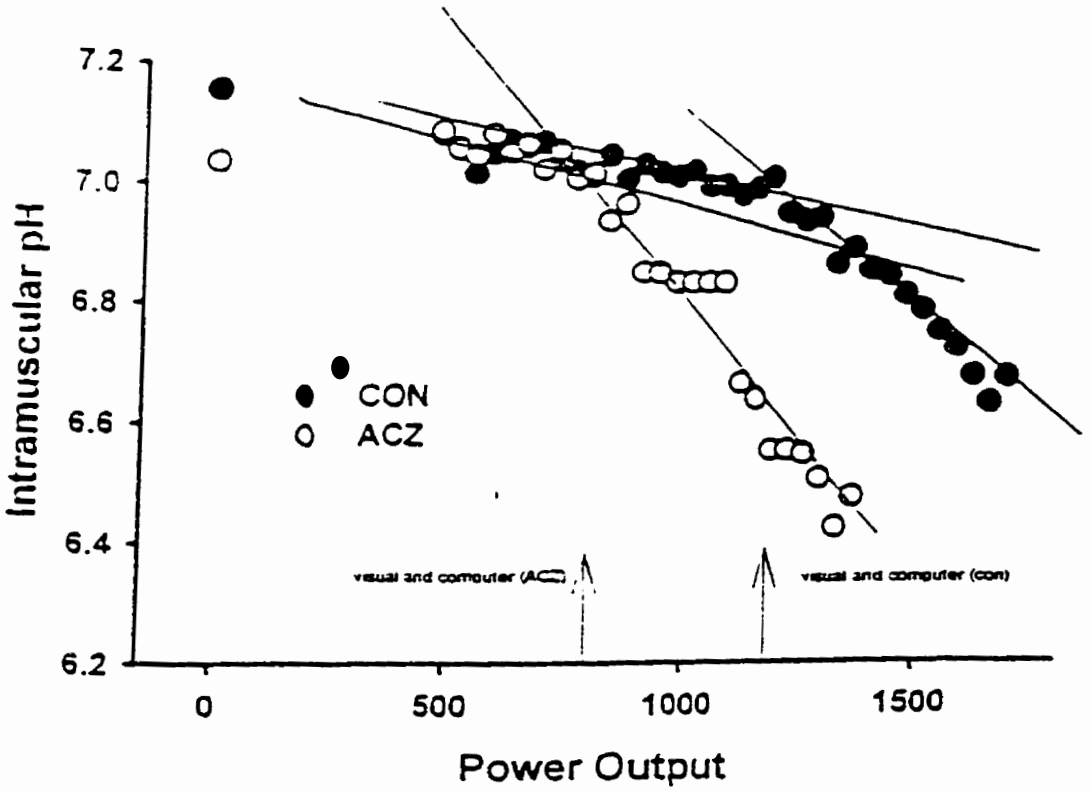

Fig 4. An example of intramuscular pH changes during progressive forearm flexion protocol and the intracellular pH thresholds (T_{pH}) determined by visual inspection and computer analysis for 1 subject.

CHAPTER 4
RESULTS

4.1 Exercise Performance

Studies were performed at the Lawson Research Institute for collection of muscle metabolic data using ^{31}phosphorus magnetic resonance spectroscopy (^{31}P-MRS) and at the Centre for Activity and Ageing for collection of blood data (BLD). During the exercise performance test resistance was increased in a ramp-like fashion with no difference in the rate of the ramp during the ^{31}P-MRS and BLD experiments, or the CON and ACZ conditions (~ 0.09 W/min;~ 200 ml H_2O/min; Appendix A). The time to fatigue and peak power output were greater ($p<0.05$) during the ramp tests performed during BLD than ^{31}P-MRS experiments (Table 2). However, in neither instance was there any difference between CON and ACZ.

4.2 Plasma Lactate and Acid-Base Status

The effect of ACZ administration on equilibrated plasma [La^-] during the forearm ramp protocol are presented in Fig. 5. Plasma [La^-] increased with increasing power output and % time to fatigue. End-exercise plasma [La^-] was greater ($p<0.05$) in CON (5.0 ±1.7 mmol/l) than ACZ (3.7 ± 1.7 mmol/l) in spite of the similar peak power output between these conditions. Plasma [La^-] was also greater ($p<0.05$) in CON than ACZ during the first 3 min of recovery, but, after this time no difference was observed between conditions. The end recovery plasma [La^-] (CON 1.93 ± 0.52 mmol/l; ACZ 1.94 ± 0.33 mmol/l) remained elevated ($p<0.05$) above resting values (CON 1.01 ± 0.43 mmol/l; ACZ 1.23± 0.36 mmol/l). The plasma La^- threshold (T_{La^-}) occurred at a lower ($p<0.05$) absolute power output in CON (1041 ± 414 mW) than ACZ (1433 ● 243 mW) (Table 3). In addition, the T_{La^-} occurred at a lower

($p<0.05$) percent peak power output in CON (53 ± 19%) than ACZ (76 ± 17%) (Table 3).

The effects of ACZ administration on equilibrated venous plasma pH during the forearm ramp protocol is presented in Fig. 6. Plasma pH was similar at rest (post-infusion) (CON, 7.41 ± 0.04; ACZ, 7.48 ± 0.13) and decreased with increasing power output and % time to fatigue, reaching a similar end-exercise plasma pH between conditions (CON, 7.29 ± 0.05; ACZ, 7.31 ± 0.06). In general, plasma pH was similar in CON and ACZ throughout recovery, except at 11 and 15 min recovery where plasma pH was lower ($p<0.05$) in ACZ.

The effects of ACZ administration on equilibrated plasma [H^+] during the forearm ramp protocol are presented in Fig. 7. Plasma [H^+] was similar at rest (CON, 40 ± 4 nmol/L; ACZ, 35 ± 10 nmol/L) and increased with increasing power output and % time to fatigue. End-exercise plasma [H^+] was similar between conditions (CON, 51 ± 7 nmol/l; ACZ, 50 ± 6 nmol/l). As it was with plasma pH, plasma [H^+] was similar for the CON and ACZ groups throughout recovery, except at 11 and 15 min where plasma [H^+] was higher ($p<0.05$) in ACZ.

The effects of ACZ administration on equilibrated venous plasma PCO_2 during the forearm ramp protocol are presented in Fig. 8. Plasma PCO_2 was similar at rest after ACZ infusion (CON, 47 ± 6 mm Hg; ACZ, 42 ± 10 mm Hg) plasma PCO_2 increased abruptly at the onset of exercise, changed very little during moderate intensity exercise, and PCO_2 increased further during heavy exercise reaching a similar end-exercise PCO_2 (CON, 59 ± 11 mm Hg; ACZ, 60 ± 11 mm Hg). Plasma PCO_2 decreased to resting levels during recovery with no difference between conditions.

4.3 Intramuscular Metabolic and Acid-Base Status

The effects of ACZ administration on intramuscular pH (pHi) during the forearm ramp

protocol are shown in Fig 9. Intramuscular pH decreased with increasing power output and % time to fatigue, with no difference observed between conditions. End-exercise pHi was also similar in the two conditions (CON, 6.43 ± 0.22; ACZ, 6.38 ± 0.10). The slope of the pHi versus power output relationship was similar between conditions but was less below T_{pHi} compared to above T_{pHi} (Table 4). During the early part of recovery (1-7 min) pHi was higher ($p<0.05$) in CON than ACZ; the pHi at end-recovery was similar between conditions (CON, 7.02 ± 0.06; ACZ, 7.04 ± 0.06) and similar to pre-exercise values (CON, 7.05 ± 0.06; ACZ, 7.07 ± 0.05). The pHi returned to similar pre-exercise values at 9 min of recovery for CON (6.98 ● 0.11) and at 15 min of recovery for ACZ (7.04 ± 0.06). The T_{pHi} occurred at a similar power output (CON, 855 ± 211 mW; ACZ, 722 ± 50 mW) and % peak power output (CON, 49 ± 11%; ACZ, 46 ± 7%) in both conditions (Table 3). Compared to the T_{La-}, the T_{pHi} occurred at a similar absolute power output (T_{pHi}, 855 ± 211 mW; T_{La-}, 1041 ± 414 mW) and % peak power output (T_{pHi}, 49 ± 11%; T_{La-}, 53 ± 19%) in CON, but a lower power output (T_{pHi}, 722 ± 50 mW; T_{La-}, 1433 ± 243 mW) and % peak power output (T_{pHi}, 46 ± 7%; T_{La-}, 76 ± 17%) in ACZ (Table 3).

The effects of ACZ administration on intramuscular [H^+] during the forearm ramp protocol are presented in Fig 10. The intramuscular [H^+] increased with increasing power output and % time to fatigue, with no difference between conditions; end-exercise intramuscular [H^+] was similar between conditions (CON, 424 ± 186 nmol/l; ACZ, 432 ± 109 nmol/l). During the early part of recovery (1-7 min) intramuscular [H^+] was lower ($p<0.05$) in CON than ACZ; the intramuscular [H^+] at end-recovery was similar between conditions (CON, 95.2 ± 14.6 nmol/l; ACZ 91.9 ± 13.0 nmol/l) and similar to pre-exercise values (CON, 84.5 ● 8.9 nmol/l; ACZ, 84.6 ± 7.2 nmol/l).

The effects of ACZ administration on log (Pi/PCr) are presented in Fig 11. Log (Pi/PCr) increased as power output and % time to fatigue increased with no difference between conditions reaching similar end-exercise values in both conditions (CON, 0.56 ± 0.51; ACZ, 0.91± 0.54). The slope of the log (Pi/PCr) versus power output relationship was similar between conditions but was less below $T_{logpi/PCr}$ compared to above $T_{logpi/PCr}$ (Table 5). During recovery, log (Pi/PCr) decreased to pre-exercise levels with no difference between conditions. The absolute power output (CON, 835 ± 235 mW; ACZ, 796 ± 75 mW) and % peak power output (CON, 53 ± 11%; ACZ, 51 ± 6%) corresponding to the $T_{log(Pi/PCr)}$ were similar between conditions and similar to the T_{pHi} within each condition. While no difference was observed between $T_{log(Pi/PCr)}$ and T_{La-} in CON, the $T_{log(Pi/PCr)}$ occurred at a lower power output ($T_{log\ (Pi/PCr)}$ 796 ± 75 mW; T_{La-} 1433 ± 243 mW) and % peak power output ($T_{log\ (Pi/PCr)}$ 51 ± 6%; T_{La-} 76 ± 17%) than the T_{La-} in ACZ (Table 3).

Table 2 Time to fatigue (min) and peak power output (mW) during the progressive ramp exercise tests to fatigue for control (CON) and acetazolamide (ACZ) during studies using magnetic resonance spectroscopy (MRS) and blood collection (BLD).

	CON		ACZ	
	Time to Fatigue (min)	peak PO (mW)	Time to Fatigue (min)	peak PO (mW)
MRS				
1	13.2	1640	11.6	1501
2	13.6	1640	10.4	1417
3	13.2	1647	13.2	1651
4	10.8	1479	14.0	1642
5	11.2	1497	15.2	1770
6	14.8	1708	12.8	1611
7	11.6	1554	14.4	1779
8	14.0	1728	10.4	1417
9	9.2	1188	11.2	1428
MEAN	12.4*	1565*	12.6*	1580*
SD	1.8	166	1.8	145
BLD				
1	---	---	---	---
2	19.0	2194	16.0	1920
3	16.0	1911	16.0	1907
4	12.0	1532	13.0	1620
5	16.0	1903	20.0	2207
6	16.0	1911	17.0	1973
7	19.0	2172	20.0	2238
8	16.0	1929	16.0	1876
9	16.3	1934	13.0	1642
MEAN	16.3*	1936*	16.4*	1923*
SD	2.2	203	2.7	225

* significant difference ($p<0.05$) between MRS and BLD

Table 3 Intracellular threshold for log (Pi/PCr) ($T_{log(Pi/PCr)}$) and pHi (T_{pHi}), and plasma La^- threshold (T_{La^-}) for Control (CON) and Acetazolamide (ACZ).

	T_{La^-}			T_{pHi}			$T_{log(Pi/PCr)}$		
	La- (mmol/l)	PO (mW)	%peakPO (%)	pHi	PO (mW)	%peakPO (%)	log(Pi/PCr)	PO (mW)	%peakPO (%)
CON:									
1	—	—	—	7.08	808	49	-0.6	843	51
2	2.7	1267	58	6.93	843	51	-0.3	949	58
3	1.6	1355	71	6.98	737	45	-0.6	808	49
4	1.0	649	42	7.00	773	52	-0.5	773	52
5	1.3	605	32	7.06	737	49	-0.9	561	37
6	1.7	472	25	6.92	1196	28	-0.4	1161	68
7	2.5	1355	62	6.94	843	54	-0.7	702	45
8	1.9	1046	54	6.98	1196	69	-0.2	1196	69
9	1.7	1576	81	6.96	561	47	-0.5	525	44
MEAN	1.8	1041*	53	6.98	855	49	-0.5	835	53
SD	0.6	414	19	0.1	211	11	0.2	235	11
ACZ:									
1	—	—	—	7.06	773	51	-0.5	773	51
2	2.5	1311	68	6.92	773	55	-0.2	773	55
3	2.2	1399	73	7.03	773	47	-0.5	914	55
4	2.0	1532	95	7.02	631	38	-0.4	702	43
5	1.8	914	41	6.98	702	40	-0.4	879	50
6	2.4	1444	73	7.08	702	44	-0.5	773	48
7	1.8	1664	74	6.97	667	37	-0.3	737	41
8	2.3	1664	89	7.06	737	52	-0.3	737	52
9	2.2	1532	93	6.91	737	52	-0.1	879	62
MEAN	2.2	1433*+	76*+	7.00	722*	46	-0.4	796*	51
SD	0.3	243	17	0.1	50	7	0.1	75	6

* significant difference ($p<0.05$) between CON and ACZ.
+ significant difference ($p<0.05$) between plasma and intracellular thresholds.

Table 4 Regression slope (m), intercept (b), and correlation coefficient (r) for pHi vs. power output for control (CON) and acetazolamide (ACZ)

CON	BELOW			ABOVE		
	m	b	r	m	b	r
1	1.62×10^{-4}	6.99	0.78	7.95×10^{-4}	7.76	0.95
2	3.59×10^{-4}	7.18	0.88	9.57×10^{-3}	7.82	0.95
3	1.39×10^{-4}	7.09	0.67	8.08×10^{-4}	7.60	0.98
4	6.13×10^{-6}	7.05	0.04	1.07×10^{-3}	7.82	0.99
5	6.40×10^{-5}	7.03	0.24	2.23×10^{-4}	7.23	0.84
6	1.26×10^{-4}	7.13	0.69	5.53×10^{-4}	7.68	0.83
7	1.06×10^{-4}	7.09	0.72	1.2×10^{-3}	8.00	0.98
8	1.34×10^{-4}	7.14	0.91	7.02×10^{-4}	7.83	0.98
9	2.10×10^{-5}	7.03	0.28	7.52×10^{-4}	7.43	0.96
MEAN	1.27×10^{-4}*	7.08*	0.58*	7.84×10^{-4}*	7.69*	0.94*
±SD	1.00×10^{-4}	0.06	0.31	2.88×10^{-4}	0.24	0.06
ACZ						
1	5.25×10^{-6}	7.07	0.04	1.45×10^{-3}	8.24	0.98
2	1.41×10^{-4}	7.11	0.56	1.13×10^{-3}	7.86	0.96
3	5.29×10^{-5}	7.08	0.43	7.92×10^{-4}	7.67	0.97
4	1.96×10^{-4}	7.17	0.89	6.99×10^{-4}	7.48	0.99
5	2.22×10^{-4}	7.13	0.91	5.41×10^{-4}	7.37	0.95
6	6.66×10^{-6}	7.10	0.04	8.90×10^{-4}	7.79	0.95
7	5.39×10^{-5}	7.09	0.45	4.69×10^{-4}	7.34	0.94
8	8.56×10^{-6}	7.05	0.09	9.85×10^{-4}	7.79	0.98
9	2.28×10^{-4}	7.08	0.84	6.49×10^{-4}	7.40	0.97
MEAN	1.10×10^{-4}*	7.09*	0.47*	8.45×10^{-4}*	7.66*	0.97*
±SD	8.90×10^{-5}	0.04	0.36	3.10×10^{-4}	0.30	0.02

* significant difference ($p<0.05$) between below and above thresholds

Table 5 Regression slope (m), intercept (b), and correlation coefficient (r) for log (Pi/PC)r vs. power output for control (CON) and acetazolamide (ACZ).

CON	BELOW			ABOVE		
	m	b	r	m	b	r
1	4.23×10^{-4}	-0.96	0.71	1.51×10^{-3}	-1.92	0.98
2	9.39×10^{-4}	-1.15	0.91	1.10×10^{-3}	-1.16	0.38
3	6.63×10^{-4}	-1.09	0.85	2.08×10^{-3}	-2.33	0.97
4	6.75×10^{-4}	-1.07	0.92	1.96×10^{-3}	-2.02	0.98
5	1.62×10^{-4}	-0.79	0.86	7.86×10^{-4}	-1.31	0.93
6	4.62×10^{-4}	-0.85	0.80	1.79×10^{-3}	-2.50	0.91
7	2.56×10^{-4}	-0.81	0.64	1.69×10^{-3}	-1.75	0.97
8	5.49×10^{-4}	-0.92	0.88	2.01×10^{-3}	-2.71	0.98
9	1.13×10^{-3}	-1.06	1.0	5.77×10^{-4}	-0.67	0.76
MEAN	5.84×10^{-4}*	-0.97*	0.84*	1.50×10^{-3}*	-1.82*	0.87*
±SD	3.10×10^{-4}	0.13	0.11	5.50×10^{-4}	0.67	0.20
ACZ						
1	6.45×10^{-4}	-1.03	0.90	9.8×10^{-4}	-1.17	0.84
2	1.85×10^{-4}	-0.55	0.30	2.32×10^{-3}	-2.14	0.97
3	6.18×10^{-4}	-1.04	0.77	1.65×10^{-3}	-2.08	0.98
4	4.55×10^{-4}	-0.78	0.82	1.59×10^{-3}	-1.65	0.91
5	6.43×10^{-4}	-0.84	0.93	1.29×10^{-3}	-1.48	0.95
6	5.96×10^{-4}	-0.95	0.86	2.16×10^{-3}	-2.31	0.97
7	1.04×10^{-3}	-1.15	0.94	1.12×10^{-3}	-1.26	0.86
8	5.35×10^{-4}	-0.76	0.84	2.52×10^{-3}	-2.32	0.97
9	9.90×10^{-4}	-0.92	0.89	1.64×10^{-3}	-1.58	0.91
MEAN	6.34×10^{-4}*	-0.89*	0.81*	1.70×10^{-3}*	-1.78*	0.93*
±SD	2.60×10^{-4}	0.18	0.20	5.40×10^{-4}	0.45	0.05

* significant difference ($p<0.05$) between below and above thresholds

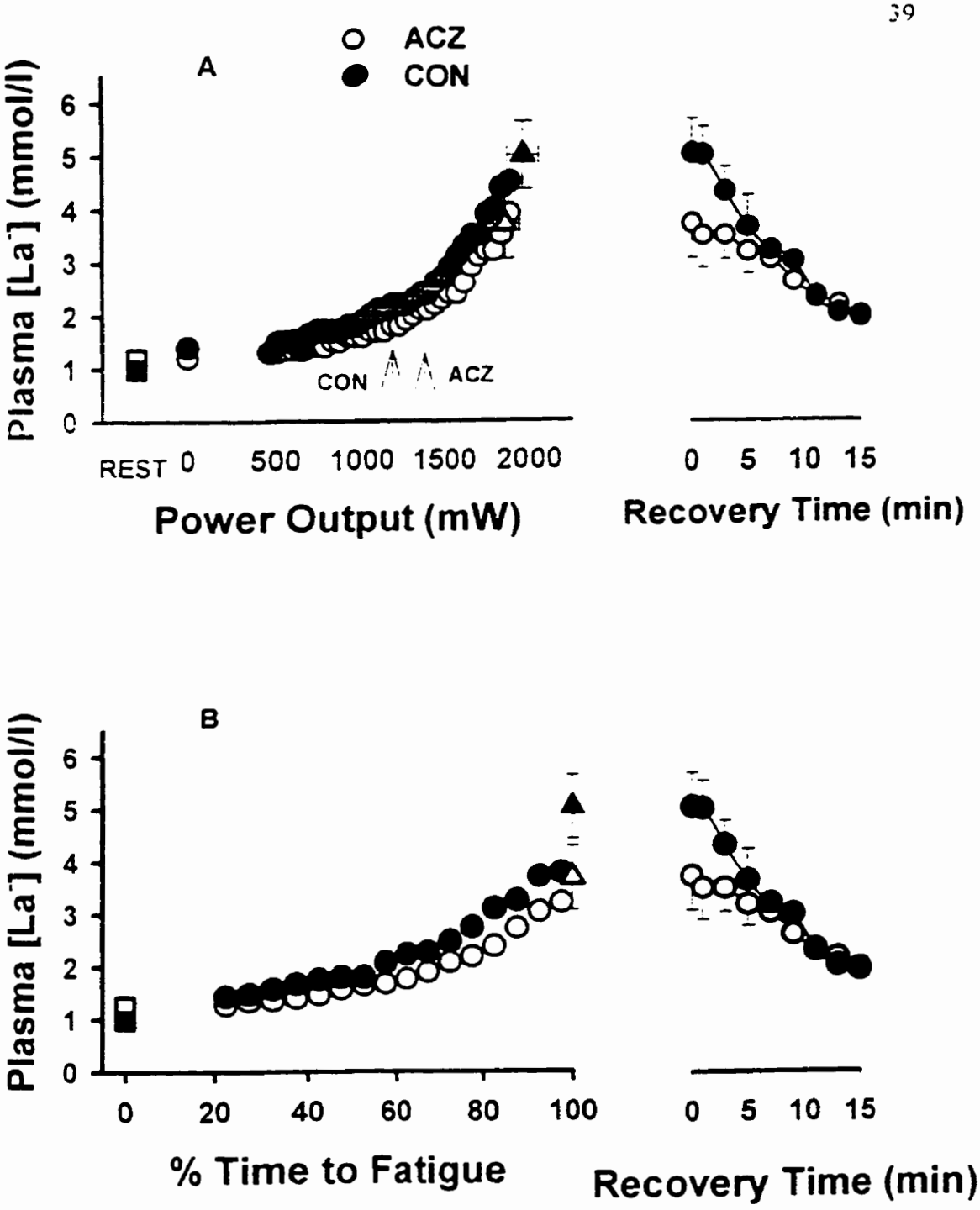

Fig 5 A. The effect of acetazolamide (ACZ) administration on plasma [La⁻] relative to power output (mW) during incremental forearm wrist flexion exercise and recovery time. B. The effect of acetazolamide (ACZ) administration on plasma [La⁻] relative to % time to fatigue during incremental forearm wrist flexion exercise and recovery time. Condition: CON, closed symbols; ACZ, open symbols. Time: Rest (□,■); Exercise and Recovery, where each point corresponds to the mean of 5 or more subjects (○,●) [fig 5a] or 8 subjects [fig 5b]; Fatigue, where each point corresponds to the mean of all 8 subjects (△,▲). Arrows correspond to the lactate threshold (T_{La}) forced condition. Error bars represent the SEM.

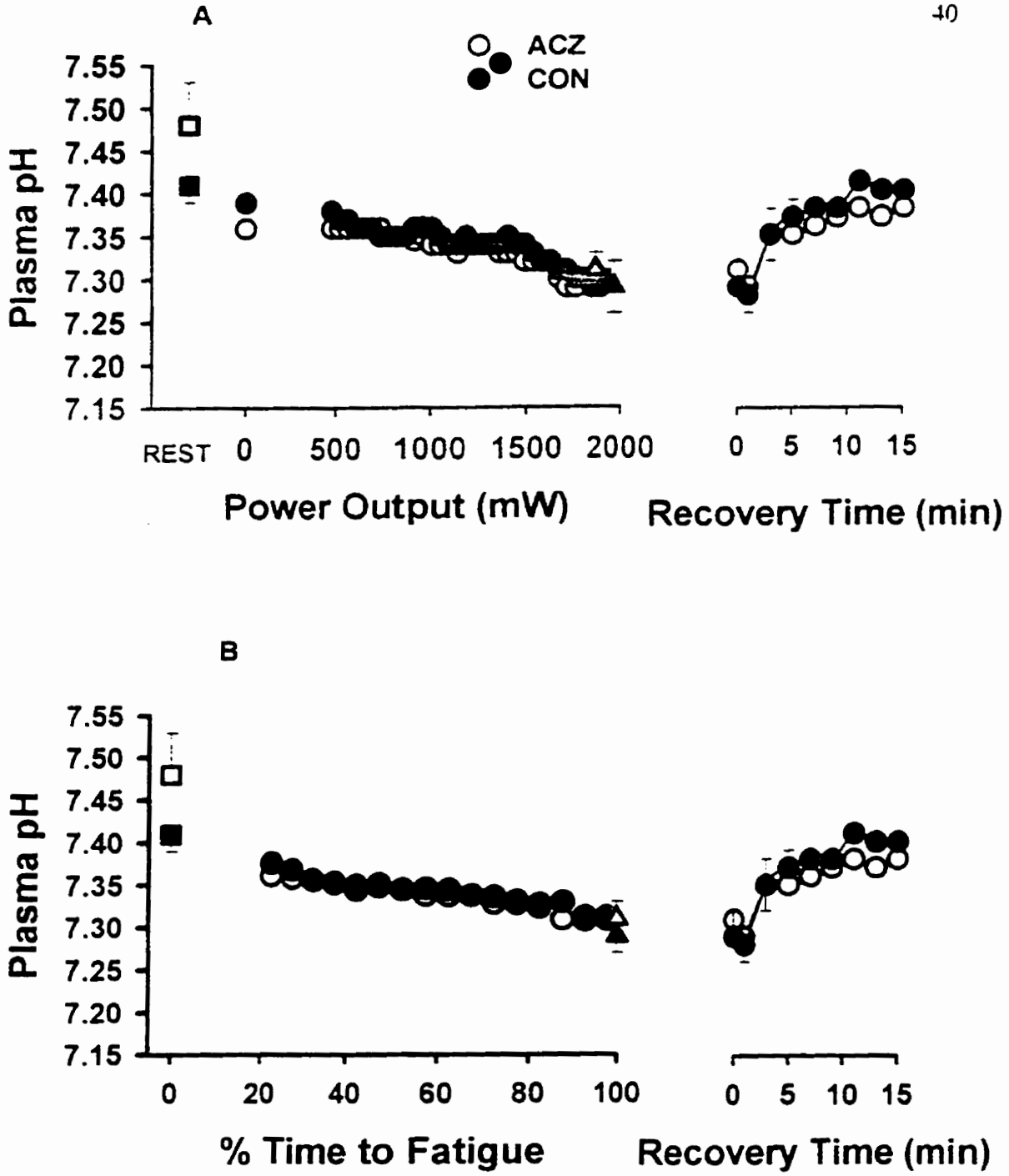

Fig 6. A. The effect of acetazolamide (ACZ) administration on plasma pH relative to power output (mW) during incremental forearm wrist flexion exercise and recovery time. B. The effect of acetazolamide (ACZ) administration on plasma pH relative to % time to fatigue during incremental forearm wrist flexion exercise and recovery time. Condition: CON, closed symbols; ACZ, open symbols. Time: Rest (▫,▪); Exercise and Recovery, where each point corresponds to the mean of 5 or more subjects (○,●) [fig 6a] or 8 subjects [fig 6b]; Fatigue, where each point corresponds to the mean of all 8 subjects (△,▲). Error bars represent the SEM.

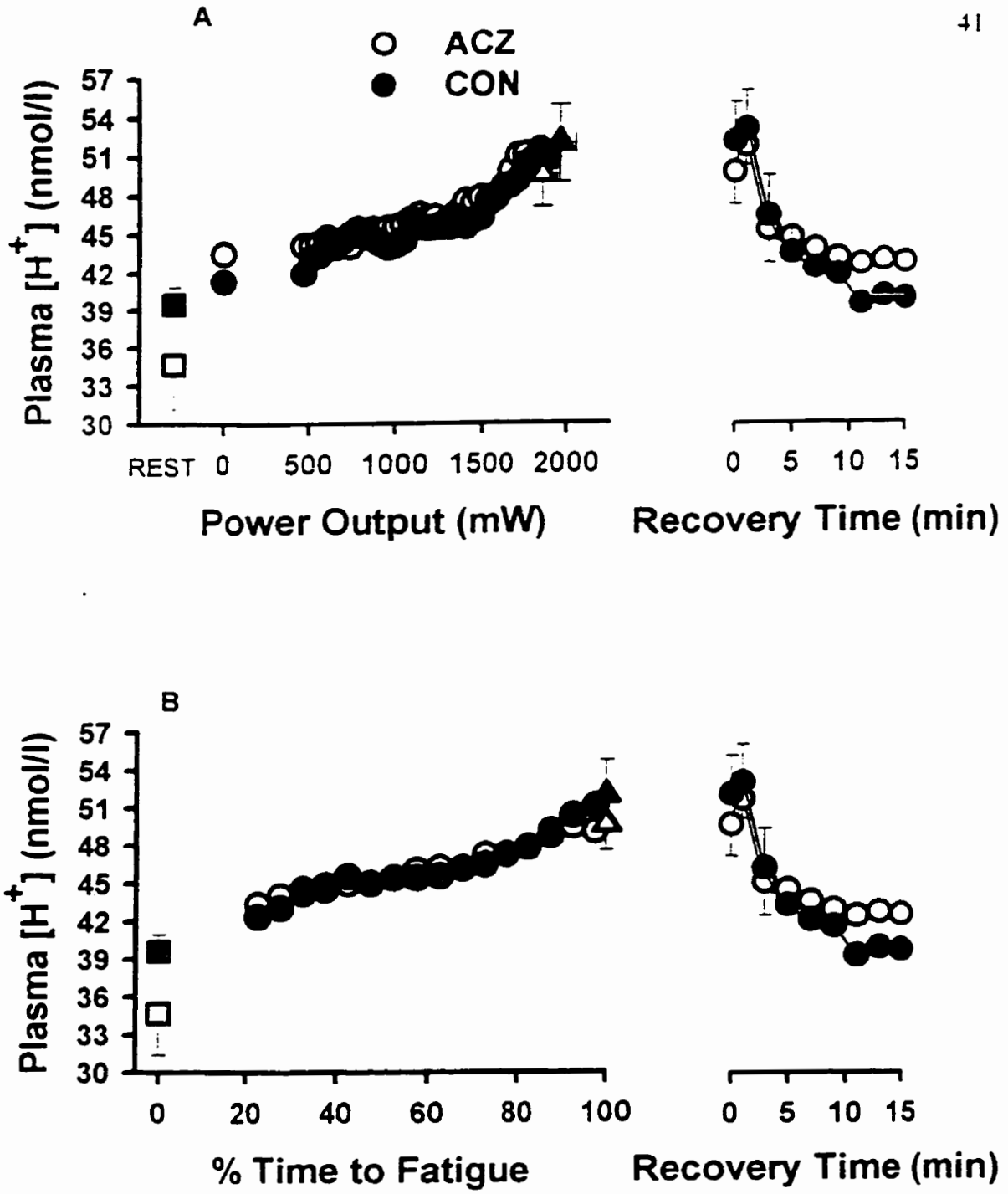

Fig 7. A. The effect of acetazolamide (ACZ) administration on plasma [H^+] relative to power output (mW) during incremental forearm wrist flexion exercise and recovery time. B. The effect of acetazolamide (ACZ) administration on plasma [H^+] relative to % time to fatigue during incremental forearm wrist flexion exercise and recovery time. Condition: CON, closed symbols: ACZ, open symbols. Time: Rest (▫,▪); Exercise and Recovery, where each point corresponds to the mean of 5 or more subjects (○,•) [fig 7a] or 8 subjects [fig 7b]; Fatigue, where each point corresponds to the mean of all 8 subjects (△,▲). Error bars represent the SEM.

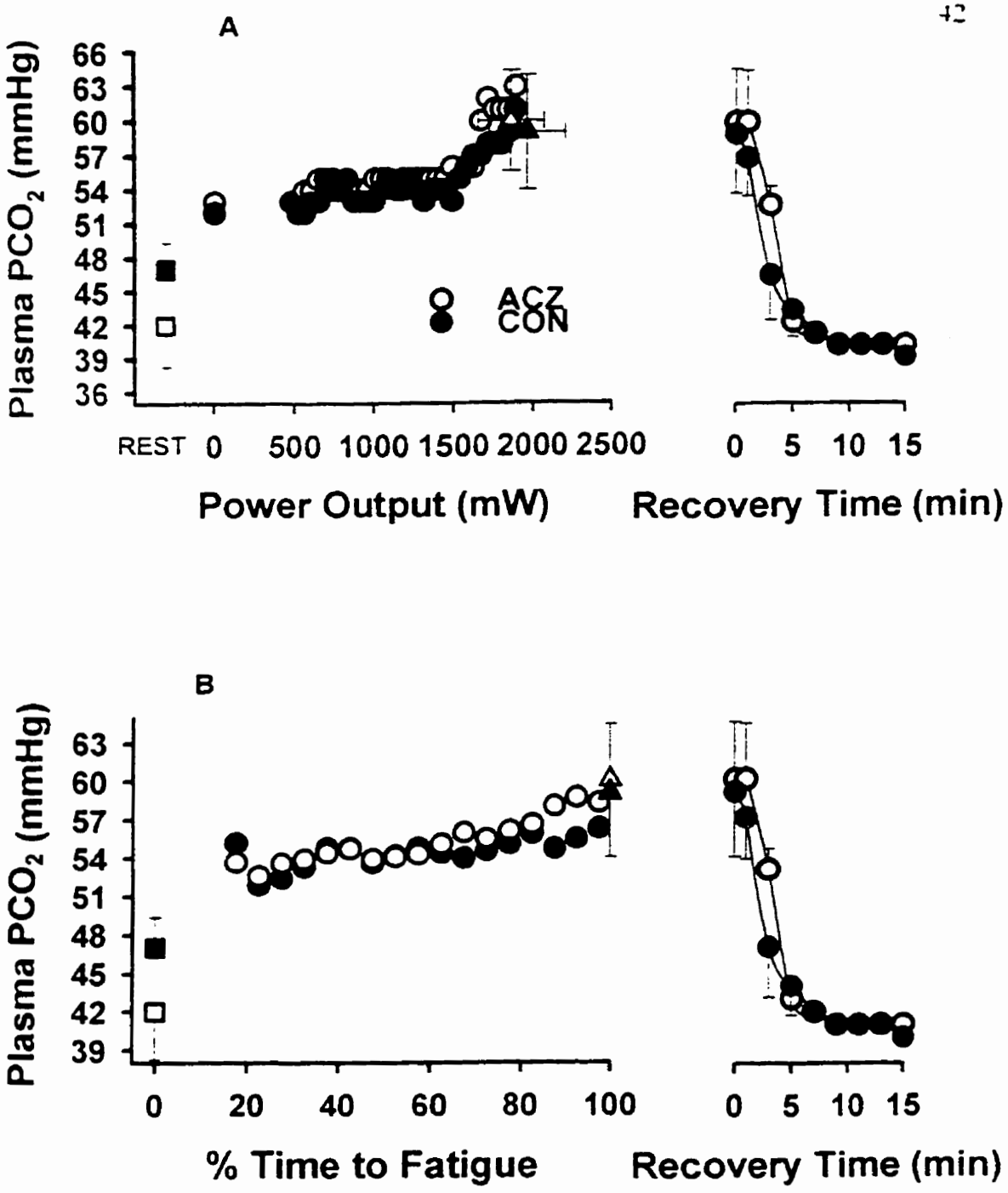

Fig 8. A. The effect of acetazolamide (ACZ) administration on plasma PCO_2 relative to power output (mW) during incremental forearm wrist flexion exercise and recovery time. B. The effect of acetazolamide (ACZ) administration on plasma PCO_2 relative to % time to fatigue during incremental forearm wrist flexion exercise and recovery time. Condition: CON, closed symbols; ACZ, open symbols. Time: Rest (□,■); Exercise and Recovery, where each point corresponds to the mean of 5 or more subjects (○,•) [fig 8a] or 8 subjects [fig 8b]; Fatigue, where each point corresponds to the mean of all 8 subjects (△,▲). Error bars represent the SEM.

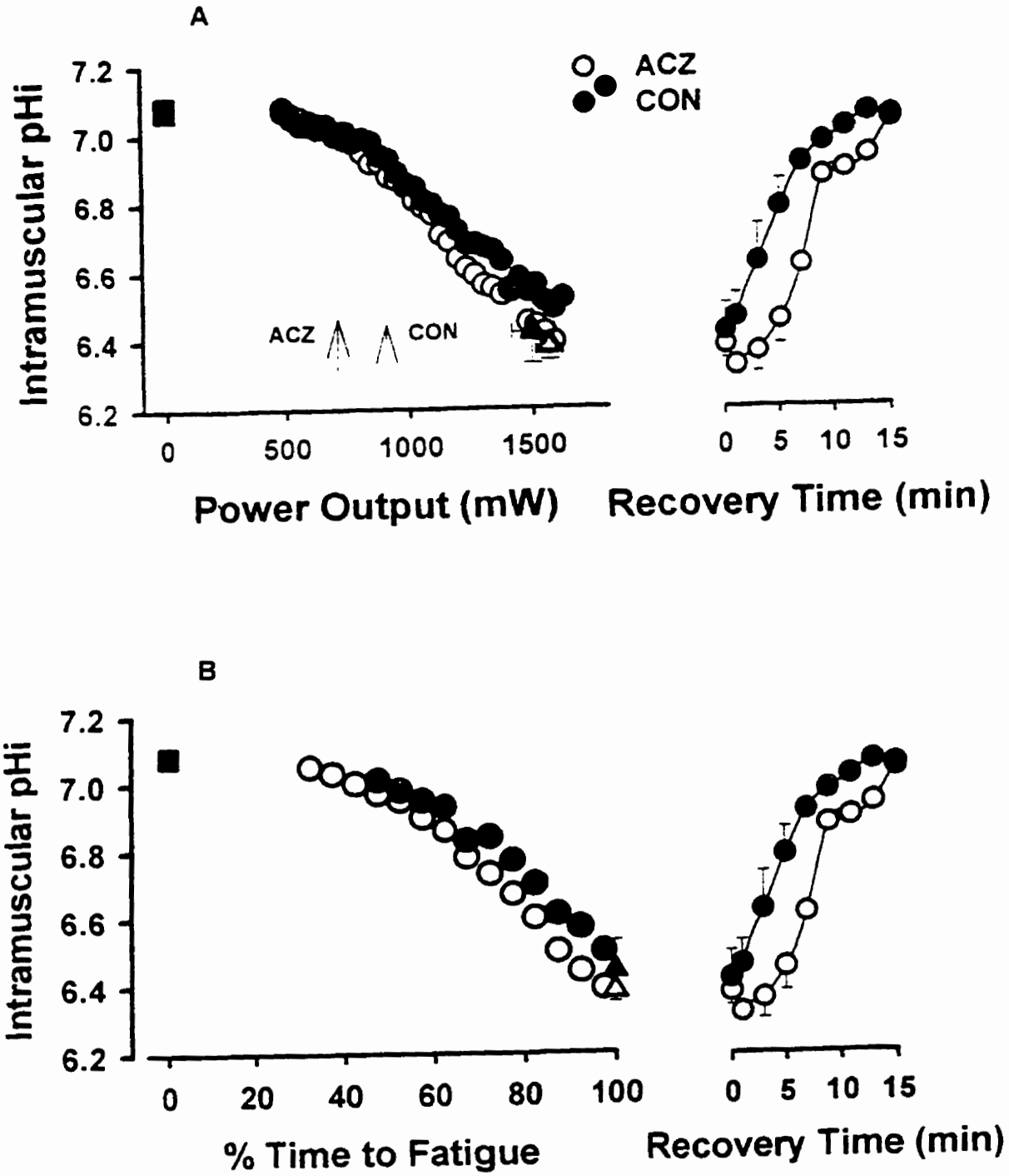

Fig 9. A. The effect of acetazolamide (ACZ) administration on intramuscular pHi relative to power output (mW) during incremental forearm wrist flexion exercise and recovery time. B. The effect of acetazolamide (ACZ) administration on intramuscular pHi relative to % time to fatigue during incremental forearm wrist flexion exercise and recovery time. Condition: CON, closed symbols; ACZ, open symbols. Time: Rest (▫,▪); Exercise and Recovery, where each point corresponds to the mean of 5 or more subjects (○,●) [fig 9a] or 8 subjects [fig 9b]; Fatigue, where each point corresponds to the mean of all 8 subjects (△,▲). Arrows correspond to the intramuscular pHi threshold (T_{pHi}). Error bars represent the SEM.

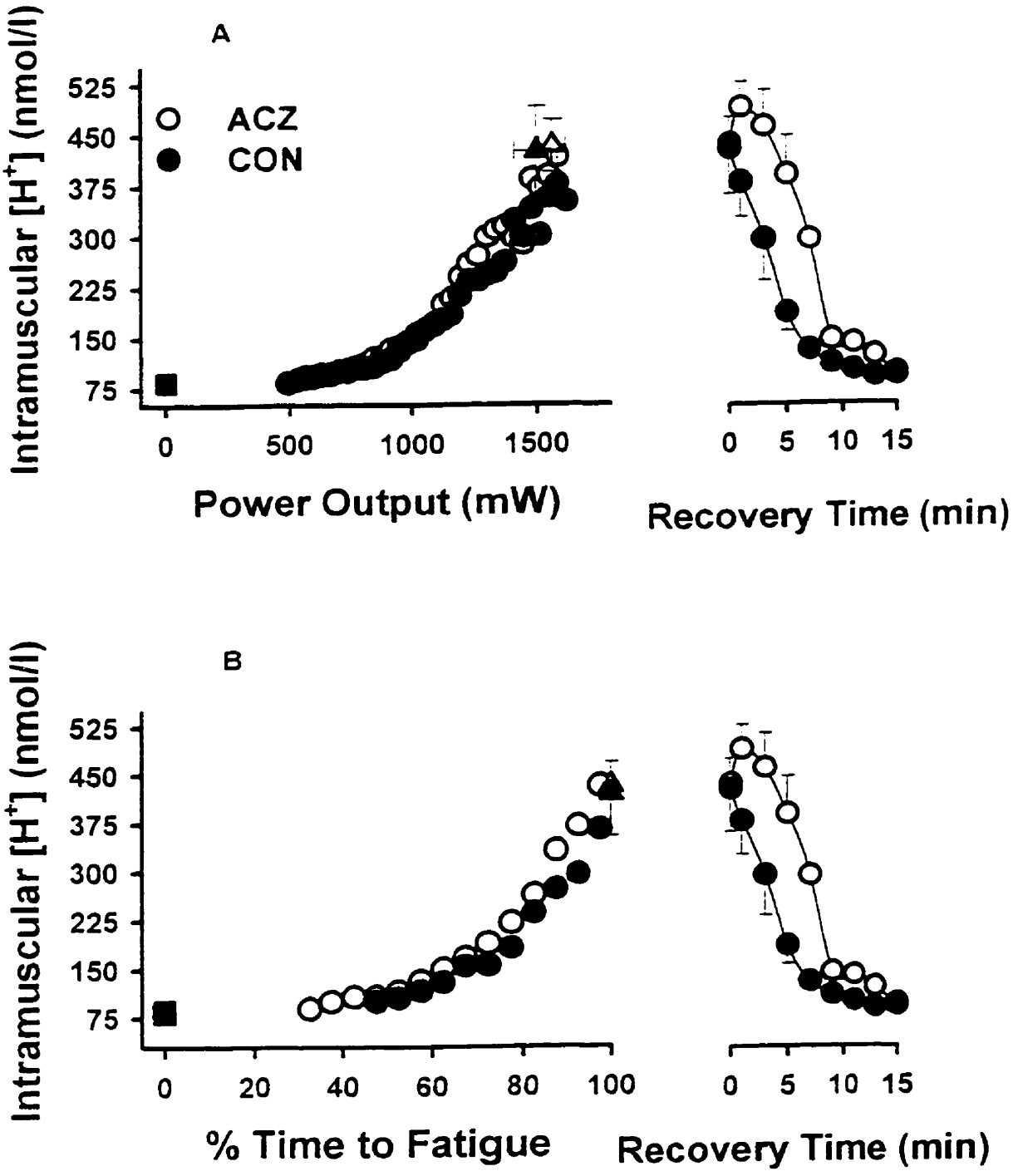

Fig 10. A. The effect of acetazolamide (ACZ) administration on intramuscular [H$^+$] relative to power output (mW) during incremental forearm wrist flexion exercise and recovery time. B. The effect of acetazolamide (ACZ) administration on intramuscular [H$^+$] relative to % time to fatigue during incremental forearm wrist flexion exercise and recovery time. Condition: CON, closed symbols: ACZ, open symbols. Time: Rest (□,■); Exercise and Recovery, where each point corresponds to the mean of 5 or more subjects (○,●) [fig 10a] or 8 subjects [fig 10b]; Fatigue, where each point corresponds to the mean of all 8 subjects (△,▲). Error bars represent the SEM.

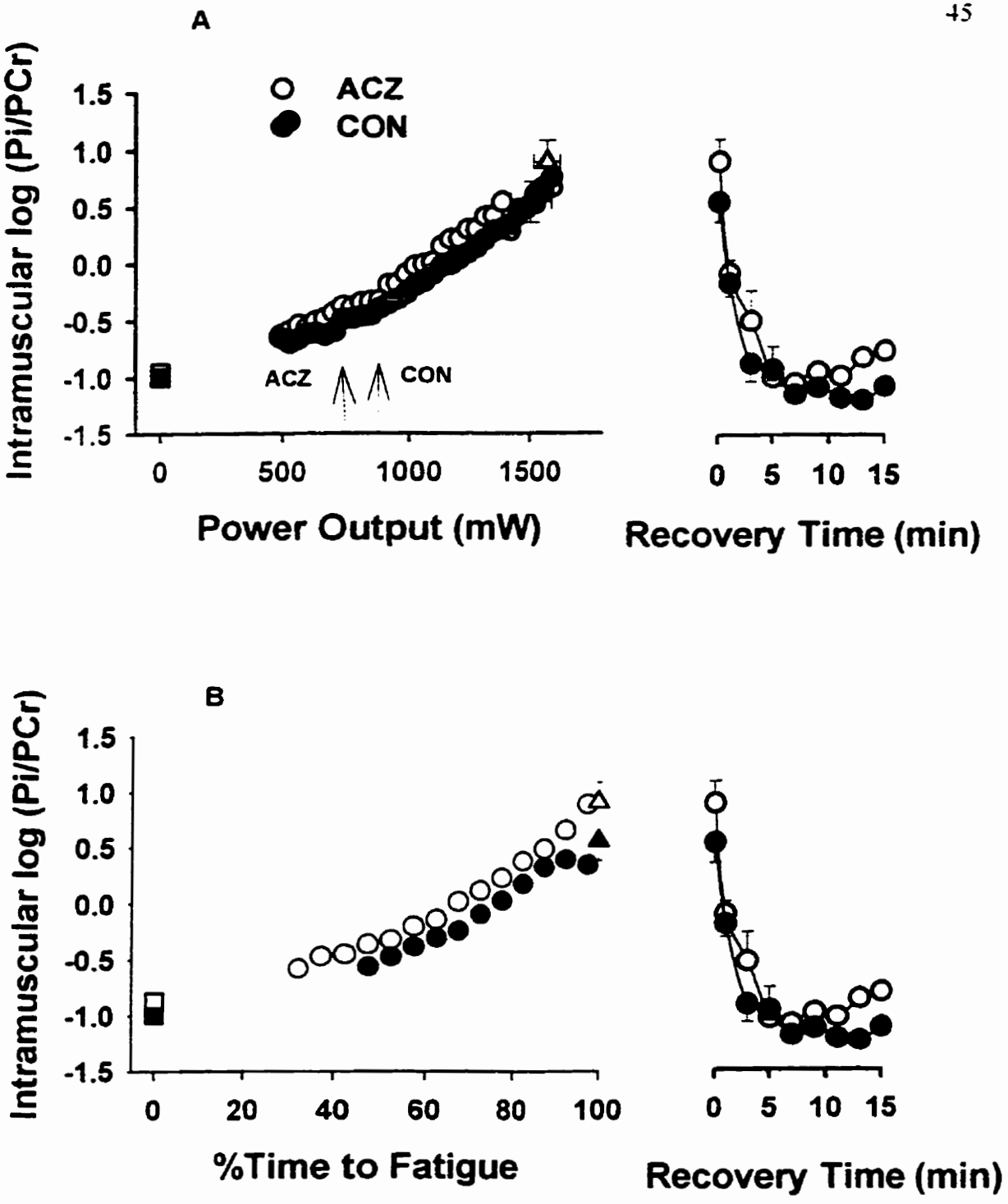

Fig 11. A. The effect of acetazolamide (ACZ) administration on intramuscular log Pi/PCr relative to power output (mW) during incremental forearm wrist flexion exercise and recovery time. B. The effect of acetazolamide (ACZ) administration on intramuscular log Pi/PCr relative to % time to fatigue during incremental forearm wrist flexion exercise and recovery time. Condition: CON, closed symbols; ACZ, open symbols. Time: Rest (▫,▪); Exercise and Recovery, where each point corresponds to the mean of 5 or more subjects (○,●) [fig 11a] or 8 subjects [fig 11b]; Fatigue, where each point corresponds to the mean of all 8 subjects (△,▲). Arrows correspond to the intramuscular log Pi/PCr threshold ($T_{log Pi/PCr}$). Error bars represent the SEM.

CHAPTER 5
DISCUSSION

This study examined the muscle and venous plasma metabolic and acid-base response to progressive dynamic forearm flexion to fatigue before and following ACZ - induced carbonic anhydrase (CA) inhibition. Muscle and venous plasma metabolic and acid-base responses were assessed using a combination of ^{31}phosphorus magnetic resonance spectroscopy (^{31}P-MRS) and blood-sampling (BLD) techniques. The major findings of this study were i) the plasma [La^-] - power output relationship was shifted to the right with ACZ such that the venous plasma [La^-] was lower at fatigue and during early recovery in ACZ compared to CON; ii) the plasma La^- threshold (T_{La^-}) occurred at a higher power output and % peak power output in ACZ compared to CON; iii) the intracellular threshold for pHi (T_{pHi}) and log Pi/PCr ($T_{logPi/PCr}$) were similar in CON and ACZ, and similar to each other; iv) the T_{pHi} and $T_{logPi/PCr}$ were similar to the T_{La^-} in CON but occurred at a lower power output and % peak power output compared to the T_{La^-} in ACZ; and v) recovery of pHi following progressive exercise to fatigue was faster in CON than ACZ.

Progressive dynamic forearm flexion was performed 45 min post ACZ infusion, thus avoiding the significant plasma acidosis that normally accompanies prolonged administration of ACZ (Kowalchuk et al., 1992, 1994). The resting plasma [H^+] in CON (40 ● 4 nmol/L) and ACZ (35 ± 9 nmol/L) were not significantly different confirming that an acidosis was not established prior to the exercise test. In the present study ACZ was infused at a dose of 10 mg/kg BM, and while CA activity was not measured, Maren (1967) reported that CA inhibition is essentially complete at doses of 5-20 mg ACZ/kg. In addition studies from our laboratory using a similar dose of ACZ have demonstrated lower blood [La^-] and slower

VCO_2 kinetics at the onset of constant-load exercise below and above the ventilatory threshold (Scheuermann et al., 1998b, unpublished observation), and a lower plasma [La^-], and a rightward shift in T_{La^-} during progressive leg cycling exercise to fatigue (Scheuermann et al., 1998a,b,c,d, unpublished observation), thus confirming that the dose and protocol used in this study effectively inhibited CA activity.

The time to fatigue and the peak power output achieved during the ramp tests were similar between conditions, however both were greater during BLD (CON 16.3 ± 2.2 min; ACZ 16.4 ± 2.7 min) than ^{31}P-MRS (CON 12.4 ± 1.8 min; ACZ 12.6 ± 1.8 min). It is not apparent why the time to fatigue was longer during BLD than ^{31}P-MRS as the protocols were similar for each of the series. It is possible that the level of discomfort was greater with exercise in the ^{31}P-MRS unit. Although not documented at the time, the subjects did complain of being uncomfortable while positioned in the ^{31}P-MRS unit. Complaints of slight paraesthesia and numbing of the hand suggest that there may have been local nerve compression while positioned in the ^{31}P-MRS unit. This was specific to the 31 P-MRS studies and we were unable to recreate for the BLD studies. While we tried to insure that blood flow to the working limb was adequate during each of the trials, the need to wrap the arm in foil at the shoulder in order to ground the arm and improve the signal to noise ratio while in the ^{31}P-MRS unit may have inadvertently impaired blood flow during time trials. While this may have caused the subject to fatigue prematurely in the ^{31}P-MRS compared to BLD studies, it would not affect comparisons between CON and ACZ.

5.1 Plasma [La^-]

The present study found that the ACZ treatment was associated with a rightward-shift in the plasma [La^-]-power output relationship, with a marked reduction in end-exercise and

recovery plasma [La^-] and a higher power output corresponding to the T_{La^-}. These results are consistent with the findings from previous studies (Kowalchuk et al., 1992; Kowalchuk et al., 1994; Scheuermann et al., 1998a,b,c,d, unpublished observations; Rose et al., 1990; McLellan et al., 1988).

The plasma [La^-] represents a balance between La^- release into plasma from La^--producing tissues and La^- removal from plasma by La^--utilizing tissues. La^- release is related to the La^- production in muscle and the La^- efflux from the intramuscular to the interstitial and extracellular compartments. Kowalchuk et al. (1992; 1994) demonstrated that the arterial-venous [La^-] difference across an inactive forearm muscle was reduced during acute and chronic ACZ administration and was related to the arterial plasma [La^-] (i.e. similar fractional uptake of La^-) suggesting that La^- uptake by inactive muscle (and perhaps other tissues) was reduced, not increased. Thus La^- appearance in blood probably accounts for the lower plasma [La^-] observed following ACZ administration and would be related to either a decreased rate of glycolysis and pyruvate production, an increased rate of pyruvate oxidation, or a decreased rate of La^- release from muscle. To further understand the mechanism for a decrease in plasma [La^-] following CA inhibition the ^{31}P-MRS was employed to examine the components of the intramuscular phosphate pool, intramuscular pH (pHi), and intracellular thresholds identifying the increase of intramuscular glycolysis.

5.2 Intramuscular pH (pHi) and Intramuscular pH Threshold (T_{pHi})

^{31}P-MRS was used to study the effect of ACZ administration on muscle metabolism, specifically the intramuscular pH (pHi) and intramuscular pH threshold (T_{pHi}), during progressive forearm exercise to fatigue. Inhibition of CA is associated with an increase in plasma and tissue PCO_2 (Cardenas et al., 1998; Taki et al., 1993). According to

physicochemical principles, the PCO_2 is a major determinant of [H^+] in biological fluids (Stewart, 1981). Therefore, an increase in plasma and tissue PCO_2 following CA inhibition would be expected to contribute to a rise in plasma and tissue [H^+]. Geers & Gros (1988a, 1988, 1990) observed a lower pHi in isolated, nonstimulated muscle when inhibited with chlorzolamide ($5*10^{-4}$ M) (inhibiting CA III). A study in horses treated chronically (3 days) with ACZ (30 mg/kg twice daily) found a lower muscle pH (determined using the muscle homogenate technique) at rest and after maximal exercise (Rose et al., 1990). Côté et al. (1997) used methazolamide (1mM) to inhibit the intramuscular CA III isozyme and did not observe a significant decrease in pHi of the isolated nonstimulated muscle preparation. Therefore, pHi was examined in the present study to determine whether it was affected by CA inhibition with ACZ. A more acidotic environment in the cell following ACZ treatment could potentially contribute to an inhibition of muscle glycolysis and La^- production and thus contribute, in part, to a lower plasma [La^-]. In the present study, pHi, determined using ^{31}P-MRS, was not affected by ACZ treatment at rest or during exercise. However, recovery of pHi after exercise in the ACZ-treated subjects was slowed compared to CON. CA plays a role in facilitating the transport of CO_2 through biological solutions (Geers & Gros, 1991). Inhibition of either intracellular (cytosolic CA III isozyme), extracellular (membrane bound CA IV isozyme) or both types of CA isozymes may lead to a slower transport of CO_2 out of the cell and thus lead to a slower rate of recovery of pHi. In addition, a slowing of the transport of other H^+ -equivalents (mainly La^-) out of the cell may also contribute to a slower rate of pHi recovery.

The T_{pHi} was examined to determine the onset of glycolytic metabolism in muscle. During progressive exercise, the T_{pHi} indicates the exercise intensity or power output

associated with an increase in the rate of glycolytic metabolism (Marsh et al., 1991; Kent-Braun et al., 1993; Systrom et al., 1990). In theory, during light to moderate intensities of exercise (i.e. not associated with significant La^- production) the rate of pyruvate formation is balanced by pyruvate oxidation in the mitochondria. As exercise intensity increases from moderate to heavy, pyruvate oxidation no longer keeps pace with production, and lactic acid (HLa) formation increases, leading to the production of H^+ and decrease in pHi. Thus the onset of the increase in glycolysis is associated with an increase in the rate at which pHi falls in muscle, and is identified as an increase in slope of the pHi - power output relationship (i.e. T_{pHi}). In this study no difference was found in the power output or % peak power output corresponding to T_{pHi} between conditions, nor was there a difference in the slope of the pHi-power output relationship above the T_{pHi}. In addition, the pHi at the point of fatigue was similar between conditions. These findings suggest that the increase of glycolysis and the rate of glycolysis (and presumably La^- production), once initiated, were not affected by ACZ. Accordingly, these data suggest that the lower plasma [La^-] observed in ACZ most probably occurs as a consequence of an impairment in La^- efflux from muscle, in spite of a similar rate of La^- production.

pHi recovery after exercise was slowed in ACZ suggesting that removal of protons or H^+ -equivalents (i.e. the independent variables determining the intramuscular [H^+] such as strong ions, including La^-, PCO_2, and weak acids) were slowed following ACZ administration. If, during exercise, extrusion of one or more of these factors was impaired, it is possible that the intramuscular accumulation of these factors may have been similar between conditions in spite of a lower rate of production (of La^- or CO_2), the manifestation of which may be that the pHi is seen to fall at a similar rate with increasing power output in ACZ and CON. In this

case, the lower plasma [La^-] observed in ACZ may occur because of a combination of lower rate of La^- production and impaired La^- efflux from muscle.

These data do not permit us to discriminate between the two mechanisms. Alternatively, the similarity in pHi during the heavy exercise intensities in CON and ACZ may be related to a greater hydrolysis of PCr during ACZ as it is known that protons are consumed in the CPKinase reaction. Although the logPi/PCr was not significantly different between conditions, there was a tendency ($p=0.065$) for a higher log Pi/PCr in ACZ suggesting a greater PCr breakdown. Côté et al. (1997) found a greater rate of PCr hydrolysis early in exercise when the cytosolic CA III isozyme was inhibited and, unlike the control condition where pHi decreased with stimulation, pHi was at pre-exercise levels for a longer period of time. However, Scheuermann et al. (1998d, unpublished observations) found that PCr breakdown was similar during heavy intensity, constant-load exercise in CON and ACZ treated subjects, although PCr kinetics were not examined, nor was [PCr] determined at earlier time points.

5.3 Intracellular Pi/PCr Ratio and Intracellular Pi/PCr Threshold ($T_{logPi/PCr}$)

The Pi/PCr ratio represents the energy state of a muscle. The initial slope of Pi/PCr - power output relationship can be used to estimate the oxidative potential of a muscle (Chance et al., 1985). A steep initial Pi/PCr - power output slope would indicate lesser increase in Pi/PCr at any given relative workload, implying that there is an improved ability to keep pace with energy needs by means of oxidative phosphorylation (Chance et al., 1985). Stable values of Pi/PCr indicate an adequate energy status (balanced ATP production), while rapid changes in this ratio show that energy homeostasis (unbalanced ATP production) cannot be maintained (Marsh et al., 1991). An increase in Pi/PCr at power outputs above $T_{logPi/PCr}$

are an indication of an inability to provide ATP through oxidative processes alone, thus requiring the activation of glycolysis (Marsh et al., 1991; Kent-Braun et al., 1993; Chance et al., 1981). Thus an alternate means of identifying the onset of a rapid increase in muscle glycolysis is by a rapid increase in Pi/PCr ratio - power output relationship (or log Pi/PCr ratio as used in this study).

In the present study, no differences were found in the power output or % peak power output corresponding to the log Pi/PCr between conditions, nor were differences found in the log Pi/PCr ratio at any time, or in the slope of the log Pi/PCr ratio - power output relationship below or above the $T_{log\ Pi/PCr}$ between conditions. These findings suggest that the power output corresponding to the rapid onset of glycolysis was not affected by ACZ, supporting the findings of the pHi data. These data further support the suggestion that the glycolytic rate above the $T_{logPi/PCr}$ (andT_{pHi}) was similar in ACZ and CON, and not reduced as alternatively suggested above because the slopes of the log Pi/PCr ratio (both below and above the $T_{log\ Pi/PCr}$) were similar between conditions. In agreement, Scheuermann et al. (1998d, unpublished observations) using a similar drug protocol, observed that although the plasma [La^-] was lower in ACZ than CON after 6 min heavy intensity, constant-load exercise, muscle glycogen breakdown was similar between conditions, and the muscle-to-plasma La^- concentration ratio tended to be higher in ACZ, suggesting that glycogen breakdown and La^- production were similar but that La^- efflux from muscle was impaired.

5.4 Relationship Between the T_{pHi}, $T_{logPi/PCr}$, and T_{La^-}

Studies have found a close approximation between the $T_{logPi/PCr}$ and T_{pHi} (Marsh et al., 1991; Kent-Braun et al., 1993) and the T_{pHi}, T_{La^-} and the gas exchange threshold (T_{vent}) (Systrom et al., 1990) which suggests that all three measures describe a common

intramuscular threshold in control conditions. This study found an agreement between the $T_{logPi/PCr}$ and T_{pHi} and there were no differences in the power output or % peak power output between conditions suggesting that the two measures could be describing a similar intramuscular process.

T_{La^-}, T_{pHi}, and $T_{logPi/PCr}$ occurred at similar power outputs and (% peak power outputs) during CON. However, a difference was found between the power output (and % peak power output) corresponding to the T_{La^-} and T_{pHi}, and T_{La^-} and $T_{logPi/PCr}$ following ACZ-administration, with the T_{La^-} occurring at a higher power output than the power output (and % peak power output) corresponding to the T_{pHi} and $T_{log\ Pi/PCr}$ condition. In ACZ, however, the T_{La^-} occurred at a higher absolute power output and % peak power output than T_{pHi} and $T_{logPi\ PCr}$ suggesting that the La^- appearance in blood was attenuated with little or no change in the onset or rate of glycolysis and La^- production in muscle. The similarity of the T_{pHi} and $T_{logPi/PCr}$ between conditions, along with the similarity in slope of the pHi- and logPi/PCr - power output relationships above their respective thresholds, as well as the muscle biopsy data of Scheuermann et al. (1998d, unpublished observations) showing a similar glycogen breakdown between conditions, strongly suggest that the onset and the rate of muscle glycolysis and La^- production are probably not affected by ACZ. The delayed appearance of La^- and the higher T_{La^-} observed in ACZ (along with a slower rate of pHi recovery) in this study are probably related to a slower rate of La^- efflux from muscle. This is supported by a higher muscle-to-plasma [La^-] ratio after exercise following acute (Scheuermann et al., 1998d, unpublished observations) and chronic (McLellan et al., 1988) ACZ treatment.

Release of La^- from the muscle into blood is influenced by the extracellular acid-base status (Jones et al., 1977). ACZ administration may produce an initial respiratory acidosis

followed by a metabolic acidosis, which could contribute to an impaired efflux of La^- from muscle. However, in the present study, ACZ was administered acutely to avoid any significant plasma acidosis. The venous plasma pH data demonstrate this to be the case as plasma pH was similar between conditions at rest and during exercise. In previous studies from our laboratory ACZ treatment has been shown to produce a small but significant increase (≈ 2-3 nmol/l) in arterial plasma [H^+] during loadless cycling which is maintained throughout exercise (Scheuermann et al., 1998d, unpublished observations). In the studies of Jones, Sutton et al. (1977; 1981) plasma [La^-] was reduced during exercise in an NH_4Cl-induced acidosis compared to a $CaCO_2$ placebo trial, but in those studies, the difference in plasma [H^+] between the placebo and acidosis trials was ≈20 nmol/l, much greater than found in this or other studies from our laboratory. Thus the lower plasma [La-] found in this study is probably not related to an ACZ-induced plasma acidosis.

DeHemptinne et al. (1986) demonstrated that ACZ-induced inhibition of the sarcolemmal CA IV isozyme was associated with a greater decrease in muscle surface pH and a longer time to recover from a propionic acid-induced intracellular acid load in an isolated muscle preparation. The increased acidity on the surface membrane was independent of the acid-base status of the bulk solution perfusing the muscle, which was kept constant. The greater decrease in surface membrane pH during recovery in ACZ was attributed to the accumulation of protons on the muscle surface as inhibition of the CA impaired the facilitated removal of protons as CO_2. Lactate removal from muscle occurs via diffusion of undissociated lactic acid (HLa) and lactate transport via the monocarboxylate transporter (MCT) found in the sarcolemma (Juel, 1997). The muscle surface membrane pH may be important in that it represents the pH "sensed" by the MCT. Therefore, in the present study

as HLa diffusion or coupled H^+-La^- co-transport occurs, the rise in extracellular [H^+] on the membrane surface is prevented by the CA-catalyzed dehydration of carbonic acid. Inhibition of CA with ACZ interferes, with this process, leading to an accumulation of protons on the membrane and impairing the removal of both La^- and H^+ from the muscle.

5.5 Conclusion

The present study examined the muscle and venous plasma metabolic and acid-base responses to progressive forearm flexion to fatigue before and following acetazolamide (ACZ) -induced carbonic anhydrase (CA) inhibition. Muscle and venous plasma metabolic and acid-base responses were assessed using a combination of ^{31}phosphorus magnetic resonance spectroscopy and blood sampling techniques. ACZ-induced CA inhibition was associated with a rightward shift in the T_{La^-} which occurred at a higher power output, and a lower plasma [La^-] at end exercise and during recovery. The pHi and logPi/PCr during exercise, and the power outputs corresponding to the T_{pHi} and $T_{logPi/PCr}$ were similar between conditions. These data suggest that the lower plasma [La^-] observed during exercise is not related to a decrease in La^- production in muscle, but to an inhibition of La^- efflux from muscle into blood.

APPENDIX A

Total cylinder volume (ml) and rate of flow (ml/min) during the progressive ramp exercise tests to fatigue for control (CON) and acetazolamide (ACZ) during studies using magnetic resonance spectroscopy (MRS) and blood collected (BLD).

	CON		ACZ	
	Total cylinder Volume (ml)	Rate of Flow (ml/min)	Total Cylinder Volume (ml)	Rate of Flow (ml/min)
MRS				
1	2645	200	2330	201
2	2645	194	2140	206
3	2660	201	2670	202
4	2280	211	2650	189
5	2320	207	2940	193
6	2800	189	2580	201
7	2450	211	2960	205
8	2845	203	2140	205
9	1620	176	2165	193
MEAN	2474	199	2508	199
SD	376	11	328	6
BLD				
1	----	---	----	---
2	3900	205	3280	205
3	3260	204	3250	203
4	2400	200	2600	200
5	3240	203	3930	197
6	3260	204	3400	200
7	3850	203	4000	200
8	3300	206	3180	199
9	3310	202	2650	204
MEAN	3315	204	3286	201
SD	459	2	511	3

Note: We were unable to collect blood from Subject #1 and therefore he was not included in the BLD studies.

APPENDIX B.1
STATISTICAL TABLES

Plasma [La^-] vs % Time of Exercise (TTF)
Two Way Repeated Measures Analysis of Variance

Source of Variation	DF	SS	MS	F	P
Cond (CON;ACZ)	1	12.997	12.997	2.370	0.168
%TTF	17	215.126	12.654	26.865	<0.001
Cond x %TTF	17	8.035	0.473	2.450	0.003

Plasma [La^-] vs Time at Recovery
Two Way Repeated Measures Analysis of Variance

Source of Variation	DF	SS	MS	F	P
Cond (CON;ACZ)	1	2.623	2.623	0.428	0.537
Time	8	85.670	10.709	22.663	<0.001
Cond x Time	8	10.405	1.301	5.658	<0.001

APPENDIX B.2

Plasma La^- Threshold Power Output
One Way Repeated Measures Analysis of Variance

Group	Mean	Std Dev	SEM	P
CON	1040.625	414.391	146.509	0.025
ACZ	1432.500	242.559	85.758	

Plasma La^- Threshold % Peak Power Output
One Way Repeated Measures Analysis of Variance

Group	Mean	Std. Dev	SEM
CON	55.250	21.117	7.466
ACZ	75.750	17.442	6.167

Source of Variation	DF	SS	MS	F	P
Between Treatments	1	1681.00	1681.00	7.136	0.032

APPENDIX B.3

Plasma pH vs % Time of Exercise (TTF)
Two Way Repeated Measures Analysis of Variance

Source of Variation	DF	SS	MS	F	P
Cond(CON;ACZ)	1	0.0000269	0.0000269	0.00152	0.970
%TTF	17	0.259	0.0153	12.947	<0.001
Cond x %TTF	17	0.0257	0.00151	1.765	0.040

Plasma pH vs Time at Recovery
Two Way Repeated Measures Analysis of Variance

Source of Variation	DF	SS	MS	F	P
Cond (CON;ACZ)	1	0.00711	0.00711	0.925	0.373
Time	8	0.168	0.0210	28.126	<0.001
Cond x Time	8	0.0126	0.00158	4.189	<0.001

APPENDIX B.4

Plasma [H^+] vs % Time of Exercise (TTF)
Two Way Repeated Measures Analysis of Variance

Source of Variation	DF	SS	MS	F	P
Cond (CON;ACZ)	1	9.753	9.753	0.0519	0.826
%TTF	17	2707.726	159.278	14.479	<0.001
Cond x %TTF	17	147.183	8.658	1.373	0.162

Plasma [H^+] vs Time at Recovery
Two Way Repeated Measures Analysis of Variance

Source of Variation	DF	SS	MS	F	P
Cond (CON;ACZ)	1	61.333	61.333	0.858	0.390
Time	8	1956.134	244.517	20.379	<0.001
Cond x Time	8	145.199	18.150	3.528	0.003

APPENDIX B.5

Plasma PCO_2 vs % Time of Exercise (TTF)
Two Way Repeated Measures Analysis of Variance

Source of Variation	DF	SS	MS	F	P
Cond(CON;ACZ)	1	53.820	53.820	0.277	0.615
%TTF	17	1750.559	102.974	4.994	<0.001
Cond x %TTF	17	88.255	5.191	0.754	0.741

Plasma PCO_2 vs Time at Recovery
Two Way Repeated Measures Analysis of Variance

Source of Variation	DF	SS	MS	F	P
Cond(CON;ACZ)	1	24.988	24.988	0.169	0.695
Time	8	5936.917	742.115	25.654	<0.001
Cond x Time	8	55.743	6.968	0.307	0.959

APPENDIX B.6

Intramuscular pH vs % Time of Exercise
Two Way Repeated Measures Analysis of Variance

Source of Variation	DF	SS	MS	F	P
Cond (CON;ACZ)	1	0.300	0.300	2.250	0.172
Time	12	10.225	0.852	90.336	<0.001
Cond x Time	12	0.112	0.00934	1.261	0.254

Intramuscular pH vs Time at Recovery
Two Way Repeated Measures Analysis of Variance

Source of Variation	DF	SS	MS	F	P
Cond (CON;ACZ)	1	0.887	0.887	36.321	<0.001
Time	8	8.560	1.070	55.554	<0.001
Cond x Time	8	0.408	0.0510	3.224	0.004

APPENDIX B.7

Intramuscular pH Threshold Power Output
One Way Repeated Measures Analysis of Variance

Group	Mean	Std. Dev	SEM	P
CON	854.889	210.970	70.323	0.106
ACZ	721.667	50.449	16.815	

Intramuscular pH Threshold % Peak Power Output
One Way Repeated Measures Analysis

Group	Mean	Std. Dev	SEM
CON	49.667	9.862	3.287
ACZ	46.222	6.685	2.228

Source of Variation	DF	SS	MS	F	P
Between Treatments	1	53.389	53.389	0.886	0.374

APPENDIX B.8

Intramuscular [H^+] vs % Time of Exercise (TTF)
Two Way Repeated Measures Analysis of Variance

Source of Variation	DF	SS	MS	F	P
Cond (CON;ACZ)	1	60644.856	1.588	1.588	0.243
%TTF	12	2942391.726	245199.310	46.497	<0.001
Cond x Time	12	51389.317	4282.443	1.258	0.257

Intramuscular [H^+] vs Time at Recovery
Two Way Repeated Measures Analysis of Variance

Source of Variation	DF	SS	MS	F	P
Cond (CON;ACZ)	1	240162.804	240162.804	13.417	0.003
Time	8	2589326.451	323665.806	31.061	<0.001
Cond x Time	8	190696.645	23837.081	0.298	0.964

APPENDIX B.9

Intramuscular [Log (Pi/PCr)] vs % Time of Exercise (TTF)
Two Way Repeated Measures Analysis of Variance

Source of Variation	DF	SS	MS	F	P
Cond (CON;ACZ)	1	3.104	3.104	4.558	0.065
Time	12	51.165	4.264	95.354	<0.001
Cond x Time	12	0.632	0.0527	1.079	0.386

Intramuscular [Log (Pi/PCr)] vs Time at Recovery
Two Way Repeated Measures Analysis of Variance

Source of Variation	DF	SS	MS	F	P
Cond (CON;ACZ)	1	1.742	1.742	2.091	0.185
Time	8	45.013	5.627	26.132	<0.001
Cond x Time	8	1.924	0.240	1.045	0.414

APPENDIX B.10

Intramuscular Log (Pi/PCr) Threshold Power Output
One Way Repeated Measures Analysis of Variance

Group	Mean	Std. Dev	SEM	P
CON	835.333	235.344	78.448	0.687
ACZ	796.333	75.072	25.024	

Intramuscular Log (Pi/PCr) Threshold % Peak Power Output
One Way Repeated Measures Analysis of Variance

Group	Mean	Std. Dev	SEM	P
CON	52.556	10.760	3.587	0.686
ACZ	50.778	6.399	2.133	

Source Of Variation	DF	SS	MS	F	P
Between Treatments	1	14.222	14.222	0.176	0.686

APPENDIX B. 11

Time to Exhaustion
Two Way Repeated Measures Analysis

Source of Variation	DF	SS	MS	F	P
Cond(CON;ACZ)	1	0.475	0.475	0.0894	0.774
Between Labs (MRS;BLD)	1	116.358	116.358	29.567	<0.001
Cond x Labs	1	0.195	0.195	0.0953	0.767

Power Output At Exhaustion
Two Way Repeated Measures Analysis

Source of Variation	DF	SS	MS	F	P
Cond(CON;ACZ)	1	0.0119	0.0119	0.263	0.624
Between Labs (MRS;BLD)	1	0.867	0.867	34.516	<0.001
Cond x Labs	1	0.000153	0.000153	0.00936	0.926

Rate of Flow
Two Way Repeated Measures Analysis of Variance

Source of Variation	DF	SS	MS	F	P
Cond (CON;ACZ)	1	9.031	9.031	0.166	0.696
Between Labs (MRS;BLD)	1	75.031	75.031	1.348	0.284
Cond x Labs	1	13.781	13.781	0.318	0.590

The UNIVERSITY *of* WESTERN ONTARIO

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THE REVIEW BOARD HAS EXAMINED THE RESEARCH PROJECT ENTITLED:
"The effect of carbonic anhydrase inhibition on muscle metabolism as assessed by phosphorus magnetic resonance spectroscopy (P-MRS)."

REVIEW NO: 5728

AS SUBMITTED BY: Dr. Dr. J.M. Kowalchuk, Kinesiology, Thames Hall

AND CONSIDERS IT TO BE ACCEPTABLE ON ETHICAL GROUNDS FOR RESEARCH INVOLVING HUMAN SUBJECTS UNDER CONDITIONS OF THE UNIVERSITY'S POLICY ON RESEARCH INVOLVING HUMAN SUBJECTS.

APPROVAL DATE: 10 February 1997 (UWO Protocol, Letter of Information & Consent)

AGENCY: NSERC

TITLE:

Bessie Borwein

Bessie Borwein, Chairman

c.c. Hospital Administration

Letter of Information

The effect of Carbonic Anhydrase inhibition on muscle metabolism as assessed by phosphorus magnetic resonsance spectroscopy (P-MRS)

Principal Investigator: John M. Kowalchuk, Ph.D.

You are being asked to participate in a study that examines muscle metabolism and control of lactate production during exercise. In particular, this study will determine whether a specific enzyme, Carbonic Anhydrase, plays an important role in controlling the production of lactate in muscles.

If you agree to participate, you will be required to come to the laboratory on 4-12 different occasions for approximately 1.5 hours per visit. The exact number of visits will depend on whether you are asked to participate in the entire sequence of testing, or just a specific aspect of the testing. On your first visit to the laboratory you will be asked your medical history and then undergo a progressive exercise test to voluntary exhaustion while exercising on a cycle ergometer.

On the remainder of the visits you will be asked to perform an exercise protocol consisting of dynamic forearm (wrist) flexion performed to fatigue using a wrist flexor dynamometer. The exercise test is performed in the supine position (i.e. lying on your back) with your arm extended out to the side and your hand secured in the dynamometer. The exercise test will begin by having you contract your forearm once every 2 seconds, with no resistance on the dynamometer. After 4 minutes the resistance will increase gradually until you are unable to move the weight. This will be followed by 15 minutes of resting recovery. Three test sessions will be held at St. Joseph's Health Centre where you will perform this exercise test in the magnetic resonance spectroscopy unit, and the remainder of the tests will be performed at the Centre for Activity and Ageing.

Any intensity of exercise may be uncomfortable if you are unfit or not used to exercise. There may be some discomfort during the exercise testing, and you may experience some local fatigue from the handgrip exercise, although this should not last for any length of time. The techniques for examining muscle metabolism (i.e. magnetic resonance spectroscopy) and muscle blood flow (i.e. Doppler ultrasound) are non-invasive and you should not experience any uncomfortable sensations because of these measurements.

The exercise tests will be performed without any drugs, and after you have taken a drug called Acetazolamide (or Diamox). This is a very common drug used in clinical and research settings. This drug will be given to you in two ways: 1) by infusion, where the drug is infused into your vein under medical supervision, and 2) by oral administration, where you will take the drug by mouth two times per day for 2 days. When you come to the laboratory the Acetazolamide will be infused (acute study) and then you will perform the exercise test. You may then be asked to perform additional testing. If you agree, you will take Acetazolamide by mouth for two days, and return to the laboratory where Acetazolamide will be infused (chronic study) and then you will again perform the exercise test. The actual exercise testing that you perform in the laboratory is the same in each instance. This drug may cause drowsiness, headache, and loss of appetite. Carbonated beverages may taste flat. You may find that you have to urinate more than usual. You may become aware of your breathing if you exercise or do any heavy work. It is important to drink plenty of fluids while taking Acetazolamide.

During each exercise test involving the drug, and in one of the non-drug tests you will be required to have a catheter placed into a vein in the region of your elbow. This will be done by the investigator. There may be some pain experienced when the catheter is placed into your vein (no more than when you get a needle in your arm), after which you should feel no pain or discomfort. In the 3 tests when blood sampling will take place, the catheter will remain in your vein during the entire test and will be used to sample blood. The amount of blood taken during each test should not amount to more than 120 ml (24 tsp; 4 oz). In the remaining tests involving the drug, the catheter

will be removed as soon as the drug has been infused. Minor bruising is common following venous catheterization, but it generally fades after a few days.

You are encouraged to ask questions of the investigator regarding the purpose of the study and the outcome of your exercise test. Participation in this study is voluntary. You may refuse to participate or withdraw from the study at any time. Records from the studies are confidential and securely stored. The records are listed according to an identification number rather than your name. Published reports resulting from these studies will not identify you by name.

If you have any questions regarding the study, please contact: Dr. John M. Kowalchuk (661-1607) or Shelly Smith (661-1646), Centre for Activity and Ageing, Saint Joseph's Health Centre Annex.

Letter of Informed Consent

The effect of Carbonic Anhydrase inhibition on muscle metabolism as assessed by phosphorus magnetic resonsance spectroscopy (P-MRS)

Principal Investigator: John M. Kowalchuk, Ph.D.

I have carefully read the accompanying "Letter of Information", and have had the nature of the study and the procedures satisfactorily explained to me. All my questions have been answered to my satisfaction.

By signing below, I agree to participate in this study.

_____

Name (please print)

_____

Signature

_____

Date

Example of Advertisement for Recruiting Subjects

WANTED: SUBJECTS FOR EXERCISE PHYSIOLOGY EXPERIMENT

Persons (Males; aged 18-50 years) wishing to participate in an Exercise Physiology experiment are asked to contact Dr. John Kowalchuk (661-1607) or Shelly Smith (661-1646) at the Centre for Activity and Ageing.

The research study will examine the effects of Carbonic Anhydrase inhibition with Acetazolamide on the control of muscle metabolism and lactate production during exercise. In particular, this study will determine whether Carbonic Anhydrase plays an important role in the production of lactate in your muscles.

Tests will be conducted at the Lawson Research Centre, St. Joseph's Health Centre, and the Centre for Activity and Ageing, Saint Joseph's Health Centre Annex, Richmond Street, London. Subjects will be required to come to the laboratory on a number of occasions:

1) The first visit will be approximately 1.5 hrs long and will involve a medical examination and an exercise stress test.

2) The remaining visits (approximately 4-12) will take approximately 1.5 hours each and will involve the different exercise tests which will be part of the study.

Subjects must be nonsmokers and will be screened for any cardiorespiratory or metabolic problems.

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IMAGE EVALUATION
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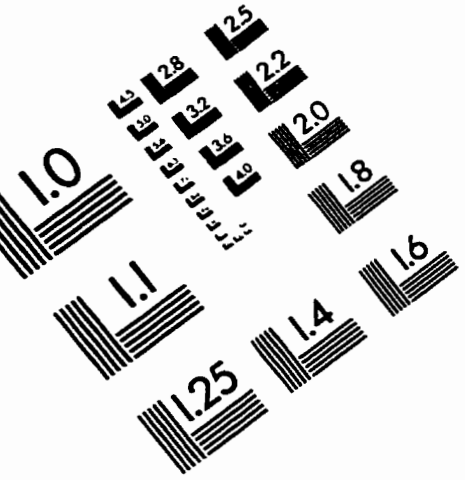

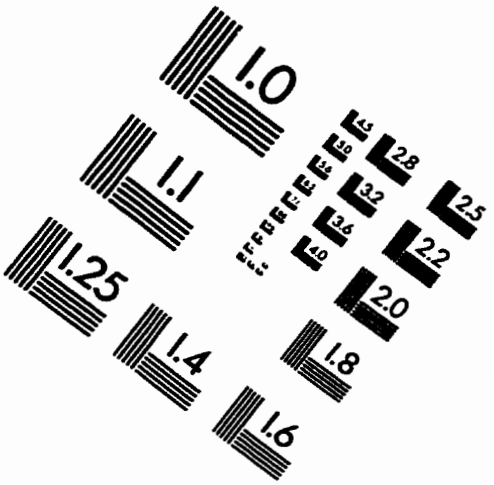

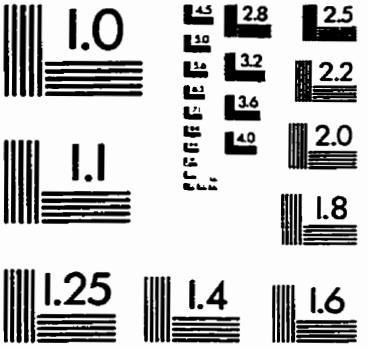

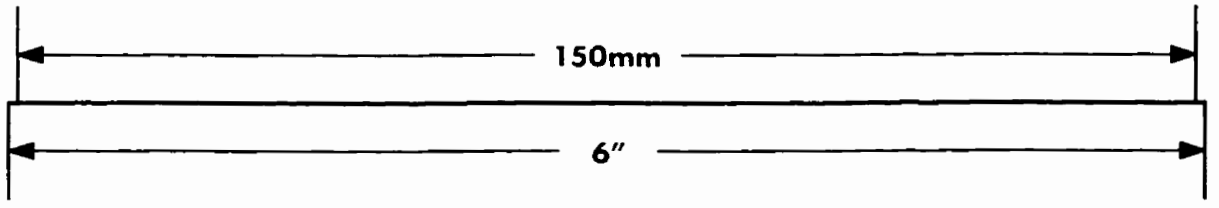

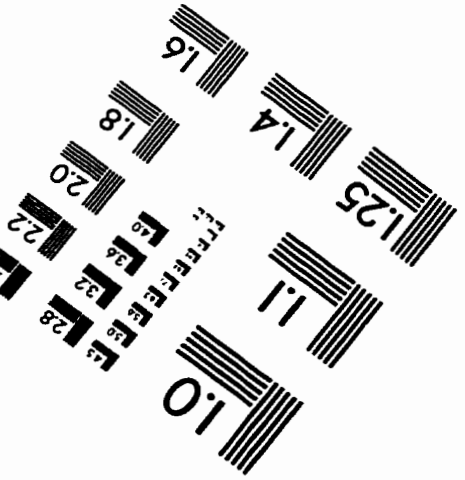

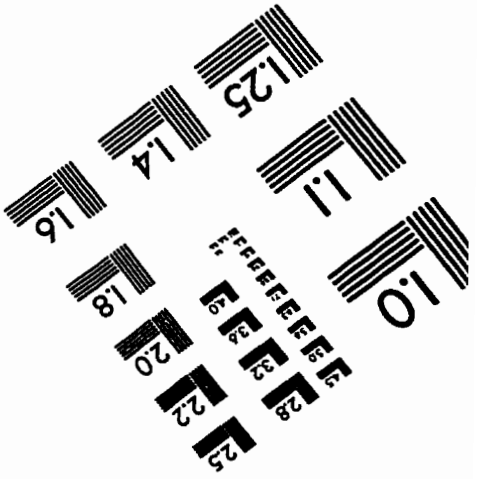

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1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989