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Gregory Rex Adams

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Ph.D. degree in Physiology

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THE METABOLIC CORRELATES OF FATIGUE IN SKELETAL MUSCLE

By

GREGORY REX ADAMS

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
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DOCTOR OF PHILOSOPHY

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ABSTRACT

THE METABOLIC CORRELATES OF FATIGUE IN SKELETAL MUSCLE

By

Gregory Rex Adams

Changes in the intracellular concentrations of lactic acid, hydrogen ion (H^+), inorganic phosphate (P_i), and diprotonated P_i ($H_2PO_4^{1-}$) have been suggested as causes of fatigue in skeletal muscle. Nuclear magnetic resonance spectroscopy (NMR) was used to measure changes in these metabolites during muscle contraction. Fast-twitch biceps and slow twitch soleus muscles were surgically isolated, arterially perfused and removed from anesthetized cats. Individual muscles were mounted in an NMR probe equipped with a force transducer. Intracellular pH and the concentrations of PCr, ATP, P_i and $H_2PO_4^{1-}$ were calculated from ^{31}P-NMR spectra. In one set of experiments interleaved ^{1}H-NMR spectra were collected and used to calculate lactic acid concentrations.

In Vivo Buffer Capacity Gated ^{31}P-NMR spectra were acquired after 9 seconds of 5 Hz stimulation in cat muscles. Net PCr hydrolysis was associated with an intracellular alkalinization of 0.08 ± 0.01 (mean$\pm$SE, n=3) pH units in the biceps and 0.05 ± 0.003 (n=3) in the soleus. The net change in $[H^+]$ expected from PCr hydrolysis was calculated from $\beta = \Delta H^+ / \Delta pH$. Buffer capacity was also estimated from titration of muscle homogenates. The contribution of P_i to total β of the homogenates was subtracted to ascertain the non-P_i β for each muscle. The non-P_i β values were added to the actual amount of P_i present in stimulated muscles to calculate a predicted β at pH 7. The apparent β calculated from PCr and pH changes in intact muscles and the predicted β were in good agreement (38 ± 9 vs. 38, cat biceps, 21 ± 7 vs. 30, cat soleus). The results indicate that changes in pH during the first few seconds of contraction can be accounted for by proton consumption via net PCr hydrolysis.

^{1}H-NMR Measurement of Lactic Acid The expected pH change resulting from ^{1}H-NMR measured changes in lactic acid was compared to the actual ΔpH measured by ^{31}P-NMR. Lactic acid production did not appear sufficient to account for the total ΔpH. Addition of albumin to solutions of lactic acid caused an attenuation of the lactic acid signal indicating that some portion might be bound and therefore NMR invisible.

Fatigue Related Metabolites Peak tetanic tension development was measured during acidosis resulting either from hypercapnia (70% CO_2) or repetitive stimulation in isolated perfused cat muscles. During repetitive stimulation both acidosis and increased [$H_2PO_4^{1-}$] were correlated with decreased force production. However, neither acidosis nor increased [$H_2PO_4^{1-}$] resulting from hypercapnia was related to changes in tetanic tension. These results show that neither acidosis nor increased [$H_2PO_4^{1-}$] directly cause muscle fatigue. The observed correlations between these parameters and force development during normocapnia appear to be coincidental to some other effect of repetitive stimulation.

To my parents, Marvin and Jean Adams

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General Introduction

Most people recognize from personal experience that sustained physical activity leads eventually to a decline in muscle performance. This fatigue has both neuro-physiological and cellular causes. On the cellular level fatigue is generally thought to result from the depletion of substrate for energy metabolism and/or the accumulation of metabolites. The metabolites most commonly suggested as agents of fatigue are lactic acid[1], hydrogen ion (H^+) and some form of inorganic phosphate (P_i).

This dissertation focuses on the cellular biochemical factors which mediate fatigue. Initial studies provide information on the relationship between changes in H^+ production, lactate production and intracellular pH. Changes in isometric tension were measured while metabolite concentrations were experimentally altered. Nuclear magnetic resonance spectroscopy (NMR) was used to measure concentration changes in fatigue related metabolites (i.e., H^+, P_i, and lactic acid) before, during and after fatiguing muscle contraction.

The remainder of this introduction will review concepts related to muscle metabolism and fatigue. The format of the sections on specific metabolites is intended to present current doctrine and to discuss possible limitations of the underlying research. The final sections introduce the specific questions asked, and the methods and technology used, in the studies presented in this dissertation. This general methodological introduction is intended to delineate the attributes of the present studies which address the research limitations discussed in the preceding historical sections.

Muscle Metabolism Many of the biochemical reactions related to muscle contraction were identified during the first half of this century. A major goal had been to identify the "primary reaction" linking the liberation of chemical energy to the mechanical generation of force. One of the earliest studies showed that muscle contraction resulted in the production of lactic acid. This observation lead to the theory that lactic acid was the link between chemical energy and mechanical force production for muscles (54,56).

[1] At physiological pH lactic acid is essentially 100% ionized. The term lactate refers to the anionic form of lactic acid and the two are often used interchangeably.

This so-called "lactate theory of contraction" required redefinition during the early 1920's when it was discovered that of phosphorylated compounds are hydrolyzed during muscle contraction (54,99). Hydrolysis of phosphocreatine (PCr) was identified as the immediate reaction associated with contraction. The lactate producing pathway was, however, still considered to be the primary provider of chemical energy for muscular contraction (54). Although, processes involved in oxidative metabolism were well known at this time, they were thought to play a role only during recovery from contraction as an adjunct to lactate production of chemical energy (99).

The discovery of the hydrolysis of adenosine triphosphate (ATP) and the regeneration of ATP at the expense of PCr was the last major refinement of the theory of energy transduction in muscle (99,100). Current theory holds that the transduction of chemical free energy into mechanical work is accomplished by hydrolysis of ATP by the actomyosin ATPase[2] during the crossbridge cycle. The resultant adenosine diphosphate (ADP) is rephosphorylated by donation of P_i from PCr via the creatine kinase reaction. In the presence of oxygen, the energy for regeneration of PCr and ATP is supplied by oxidation of carbohydrate and lipid resulting in production of water and CO_2. When there is insufficient oxygen to support energy demands via oxidation, the excess demand is met by the break down of carbohydrate with the production of lactic acid.

Fatigue The etiology of muscle fatigue has been the focus of investigation for more than one hundred years (41). Despite continuous investigation, a definitive explanation of the mechanisms underlying contractile failure has yet to be established.

In order to discuss fatigue it is necessary to first present a precise definition of it. This definition must include specific information on both intensity and duration of contractile activity. Based on these criteria two distinct forms of cellular fatigue with differing etiologies are commonly recognized. "Low intensity" fatigue is the

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[2] The contractile proteins actin and myosin form the cross bridge complex which has enzymatic activity.

depression of muscle performance resulting from prolonged, sub-maximal contraction as seen in endurance running. This form of fatigue is generally attributed to depletion of substrate for energy metabolism (8,49). "High intensity" fatigue results from short duration, near maximal contractions that might be encountered in heavy resistance training. Under these conditions muscle cells often retain significant stores of substrate for energy metabolism. This high intensity fatigue is thought to result from the accumulation of metabolites or some toxin within muscle cells (45). The work presented in this dissertation is concerned with the high intensity form of fatigue.

The immediate substrates and products of the actomyosin ATPase are ATP, ADP, P_i, H^+ and Mg^{2+}. As such these ions and compounds have become the focus of much of the research on the mechanisms of fatigue. In addition, the development of fatigue is often accompanied by an accumulation of lactic acid.

Lactate and Fatigue One of the earliest, and longest lived, hypotheses is that production and/or accumulation of lactic acid causes fatigue (38,56). From the beginning of this century to present time, many investigators have reported a direct correlation between lactate accumulation and depression of force (9,37,38,49,55,103).

Direct measurement of lactate has traditionally involved homogenization of tissue with subsequent biochemical assay (38,48). Metabolite measurements obtained by this method often include significant discrepancies due to breakdown of metabolites, release of bound molecules and loss of cellular and subcellular compartmental integrity (3,30,31,86,120).

Some experimental results have called into question the role of lactic acid accumulation in fatigue. Intracellular accumulations of 15-30 mM lactate are often associated with fatigue (48). In contrast, Mainwood and Alward have produced fatigue in superfused rat diaphragm when the biochemically measured lactate concentration was only 7mM (78). Following glucose loading, produced by preincubation with insulin and glucose, this preparation accumulated 17.5 mmol/g lactate yet exhibited the same

degree of fatigue as seen with 7 mmol/g (78). These results demonstrate an apparent dissociation of lactate accumulation and fatigue (11,78). However, superfused muscle preparations, such as those used by Mainwood and Renaud, depend on diffusion for delivery of substrate and removal of waste products. Regions of the muscle more than 2-3 cell layers thick may be functionally impaired by insufficient diffusion especially during the metabolic stress of vigorous contraction. Thus the results of this study may reflect some effect on oxidative metabolism.

Much of the current dogma on lactate production during exercise and/or ischemia is based on measurement of blood lactate levels (61,62,134). This indirect method of lactate assessment also suffers from several drawbacks which tend to confound interpretation of results (14,63,123). First the lactate content of venous blood will be the sum of the contribution and extraction of that metabolite by all the tissue traversed since the blood entered the exchange vessels (14,44,51,62,111,112). Second there is some latency in the appearance of lactate in the blood following its production and accumulation in cells (51). In addition, there have been studies showing that significant amounts of lactate are recovered as glycogen or oxidized in both the working muscles and adjacent non-working muscles (62,92).

Chase and Kushmerick studied the effect of 50mM lactate on contraction in skinned rabbit psoas muscle fibers independent of pH (21). A skinned muscle fiber consists of intact myofilaments without the sarcolemma and sarcoplasm (21,26,33,36,81,96). The investigator supplies an artificial sarcoplasm, allowing for controlled addition of metabolites inorder to study contractile behavior. When pH was held constant at either 7.1 or 6.0, 50mM lactate per se had no effect on force production. This suggests that the often-observed correlation between lactic acid accumulation and fatigue may result from the concomitant production of H^+ rather than some effect of lactate.

pH and Fatigue In 1880, Gaskel reported that acidification of cardiac muscle caused a decrease in the force of contraction (41). Subsequently, many studies have reported a direct relationship between changes in intracellular pH (pH_i) and the contractile properties of striated muscle (21,26,30,33,36,50,82,85,96,102).

The earliest measurements of pH at fatigue were made in minced muscle (40). This technique results in a loss of cellular integrity and the possibility of continued or accelerated hydrolysis of substrate and measurements may not reflect *in vivo* conditions (97). The addition of a quick freezing step, often while muscles are *in situ* and contracting, has helped to reduce the variability resulting from continued metabolic activity in homogenized muscle samples (40).

Early methods of pH measurement in intact muscle cells involved the use of pH sensitive dyes (97,117). It is difficult calibrate these dyes which limits their usefulness (80,97). A later method involved following the distribution of weak acids such as radioactive DMO (5,5-dimethyl-2,4-oxazolidine-dione-2-^{14}C). This technique is difficult to implement, destructive to the tissue, and it lacks temporal resolution (e.g., $\approx$ 1hr.in skeletal muscle) (80,97,117). In addition, recent evidence indicates that DMO measurements represent some average intracellular pH, heavily weighted toward the mitochondrial pH (1). Intracellular pH can also be measured using pH sensitive microelectrodes (2,3,117). This technique requires the penetration of single muscle cells limiting measurements to surface fibers (117). In addition, cell movement precludes the use of this method during muscle contraction (17,80).

Despite limitations in absolute quantification of pH_i at fatigue, the consensus of the majority of studies to date is that decreases in pH_i and force are directly correlated (21,26,30,33,36,48,50,82,85,96,102).

Several mechanisms have been proposed which may account for pH effects on contraction. They stem from two more general biochemical observations. First,

changes in hydrogen ion concentration ($[H^+]$[3]), in the physiologicaly relevant range, will alter the state of ionogenic groups at the active site of enzymes, thereby modulating their reactivity (122). Second, protein conformation is also sensitive to $[H^+]$, providing further opportunity for alterations in function (122). With specific regard to muscle fatigue, H^+ modulation of proteins reportedly results in: (i.) an inhibition of glycolysis (118), (ii.) the disruption of excitation-contraction coupling (36,39,60,90,97), (iii.) a decrease in the calcium (Ca^{2+}) sensitivity of actomyosin formation (21,26,33,34,36,39,65,82,91), and (iiii.) the alteration of Ca^{2+} uptake (60) or release by the sarcoplasmic reticulum (77,90).

In summary, contracting skeletal muscle produces lactic acid when energy demands exceed the capacity for oxidative regeneration of adenosine triphosphate. At physiological pH and temperature, lactic acid (pK = 3.7) dissociates producing lactate anion and hydrogen cations. Coupled with the aforementioned effects of H^+ on force, these observations provide the basis for the most widely held theory of fatigue resulting from high intensity contractile activity: i.e., an increase in intracellular hydrogen ion concentration $[H^+]$, resulting from lactic acid production, is responsible for decreases in both maximum tension development and maximum velocity of shortening (10,21,34,36,81,82,102,110,).

Buffer Capacity In biological systems H^+ production is seldom linearly related to the resulting change in pH_i. Some portion of the H^+ produced will be buffered by chemical binding to P_i and ionogenic groups on proteins and peptides (108,122). The change in pH resulting from a given increase in H^+ is a function of the buffer capacity (ß) of the cells.

In order to assess the effects of changes in $[H^+]$ on contraction both the change in H^+ and the buffer capacity of a given muscle must be known. This parameter (e.g.,ß) is of particular importance when changes in pH are calculated from the

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[3] The brackets [] indicate concentration.

expected H^+ production resulting from other metabolic changes such as increases in lactate (59,105).

The conventional method for measuring buffer capacity has been to homogenize the muscle and titrate the crude homogenate. These titrations have been conducted under widely varying conditions of temperature, presence of metabolic inhibitors, and CO_2 content. This has lead to substantial variation (i.e., from 10 to 116 Slykes) in buffer capacity values reported in the literature (3,4,13,18,28).

Inorganic Phosphate and Fatigue Contracting muscle can accumulate 25-35 mmol/g of inorganic phosphate (P_i) (30,86,115). This metabolite seems to have a paradoxical role in contracting muscle. There is evidence that an increase in P_i is necessary to activate energy metabolism (22,26,67) but will inhibit force generation (26,52,53,65).

In 1978 Dawson et al. noted an apparent correlation between increased intracellular [P_i] and decreased force (30). Subsequently, Hibberd et al. demonstrated that elevated P_i decreased maximum tension development in skinned rabbit psoas fibers (53). The P_i dependence of force generation has been demonstrated in many studies since that time (5,35,65). However, within this body of literature there is variation in the characteristics of reported P_i effects. In the intact muscle studies reported by Dawson, the major effect of P_i on force is seen when concentrations increase above 20mM, below that level only slight depression of force is noted (29,30). However, in studies employing the skinned fiber model, P_i appears to depress force as the concentration increases to 15mM at which point force levels off (5,26,65).

In Dawson's experiments with intact muscle, P_i was increased by contractile activity (29). This contractile activity also decreased the intracellular pH. In the physiological range of pH, inorganic phosphate will exist as HPO_4^{2-} or $H_2PO_4^{1-}$ with a pK of 6.75 at 37°C. The proportion of $H_2PO_4^{1-}$ present can be calculated from total P_i, pH and pK. When Dawson et al. plotted [$H_2PO_4^{1-}$] against force a highly linear relationship was observed suggesting the possibility of a cause and effect relationship

(29). This observation has received support in subsequent studies of intact muscles (89,127) and skinned muscle fibers (91). Based on these findings it has been hypothesized that decreases in pH_i indirectly cause fatigue by increasing the concentration of $H_2PO_4^{1-}$ which directly interferes with the force producing step in the crossbridge cycle (91).

In contrast, several recent skinned fiber studies have failed to find a pH dependence of P_i mediated decreases in force (21,25,41,65). Despite these contradictory findings the $H_2PO_4^{1-}$ theory is entering the dogma of fatigue mechanisms.

Summary Lactic acid accumulation during contraction is thought to cause a decrease in intracellular pH. This decreased pH appears to cause fatigue, either directly or indirectly, by alteration in the concentration of $H_2PO_4^{1-}$.

Each component of this theory suffers from some methodological limitation and/or inconsistent experimental finding. Results from muscle homogenates tend to over- or underestimate metabolite concentrations. Blood lactate levels represent a spatial and temporal composite of metabolic events upstream from the sample site. Most of the available pH measurement techniques lack temporal resolution.

Isolated, superfused muscle preparations have been used for data collection. However, insufficient diffusional movement during the high metabolic activity associated with contraction might lead to significant cell to cell metabolic heterogeneity within a muscle.

Studies of skinned fibers allow for the control of more variables than either homogenized or intact muscles. Unfortunately, the physiological relevance of data collected from a preparation lacking both sarcoplasm and a cell membrane is open to question. In particular, the substitution of a homogeneous buffer solution for the complex gel-sol sarcoplasm may significantly alter access of metabolites to the contractile proteins. This may explain the discrepancy in P_i effects seen in intact vs. skinned fibers (29,30,and present Chapter 3 vs. 5,26,65).

A more global problem which applies to most studies results from use of muscles without regard for fiber type composition. Changes seen in these studies may be a function of sampling from a nonrepresentative population and/or averaging across a heterogeneous population. For example, a given stimulation pattern may preferentially activate a particular fiber type. A measured decrease in force production from this protocol would result from fatigue of selected fibers. Metabolic measurements from the whole muscle would not accurately reflect the state of just the activated fibers.

It is clear that much of the research on the metabolic basis of fatigue has been collected under conditions of severe methodological limitation. Newer technologies hold the promise of data collection from preparations which more closely mimic *in vivo* physiological conditions.

Nuclear Magnetic Resonance (NMR) NMR is a non-destructive technique which can be used to collect data from muscles *in vivo*[4]. This method allows for multiple collections of data from the same muscle, eliminating experimental variability resulting from muscle to muscle (i.e., animal to animal) differences. The non-destructive collection of data preserves the internal structure of the muscle cells avoiding the physical mixing of compartment contents and/or a loss of compartmented function seen in earlier studies.

Standard NMR collections have temporal resolution of seconds or minutes eliminating much of the averaging seen with previous methods. In addition, gating techniques, such as those detailed in chapter one, can reduce the time for data collection into the millisecond range.

A major limitation of NMR as a tool in biological studies is its relative insensitivity. While other forms of spectroscopy (e.g., UV light or fluoresence) can be used to detect metabolite concentrations in the μM (10^{-6}M) range, biological NMR is

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[4] The term *in vivo* is used here and henceforth to mean "chemical processes occurring within cells, etc., as distinguished from those occurring in cell free extracts". -Stedman's Medical Dictionary 23 Ed.

limited to detecting mM (10^{-3} M) concentrations. This results in an inability to detect metabolites such as ADP or adenosine monophosphate which have unbound concentrations in the μM range.

Phosphorus NMR (^{31}P-NMR) ^{31}P-NMR collections provide data on the intracellular concentrations of phosphorous containing compounds and ions such as ATP, PCr and P_i. The chemical shift of P_i can be used to calculate the intracellular pH of muscles while a pH sensitive extracellular marker, phenylphosphonate, can be used to calculate extracellular pH (86). The intracellular concentration of Mg^{2+} can be calculated from the chemical shift of ATP (46,69). As noted earlier, the immediate substrates and products of the actomyosin ATPase are ATP, ADP, P_i, H^+ and Mg^{2+}. The similarity between the list of parameters amenable to measurement by ^{31}P-NMR and those of interest in contractile reactions indicates the suitability of this technique for fatigue studies.

Proton NMR As the name implies, proton NMR (^{1}H-NMR) has the potential to measure any compound containing hydrogen. Biological samples contain many such compounds in concentrations sufficient to be measured by NMR (i.e. >0.5mM). However, the high concentrations of protons in water, proteins and lipids tend to obscure signals from most other compounds. Recent advances in techniques for the subtraction or suppression of these interfering signals have made quantification of concentrations of metabolites such as lactate possible (47,49,66,67,119,125). The ability to measure lactate in a living muscle with good temporal resolution promises to overcome many of the limitations of previous methods.

Muscle Models The muscle models and techniques chosen for the studies presented in the following chapters were selected in an effort to avoid the limitations which have plagued similar research in the past. The primary models for these studies are the isolated perfused cat soleus and biceps muscles. The cat soleus consists of greater than 92% slow twitch, oxidative fibers, the biceps contains 71% fast twitch, glycolytic,

24% fast twitch, oxidative, and 5% slow twitch, oxidative fibers (86). Studies employing these two muscles provide a good comparison of effects of various experimental perturbations in the two primary fiber types (fast vs slow). The relatively pure fiber type of these muscles allows the assumption that the changes seen result from uniform effects on the majority muscle fiber population rather than a small minority population.

Controlled perfusion allows matching of flow to the metabolic needs of the muscle both at rest and during exercise. The metabolic state of the muscle can be constantly monitored by ^{31}P-NMR. These two factors allow collection of data from muscles relatively free from the impairment seen in superfused muscle preparations.

The aim of this dissertation has been the collection of both ^{1}H and ^{31}P-NMR data from intact, isolated perfused muscles in an attempt to further characterize metabolic and functional changes as they relate to the development of fatigue.

Chapter 1 presents data from experiments designed to accurately measure buffer capacity *in vivo*. The results from these experiments provide information which will allow the calculation of buffer capacity as it changes during contraction. This parameter can then be used to assess the effects of other metabolic reactions such as lactate production on intracellular pH.

The experiments detailed in Chapter 2 deal with *in vivo* measurement of lactate. The aim of this work was to calculate the change in pH_i expected for a measured accumulation of lactate. This calculation included dynamic buffer capacity values provided by the results from Chapter 1. The pH_i calculated from lactate measurements was compared to the empirically measured pH_i (^{31}P-NMR) to determine if lactate production could account for the changes seen.

In the final chapter intracellular $[H^+]$ and $[H_2PO_4^{1-}]$ were altered independent of contractile activity by hypercapnia. The question asked in this work was whether the

reported correlations between increases in these metabolites and the development of fatigue represent a cause and effect relationship.

Chapter 1

Muscle buffer capacity estimated from pH changes during rest-to-work transitions.

INTRODUCTION

One essential characteristic of muscle cells that must be known, in order to measure the effects of changes in H^+ production on contraction, is buffer capacity (ß). In biological systems H^+ production is seldom linearly related to the resulting change in pH. The difference arises from the binding of H^+ to P_i and ionogenic groups on proteins and peptides (108,122). Thus a change in pH resulting from a given increase in H^+ is a function of ß.

The conventional method for measuring buffer capacity has been to homogenize the muscle and titrate the crude homogenate. These titrations have been conducted under widely varying conditions of temperature, CO_2 content, and metabolic inhibition (12,18,93). This has lead to wide variation in buffer capacity values reported in the literature (3,4,13,18,28).

^{31}P-NMR can be used to measure intracellular pH (pH_i) in intact skeletal muscle. Given conditions where a known amount of H^+ is produced or consumed, and the concurrent change in pH_i measured by ^{31}P-NMR, the apparent ß could be calculated.

The intracellular pH of skeletal muscle becomes transiently alkaline within seconds after the onset of a series of twitch contractions (86,107,113). In many recent studies using phosphorus NMR for pH measurements (19,70,86,107,113), both the magnitude and time course of the initial alkalinization suggest that it is at least partly due to proton consumption associated with net phosphocreatine (PCr) hydrolysis :

$$ATP + H_2O \longrightarrow ADP + P_i + \alpha_a H^+$$

$$PCr + ADP + H^+ \longleftrightarrow creatine + ATP$$

$$PCr + H_20 + \alpha_p H^+ \longrightarrow Cr + P_i,$$

where the coefficient $\alpha_p = (1-\alpha_a)$ is 0.4 at pH 7 and increases at lower pH (42,70). Assuming an intracellular buffer capacity of 40 Slykes for mammalian skeletal muscle

(12,18,93), net hydrolysis of 10 μmol/g PCr at pH 7 should produce a maximum alkalinization of 0.10 pH units. This small alkalinization is consistent with what is typically observed in NMR studies, suggesting that PCr hydrolysis might be entirely responsible for the transient alkalinization.

In contrast, Connett (24) recently reported a much larger transient alkalinization (0.75 pH units) after 5 seconds of twitch contraction in dog gracilis muscle. pH was estimated by chemical assay of metabolites and the calculation of H^+ using *in vitro* equilibrium constants for 5 enzymes. An alkalinization of 0.75 pH units is too large to account for by net PCr hydrolysis alone. The alkalinization calculated in Connent's study was reversed after 15 seconds. This early alkalinization would have been missed by NMR studies in which data accumulation was averaged over the first 15 or more seconds of a contraction series (71,85).

The purpose of this study was to exploit this early alkalinization to measure ß in intact skeletal muscle. To do this it was necessary to determine whether or not the transient alkalinization observed during the first few seconds of a series of contractions can be quantitatively accounted for by net PCr hydrolysis. This was accomplished by comparing the buffer capacities (ß, Slykes = $\Delta H^+/\Delta pH$) of three different mammalian muscles calculated from titrations of muscle homogenates *in vitro* with their buffer capacities estimated from the observed alkalinization during brief series of contractions. The latter was calculated assuming that net PCr hydrolysis was the only significant metabolic reaction effecting pH, i.e., $\Delta[H^+] = \alpha_p \times \Delta[PCr]$, and hence:

$$\text{I.} \qquad ß = \alpha_p \times \Delta[PCr] \ / \ \Delta pH.$$

PCr and pH were measured at discrete times by gating acquisition of phosphorus NMR scans to specific times during and after repeated bursts of 5 Hz contractions.

The results support the assumption that net PCr hydrolysis is the only significant metabolic reaction affecting pH during this time period. The H^+ consumption of this reaction is known and thus an *in vivo* ß can be calculated from the observed changes in pH and PCr.

METHODS

Phosphorus NMR studies.

These studies were performed on three animal muscle preparations described previously: the superficial 2-3 mm (predominantly fast-twitch glycolytic) portion of the rat gastrocnemius muscle in situ (70,85), and the isolated, arterially perfused cat biceps brachii (fast-twitch) and soleus (slow-twitch) muscles (71,86).

Male Sprague-Dawley rats (300-350 g) were anesthetized with sodium pentobarbital (50mg/kg, IP), and an IP catheter inserted to allow delivery of further anesthetic as needed. The sciatic nerve was dissected free, cut, and placed within a bipolar platinum electrode. Rats were mounted in a specially designed NMR probe with the Achilles tendon attached to a force transducer as described previously (85). Stimulation voltage and muscle length were adjusted to produce maximum twitch force.

Male or female cats weighing 3-4 kg were sedated with Ketamine (11 mg/kg, IM) and anesthetized with sodium pentobarbital (30 mg/kg, iv.). The biceps (7.5 ± 0.5 g, SE, n=3) or soleus muscles (4.3 ± 0.5 g, SE, n=3) were vascularly isolated, excised, and perfused at constant flow (0.2-0.4 ml/min/g) via the arteries with a 20% suspension of sheep red blood cells in Krebs-Henseleit (1.2mM KH_2PO_4) solution containing 3.5% BSA, 5mmol glucose, 0.15mM sodium pyruvate, and 30mg/l papaverine HCl (86). Perfusion pressure was 90-110 Torr. Following muscle dissection, the cats were killed with pentobarbital. Perfused muscles were attached to a force transducer and platinum stimulation electrodes in a specially designed 7.4 cm diameter NMR probe (See Appendix). Each muscle was repeatedly lengthened and

shortened while stimulated with supramaximal voltage twitches until the length for maximum force production (L_o) was estabilshed. Stimulation voltage, L_o, and temperature (37±2°) were monitored and maintained at optimal levels during the experiments.

Prior to muscle stimulation, fully-relaxed phosphorus NMR spectra (162 MHz, 7000 Hz sweep width, 2K complex data, 90 degree nominal pulse width, interpulse interval 15 sec) were acquired from each muscle. Muscles were then stimulated with 6 (rat) or 10 (cat) second trains at 5 Hz, with 4.5 min between each train. Acquisitions of phosphorus NMR spectra were gated to specific times during and after the trains of stimulation by triggering the stimulator from the spectrometer's computer (Aspect 3000 of a Bruker AM400). Scans were acquired at 5 (rat) or 9 (cat) seconds into the train, and at 20, 60, 90, 120, 180, and 240 seconds after the trains. After an additional 15 second delay, the cycle was typically repeated to a total of 8 scans per spectrum (see Figure 1). It should be emphasized that although spectra are the average from several scans, the effective time resolution of the gated spectra is equal to the acquisition time per scan, i.e., 145 ms. Free induction decay (FID) data sets were zero-filled to 4K and multiplied by an exponential corresponding to 25 Hz linebroadening before Fourier transformation. The minimum interpulse delay used in this protocol was 20 seconds, therefore the spectra were fully-relaxed (86) and no saturation corrections are necessary. PCr, ATP and P_i peaks were integrated by an iterative Lorentzian fitting routine[5] (87), and integrals scaled to μmol/g assuming pre-stimulation ATP levels of 7.2, 7.0 and 3.8 μmol/g for rat gastrocnemius (80), and cat biceps and soleus, respectively (86).

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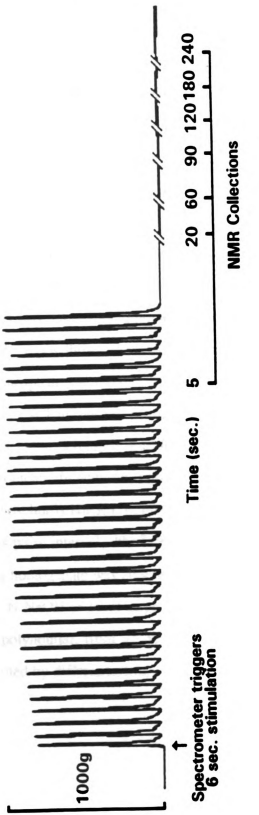
[5] This routine minimizes the error between a curve fit to the data and the equation for the ideal Lorentzian line shape ($y = 1/1-x^2$) at the designated peak location (87).

Figure 1. Twitch force record from rat gastrocnemius muscle illustrating protocol for gated NMR data collection at 5 seconds after the initiation of 5 Hz stimulation and at the indicated intervals during recovery. The cycle of stimulation and recovery was repeated eight times with each NMR collection being added to the appropriate computer memory block.


1000g
Spectrometer triggers
6 sec. stimulation
Time (sec.)
5
20 60 90 120 180 240
NMR Collections

Because of the relatively lower signal-to-noise ratio and a tendency toward non-Lorentzian lineshape of the P_i peak in spectra acquired during stimulation, the inorganic phosphate content of muscles during stimulation was estimated from the biochemically determined P_i content of resting muscle plus the P_i expected from the observed PCr hydrolysis. Intracellular pH was estimated from the chemical shift of the inorganic phosphate peak as described previously (86). The apparent buffer capacity in intact muscle was calculated according to equation I, assuming $\alpha_p = 0.4$ near pH 7.

Buffer capacity of muscle homogenates.

Animals were anesthetized as above and the muscles of interest dissected free. In each case, a 0.5 (rat) or 1 g (cat) sample roughly corresponding to the area in the sensitive volume of the NMR coils was removed and homogenized in 20 ml of 0.9% NaCl/g muscle. In order to avoid the possibility of variable metabolic changes, and in particular variable hydrolysis of phosphate metabolites, all homogenates were incubated at 37°C for 45 min before titration. A 1 ml portion of each homogenate was then frozen in liquid nitrogen and extracted in perchloric acid for measurement of inorganic phosphate (86) and protein (75) content. In addition, two perchlorate extracts of each muscle type were examined by phosphorus NMR (162MHz, 4K complex data, 8000Hz sweep width, 45 degree pulse, 1 s pulse interval, 800-2400 scans) in a standard broadband probe. The remaining homogenate was acidified to pH 6 with HCl and titrated at 37°C to pH 8 with 0.2 N NaOH in 10 μl steps. The resulting mean titration curves were fit to a fourth order polynomial, from which the slope (total ß, Slykes) over the pH range 6-8 was computed by differentiation.

RESULTS

Buffer capacity of muscle homogenates.

Titration curves of the homogenates of each muscle type appear in Figure 2. These curves were remarkably reproducible within a muscle type. For example, the pH after addition of 30 μmol of base to rat homogenates initially adjusted to pH 6 was 7.02 ± 0.04 (SE, n=6). The total buffer capacity over the range pH 6-8 computed from the slopes of polynomial fits to the mean titration data appear in Figure 3 (open symbols). At pH 7 the total buffer capacity of the fast-twitch muscles (rat gastrocnemius and cat biceps) are similar, while that of the soleus is somewhat less (Table 1). These results appear to be consistent with previous studies which reported buffer capacities in highly glycolytic muscles of around 50-60 Slykes, with somewhat lower capacities in red muscle (12,18).

However, all of the homogenates contained very high levels of free inorganic phosphate (Table 1) which was not present in intact, unstimulated muscle (Table 2), and therefore represents hydrolysis of PCr, ATP and other phosphate metabolites. This was confirmed by examination of ^{31}P-NMR spectra of perchlorate extracts of the homogenates after incubation (Figure 4). In soleus extracts, P_i was the only peak resolved in the spectra. In biceps muscles, one additional peak with area 20-25% of the area of the P_i peak was resolved. This peak resonated coincident with IMP added to the extract.

Figure 2. Titration of muscle homogenates from pH 6 to 8 in 0.2 uM steps with NaOH (mean $\pm$ SE, n=6 for each muscle type).

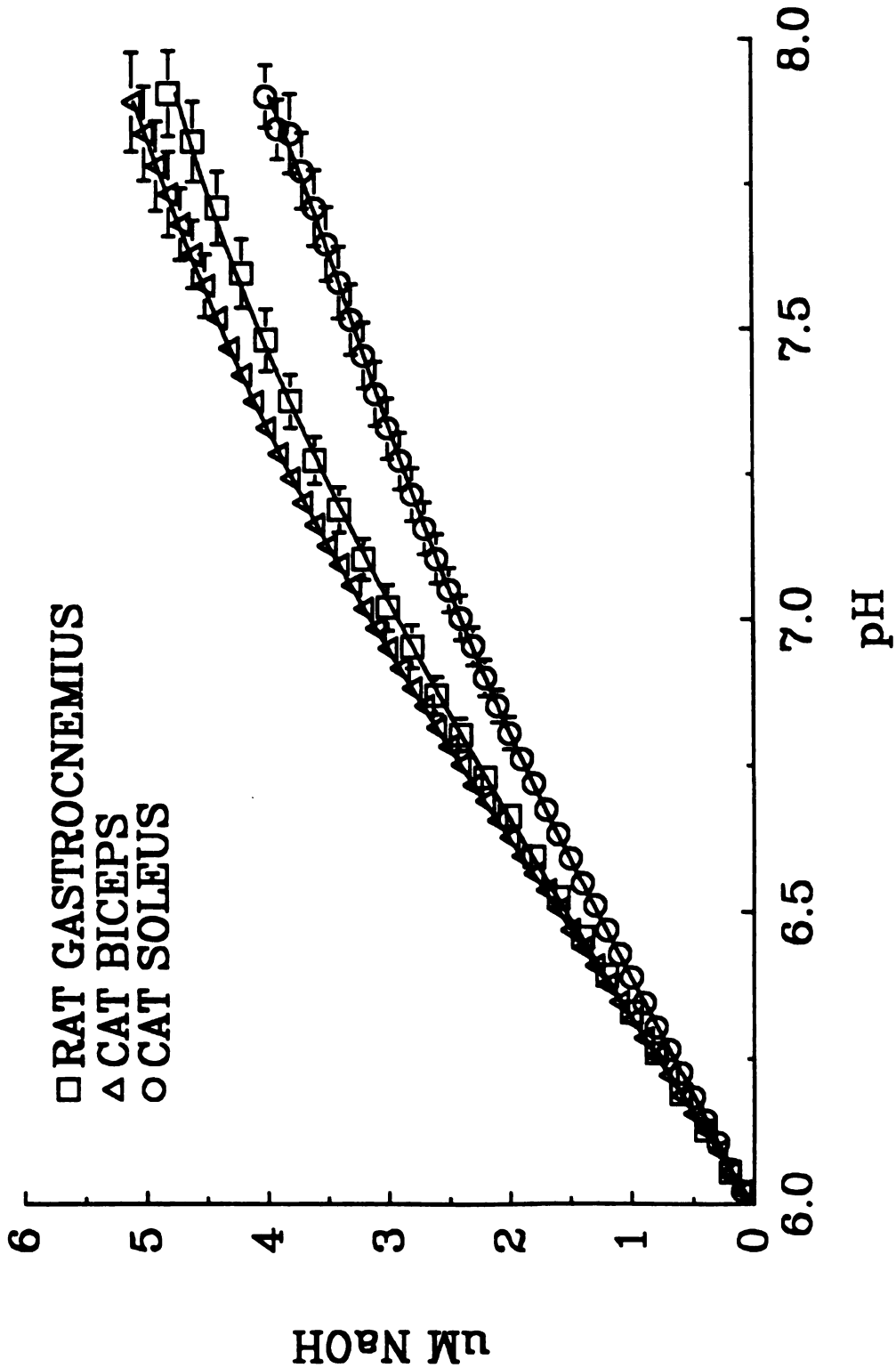

□RAT GASTROCNEMIUS
△CAT BICEPS
○CAT SOLEUS
uM NaOH
pH
0
1
2
3
4
5
6
6.0
6.5
7.0
7.5
8.0

Figure 3 Total buffer capacity (open symbols) calculated from slope of mean titration curves in Figure 2, and non-phosphate buffer capacity (closed symbols), calculated by subtracting contribution of P_i (Table 1) to total buffer capacity, of cat and rat skeletal muscle.

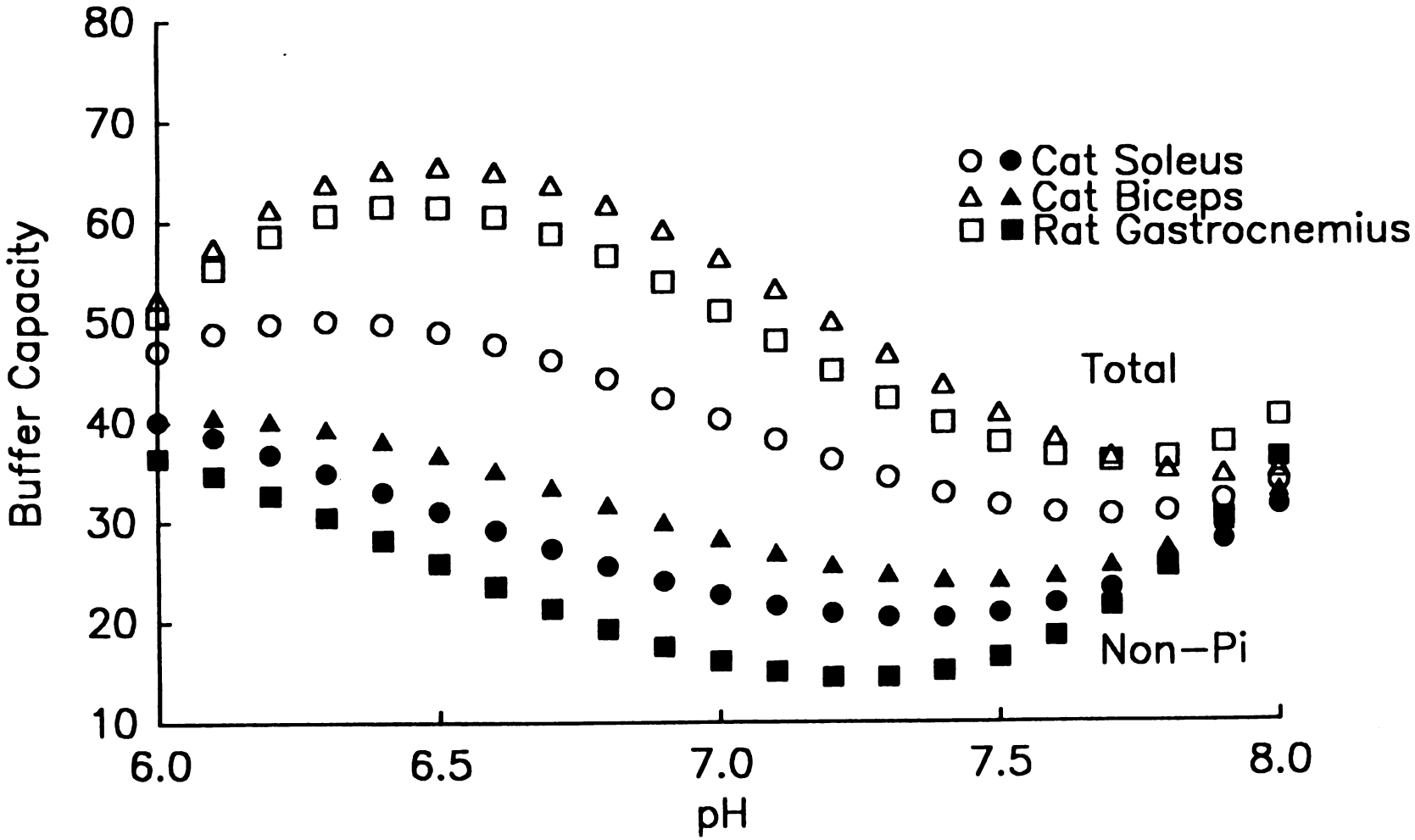

Buffer Capacity
80
70
60
50
40
30
20
10
6.0
6.5
7.0
7.5
8.0
pH
Cat Soleus
Cat Biceps
Rat Gastrocnemius
Total
Non—Pi

TABLE 1

Measurements in Muscle Homogenates.

	Muscle Protein (mg/g muscle) ($\bar{x} \pm SE$)	Phosphate (μmol/g muscle) ($\bar{x} \pm SE$)	Total ß, pH 7 (Slykes)	Non-P_i ß, pH7 (Slykes)
Cat biceps (n=6)	185±16	29.6±1.3	56	28
Cat Soleus (n=6)	174±5	18.1±1.8	40	23
Rat (n=6)	159±7	35.6±0.9	51	16

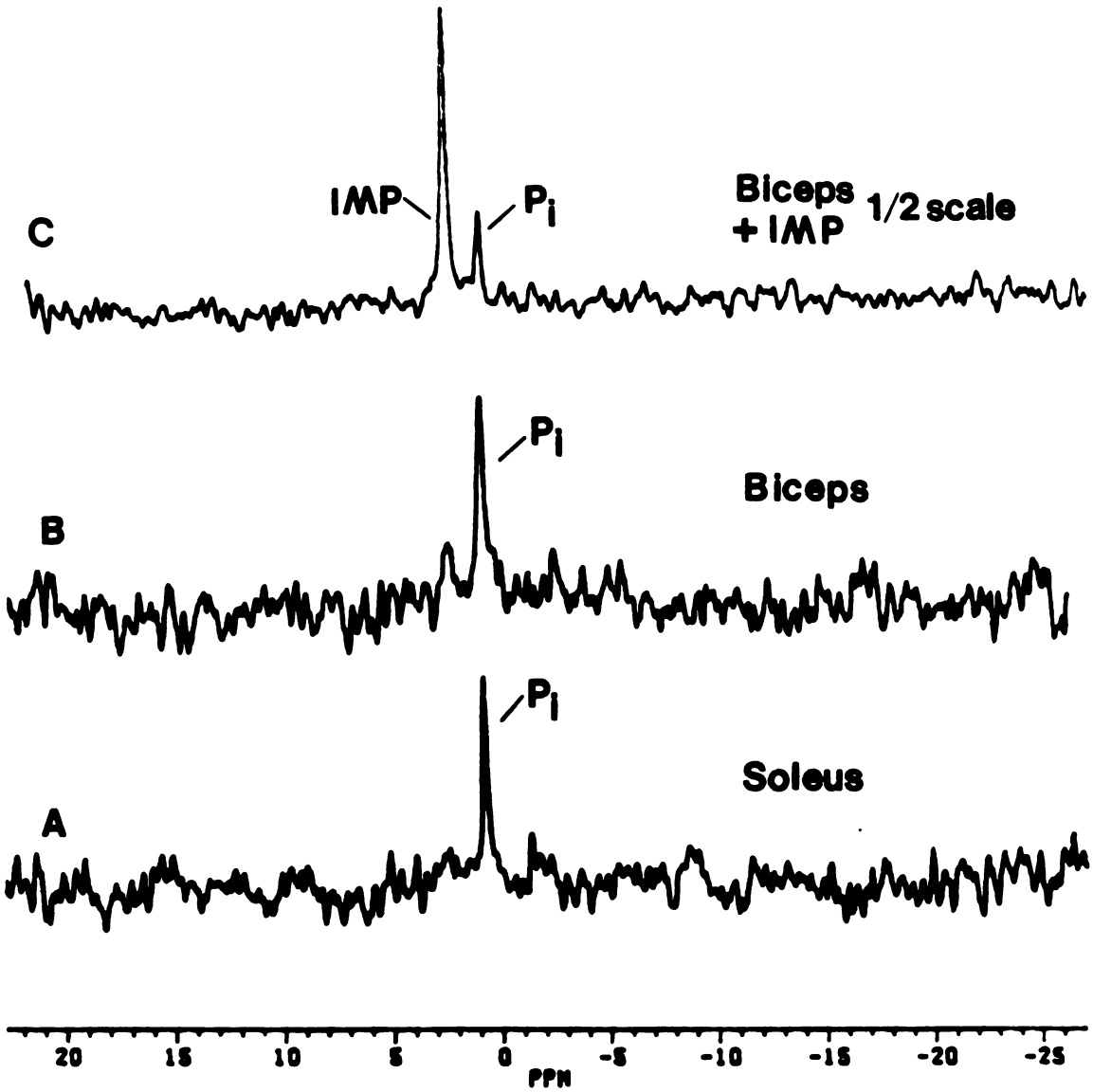
TABLE 2.

Metabolite contents of Unstimulated muscle.

	Ratio of NMR integrals (x±SE):		μmol/g:		
	P_i/ATP	PCr/ATP	ATP*	P_i Φ	PCr Φ
Biceps (n=7)	0.38±0.04	3.32±0.37	7.0	2.7	23.2
Soleus (n=4)	1.20±0.29	2.75±0.39	3.8	4.6	10.5
Rat (n=5)	0.40±0.07	3.48±0.18	7.2	2.9	25.1

* Values from references 19, 86.
Φ NMR integrals normalized to ATP.

Figure 4 ^{31}P-NMR spectra of homogenate extracts from cat soleus (A) and biceps (B) muscles. The major peak in both A and B is P_i. In spectrum C IMP was added to the biceps extract to verify that the minor peak seen in B was IMP.


C
IMP
Pi
Biceps
+ IMP
1/2 scale
B
Pi
Biceps
A
Pi
Soleus
20
15
10
5
0
-5
-10
-15
-20
-25
PPM

The pK_a of inorganic phosphate titrated under conditions identical to those used for the homogenates (i.e., muscle specific concentration, 37°C) was 6.75. Considering the high levels of inorganic phosphate in the homogenates (Table 1), P_i must make a major contribution to the total buffer capacity of the homogenates in the range pH 6.5-7.0. The solid curves in Figure 3 are the buffer capacity of the homogenates after subtracting the calculated contribution due to the inorganic phosphate present, i.e., the non-phosphate buffer capacity of the homogenates (Table 1). After this correction, the apparent correlation of buffer capacity with muscle fiber type is lost, as the rat muscle has the lowest buffer capacity while the two cat muscles are not markedly different.

Buffer capacity calculated from pH transients at the onset of stimulation.

Most muscles in these studies performed 8 serial trains of contraction with a total of 36 min between the first and last bouts. There was no significant decrease in peak twitch force over the course of the experiment in any muscle (e.g., in rat muscle mean peak force was 2.22 ± 0.3g/g (SE) body wt. during the first train and 2.32 ± 0.3g/g during the last train). In one experiment on a large cat biceps, sufficient S/N was obtained to acquire useful spectra in a single scan (Figure 5a), allowing complete data collection during and after a single 10 second train of stimuli. The results from this muscle were similar to that obtained from other muscles in which spectra were averaged over 8 cycles of stimulation (Figure 5b). These results demonstrate that the response to a brief train of stimuli is not altered by application of previous trains separated by a 4.5 min rest period.

Figure 5. Sets of phosphorus NMR spectra acquired by protocol of Figure 1. Series A is from a large biceps muscle from which the S/N was sufficient to obtain good spectra with a single scan. Series B is representative of 8 scan spectra.

Figure 6 is a set of representative spectra from a rat experiment demonstrating the downfield (alkaline) shift of the phosphate peak after 5 seconds of 5 Hz stimulation relative to the spectrum acquired 4.25 min later. There was a significant increase in pH (Figure 7) during the stimulation train which was reversed during the subsequent recovery period (Figure 7). The pH in the last spectrum of the recovery period (6.92 ± 0.04, 7.03 ± 0.01, and 7.06 ± 0.01 for rat gastrocnemius, and cat biceps and soleus, respectively) was not significantly different from pH before any stimulation. The changes in pH during stimulation were coincident with significant decreases in PCr in all muscle types (Table 3). The apparent buffer capacities (Table 3) calculated from the changes in PCr and pH using equation I lie intermediate between the total buffer capacities and the non-phosphate buffer capacities of the muscle homogenates. However, if the estimated P_i content in the muscles after 5 (rat) or 9 (cat) seconds of stimulation is added to the non-phosphate buffer capacity, then the predicted buffer capacities (Table 3) from the homogenate data agree with the apparent buffer capacities calculated from PCr and pH changes in the intact muscles.

Figure 6 Series of gated phosphorus NMR spectra (each 8 scans) from rat gastrocnemius collected as described in methods and in Figure 1. The zero time spectrum is identical to the 240 sec spectrum, and is plotted twice so that the alkaline shift of P_i is apparent. Bar on time scale represents 6 second contraction.

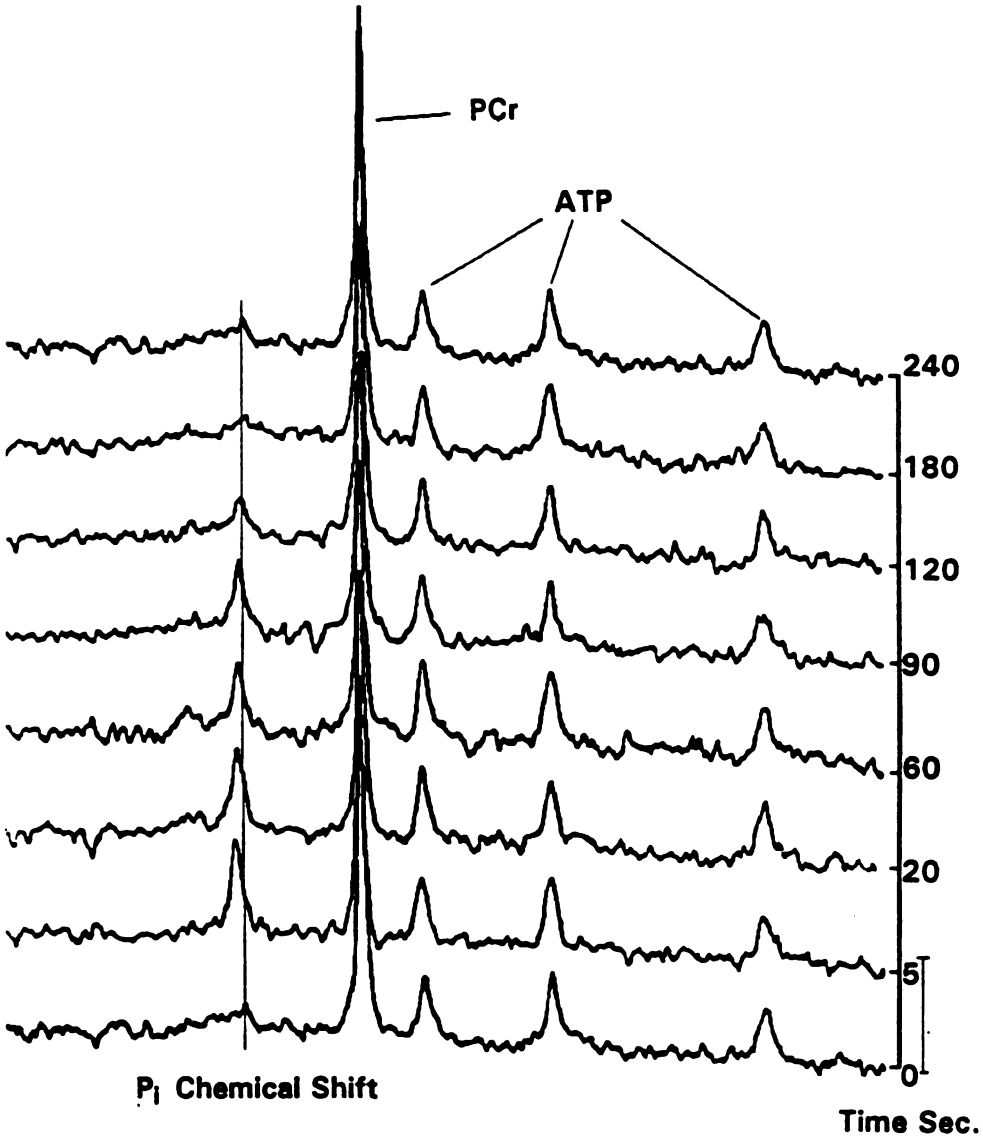

PCr
ATP
240
180
120
90
60
20
5
0
P_i Chemical Shift
Time Sec.

Figure 7. pH changes relative to the final recovery spectrum during and after muscle stimulation at 5 Hz for 6 (rat) or 10 seconds. * = significantly different from final recovery spectrum ($p < 0.05$)

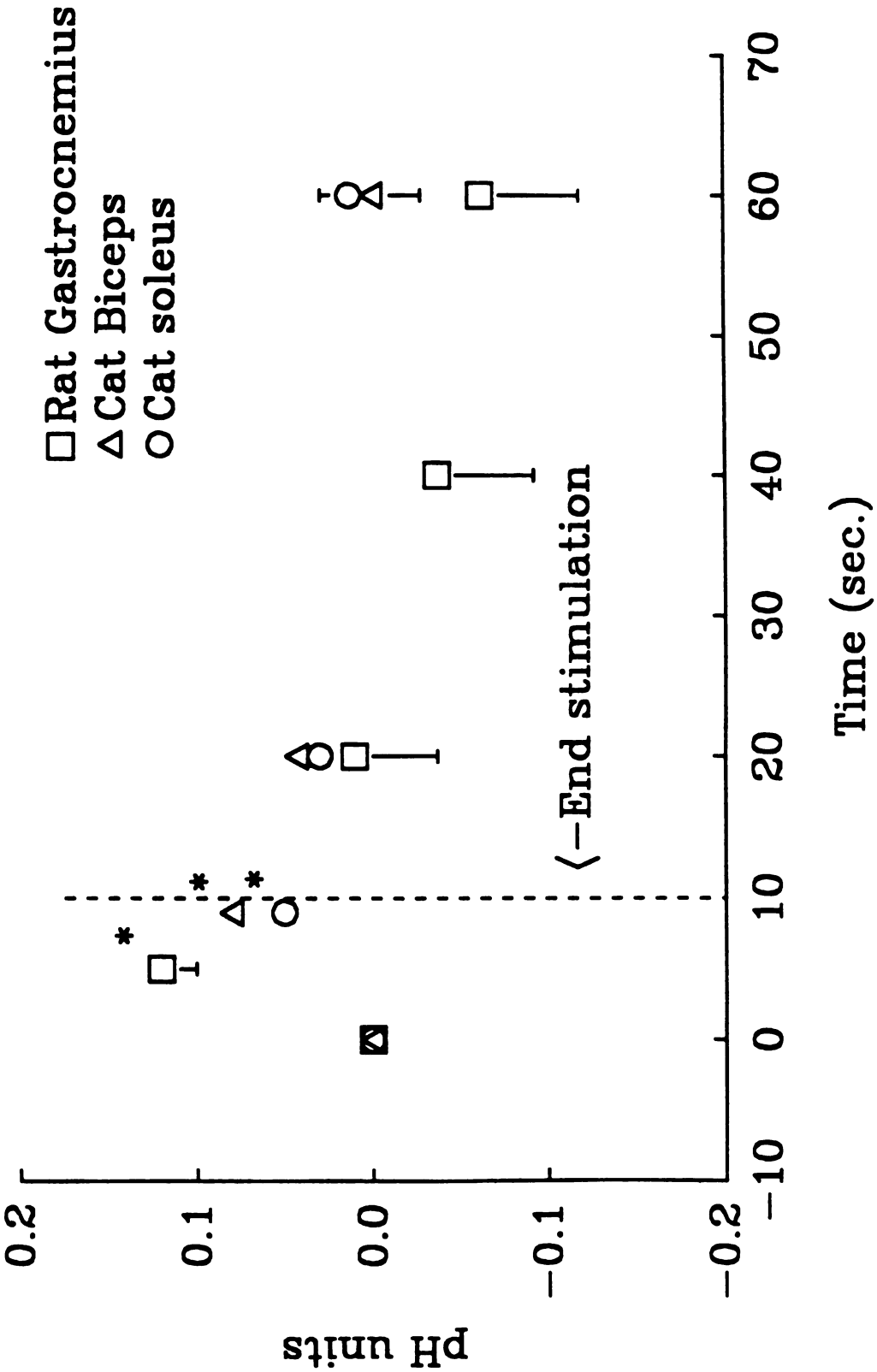

□Rat Gastrocnemius
△Cat Biceps
○Cat soleus
pH units
0.2
0.1
0.0
−0.1
−0.2
−10
0
10
20
30
40
50
60
70
Time (sec.)
<—End stimulation

TABLE 3.

Calculation of ß from in vivo measurements*:			From non-P_i ß plus P_i:	
Muscle	ΔPCr (μmol/g)	ΔpH (Slykes)	Apparent ß (Slykes)	Predicted ß
Cat biceps (n=3)	7.3±0.6	0.08±0.01	38±9	38
Cat Soleus (n=3)	2.6±0.7	0.05±0.01	21±7	30
Rat (n=3)	8.0±2.0	0.12±0.02	30±6	27

DISCUSSION

The major conclusion to be drawn from this study is that the transient alkalinization which occurs at the onset of stimulation in mammalian skeletal muscles can be quantitatively accounted for by proton consumption due to net PCr hydrolysis. If there were another quantitatively significant source or sink for protons during the first few seconds of contraction, then the buffer capacity calculated from the transient alkalinization accompanying PCr hydrolysis would differ from that predicted from titration of the homogenates. In fact, these two estimates of buffer capacity are similar in all three muscle types. Thus, it appears unlikely that there is a quantitatively significant shift of protons either extracellularly, or into subcellular organelles at the onset of muscle stimulation (24).

The fact that the observed pH changes can be fully accounted for by PCr hydrolysis also suggests that little accumulation of lactic acid had occurred in these muscles during the first few seconds of contraction. Accumulation of lactic acid during the stimulation would oppose the alkalinizing effect of PCr hydrolysis, and thus result in higher buffer capacities calculated via equation I. Although little lactic acid formation would be expected in the highly aerobic soleus muscle (15,86), stimulation of rat or cat fast-twitch muscle at 5 Hz for longer periods is well-known to result in lactic acid accumulation (87,88). Apparently, full activation of glycolysis is not achieved in these muscles until sometime after the first 5-10 seconds of stimulation.

The alkalinization observed in this study at 5 and 9 seconds is much less than that calculated by Connett (24) from metabolic data in dog gracilis muscle after 5 seconds. In that study an alkalinization from pH 7.05 to 7.8 was calculated with no significant change in PCr. Even assuming a PCr change of 10 μmol/g, the buffer capacity of dog muscle would have to be less than 6 Slykes if this alkalinization were due to PCr hydrolysis. It seems unlikely that the buffer capacity of dog muscle would be dramatically lower than that of rat and cat muscles (12,18). A more likely

explanation for the high pH calculated in Connett's study after 5 seconds is that one or more of the glycolytic reactions used to calculate pH was not near equilibrium at that time. On the other hand, the pH calculated for unstimulated muscle in Connett's study is very similar to that observed in this and previous phosphorus NMR studies, suggesting that these reactions are near equilibrium in muscle at rest. The agreement between pH measurements calculated from metabolic equilibria and phosphorus NMR in unstimulated muscle suggests that there is no complicating subcellular compartmentation of the metabolites used in the calculations (i.e., PCr, creatine, P_i, lactate, pyruvate, dihydroxyacetone phosphate and 3- phosphoglycerate (24)).

Titration of muscle homogenates is the conventional method for measuring muscle buffer capacity. Unfortunately, these titrations have been conducted under widely varying conditions of temperature, presence of metabolic inhibitors, and CO_2 content. The most quantitatively significant reactions likely to occur in muscle homogenates are lactate production and hydrolysis of high energy phosphates. Of these, the former is of no concern, since neither glycogen nor lactic acid contribute significantly to buffering above pH 6. Our results also indicate that accumulation of phosphorylated glycolytic intermediates is also of little concern, inasmuch as significant levels of these were not observed in spectra of homogenate extracts. The conversion of ATP (pK6.5, (42)) to IMP and/or AMP (pK6.2, (86)) observed in homogenates of fast muscle would reduce their buffer capacities compared to intact muscles at pH 7 by only 1.0 Slyke, assuming 7 μmol ATP converted/g muscle (19,86), and ignoring the effect of the P_i liberated. However, as our results demonstrate, the generation of inorganic phosphate from ATP and PCr in the homogenates has a major impact on the measured buffer capacity. For example, in the rat muscle, 68% of the buffering capacity of the homogenates at pH 7 was due to P_i, most of which is not present in the intact muscle. Thus, it seems quite possible that variable hydrolysis of high energy phosphates at some time during preparation of the homogenates is responsible for much of the variation in

buffer capacities reported using the titration method (12,18,93). In this study the muscle homogenates were incubated at 37°C, without metabolic inhibitors before titration. The high levels of phosphate measured after incubation (Table 1) were due to the near complete hydrolysis of PCr and ATP. Titration of these homogenates was extremely reproducible (Figure 2), and after correction for phosphate content, the results are in good agreement with the estimate from intact muscles.

Our results have two additional implications. First, because the non-phosphate buffer capacity, particularly of the rat muscle, is relatively low, the phosphate released by PCr hydrolysis during muscle stimulation contributes significantly to intracellular buffer capacity. This may be a physiologically important role of the creatine kinase reaction in highly glycolytic fast-twitch muscles (12,93,101). Second, the agreement between buffer capacities predicted from homogenate titrations vs. calculated from the intact muscle data confirms that CO_2/bicarbonate is not quantitatively important for intracellular buffering in muscle, as the homogenates were titrated at ambient CO_2 levels (12,68). Similarly, this agreement confirms that extracellular fluid does not make a significant contribution to buffer the capacity of muscle homogenates. As reviewed by others (93), the major contributors to the non-phosphate buffer capacity around pH 7 are probably proteins and peptides such as anserine and carnosine.

Chapter 2

Measurement of Lactate Accumulation in Contracting Skeletal Muscle Using ^{1}H Nuclear Magnetic Resonance Spectroscopy

INTRODUCTION

Contracting skeletal muscle produces lactic acid when energy demands exceed the capacity for oxidative regeneration of adenosine triphosphate (ATP). At physiological pH lactic acid dissociates producing lactate and hydrogen ions (H^+). Intracellular acidification resulting from this process is considered a primary cause of decreased force production seen in all muscle types during periods of prolonged, intense, contractile activity.

Many of the current concepts relating lactate accumulation to specific metabolic states have been formulated from data collected by measuring lactate levels in blood (61,62,134). However, blood will contain lactate contributed from all the tissues upstream from the collection site. In addition, the intracellular accumulation of lactate and its subsequent appearance in the blood are not temporally linear events. An alternative to this approach has been to freeze muscles and assay muscle homogenates for lactate concentrations. This method is hampered by the continued metabolism during both the freezing and assay procedures (3,6,31,86,120).

The use of nuclear magnetic resonance (NMR) spectroscopy with biological samples is non-destructive and non-invasive allowing serial collections of metabolic data (7,125). To date, most of the notable contributions of this technology in intact cells has resulted from measurements of phosphorous (^{31}P) and to a lesser extent carbon (^{13}C), nuclei.

Proton NMR (^{1}H) has much greater sensitivity than other biologically applicable nuclei (e.g., 16 times the sensitivity of ^{31}P-NMR). The chemical shift range of ^{1}H is much smaller than that of ^{31}P-NMR (10ppm vs. 30ppm) and the number of ^{1}H containing compounds present in NMR detectable concentrations is many times greater than those containing ^{31}P. These factors

lead to significant overlap in resonances. In particular, ^{1}H spectra from intact cells are dominated by signals arising from water and lipid protons.

Cell water is present in concentrations approaching 40mM. The signal from water saturates the digitizing capacity of spectrometers preventing resolution of signals from compounds present in 5 to 10 mM concentrations.

Several strategies have been employed to negate the signals arising from cell water. Selective excitation techniques avoid perturbation of the resting magnetization of water (7,58,125). Saturation or inversion recovery procedures minimize the magnetization of water during the observation period (7,58,119). A third approach, Spin-echo, takes advantage of the fast decay of the cell water signal relative to that of many metabolites (126). Spin-echo techniques refocus a decaying NMR signal. If the refocusing pulse occurs after the decay of the water signal subsequent acquisitions will contain information only from compounds with slower decaying signals.

The suppression of both water and lipid proton signals generally requires a combination of techniques. Spectral editing techniques have been used in muscle to observe the lactate methyl group which is otherwise obscured by the signals from the lipids present. In addition to spin-echo collections to suppress the water signal, compounds with spin coupled resonances are measured by collecting first a coupled then a decoupled spectrum. The decoupled spectrum will contain a peak which is 180° out of phase with the same peak in the undecoupled spectrum. Subtraction of the two spectra will leave just the peak which was out of phase. If the out of phase peak was obscured by lipid resonances it will now be resolved. Using this technique Williams et al were able to resolve a lactate peak as it increased during ischemia in rat muscles (126).

Recently, Meyer developed a pulse sequence which suppresses both water and lipid protons without the need for spectral editing (83). This technique employs spin-echo collections formed by continuous, frequency selective, pulsing of the spectral region of interest. Water protons are not excited and lipid proton signals have decayed before data collections are made. This steady state echo (SSECHO) method has been used to measure lactate formation in skeletal muscle (83) and heart (64).

In this study, interleaved SSECHO ^{1}H and ^{31}P-NMR collections were employed to quantitatively compare changes in pH and lactate in intact, contracting skeletal muscles. The results indicate that some portion of intracellular lactate may be bound and therefore not measurable using NMR.

METHODS

Muscle preparation Male or female cats were sedated with Ketamine (11 mg/Kg, IP) and anesthetized with pentobarbital (30 mg/Kg, IP). Anesthesia was maintained throughout the procedure by iv. administration of pentobarbital, following muscle excision euthanasia was achieved by an overdose of anesthetic.

Biceps muscles were dissected free and vascularly isolated, maintaining the arterial and venous flow of the muscle. The artery was then cannulated and perfusion begun at 0.2 to 0.4 ml/min/g. Perfusion pressure ranged from 90-130 Torr. The perfusate consisted of 116mM NaCl, 25mM $NaHCO_3$, 4.6mM KCl, 1.2mM KH_2PO_4, 2.5mM $CaCl_2$, 1.2mM $MgSO_4$, 5mM glucose, 0.15mM sodium pyruvate, 3.5% albumin, 30μg/ml papaverine chloride, and a 20% suspension of sheep red blood cells. The perfusate was passed through multiple windings of Silastic tubing in a sealed Plexiglass chamber to allow equilibration with 95% O_2/5% CO_2. Following excision each muscle was wrapped in

parafilm and mounted in the center of a Helmholz NMR coil in a specially designed NMR probe (see Appendix). The tendons of the muscle were attached to a variable length muscle mount / force transducer. Platinum stimulating electrodes were attached at each end of the muscle. Stimulation voltage and muscle length were adjusted to produce maximum twitch force. Muscle temperature was monitored by a thermistor and maintained at 37±2°C by circulating water through copper tubing in the probe.

NMR Collections The NMR probe used in these studies was equipped with a single Helmholz coil and isolated circuitry with both ^{31}P and ^{1}H channels. The NMR probe was inserted in the 7.4cm bore of a 9.4 Tesla, Oxford Instruments magnet. The resonant frequency of the coil was tuned to both 400MHz (^{1}H) and 162MHz (^{31}P) on separate circuits. Magnetic field homogeneity was shimmed on the proton circuit using the signal from muscle water, proton line width was less than 50 Hz. ^{31}P-NMR collection parameters were: Sweep width 7000Hz, 2K data set, 2.85 sec pulse interval, 16 scans per spectrum. Proton spectra result from the collection of 640 negative echo FID's with a 0.1 s pulse interval (83).

The acquired data sets for each spectrum were multiplied by an exponential function equivalent to 25Hz line broadening prior to Fourier transformation. The resultant spectral peaks were integrated by an iterative Lorentzian line fitting routine (87). Intracellular pH (pH_i) was calculated from the chemical shift of P_i relative to that of phosphocreatine (PCr).

Experimental Protocol Muscle stability (temperature, pH,P_i,PCr,ATP) was monitored for a minimum of sixty minutes under control perfusion conditions. Muscles were then stimulated at 1Hz for 6 min. ^{1}H- and ^{31}P-NMR collections were interleaved. Each collection required approximately one minute.

Calculations The effects of the increases in lactate on pH were calculated from:

I. $$pH = \Delta[Lactate] - \Delta[PCr] \times \alpha_p \; / \; ß_a$$

where α_p is the proton change resulting from net Δ[PCr] and $ß_a$ is the apparent buffer capacity (See Chapter 1) calculated from:

$$ß_a = \text{non-phosphate } ß + [P_i]$$

The nonphosphate ß and Δ_{H+} were corrected for pH using the ^{31}P-NMR measured value. Equation I. assumes a 1:1 production of H^+ to lactate. At each time point the [P_i] was calculated from the decrease in [PCr]. The $ß_a$ term takes into account the changing contribution of P_i to buffer capacity.

Lactate Binding SSECHO ^{1}H-NMR spectra (1280 scans) were collected on a 0.9% NaCl solution containing 20mM Lactic acid. Sucessive aliquots of 0.1ml 35% BSA ($\approx$0.039g) were added and spectra collected.

RESULTS

Figure 8 presents representative ^{1}H-NMR spectra from an isolated perfused cat biceps muscle before, during and after 1Hz stimulation. The increase in peak area at 1.36 PPM was assumed to be a build up of lactate resulting from contraction. The integrated areas of the lactate peaks were normalized to known resting levels. The changes in concentration of lactate and PCr are presented in Figure 9. Lactate increased and PCr decreased substantially as a result of contraction.

The open circles in Figure 10 represent the change in intracellular pH from two cat biceps muscles calculated from the observed change in chemical shift of the P_i peak. The calculated effects of the observed increase in lactate on pH are plotted as closed symbols in Figure 10.

Figure 8. ^{1}H-NMR SSECHO spectra from an isolated perfused cat biceps muscle stimulated at 1 Hz for 6 min. The peak at 1.36 PPM is assumed to be lactate.

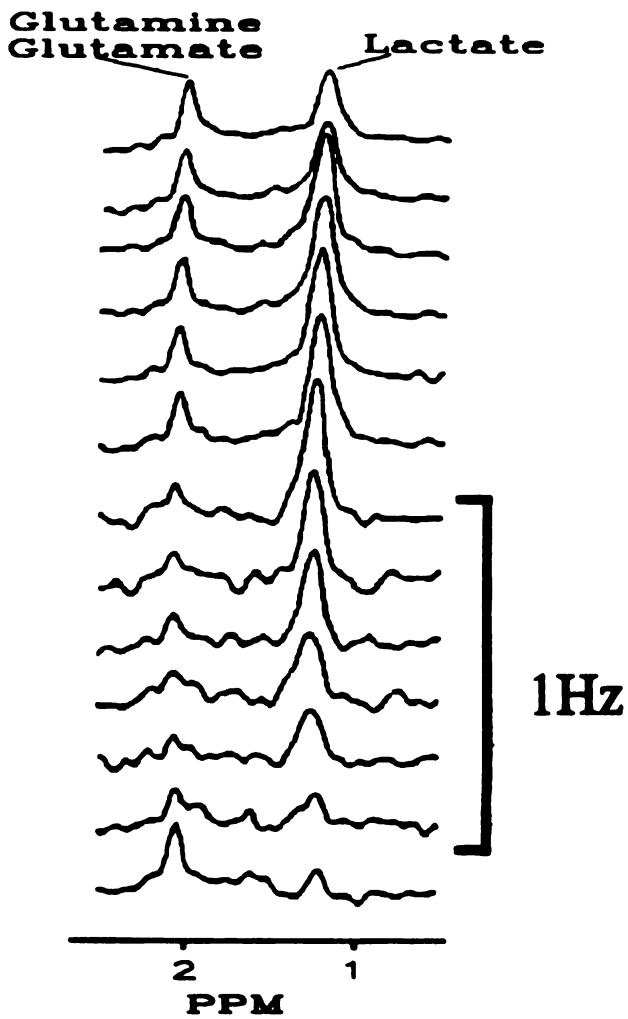

Glutamine
Glutamate
Lactate
1Hz
2
1
PPM

Figure 9. Changes in lactate (open symbols) and PCr (closed symbols) during 1Hz stimulation and recovery in two cat biceps muscles.

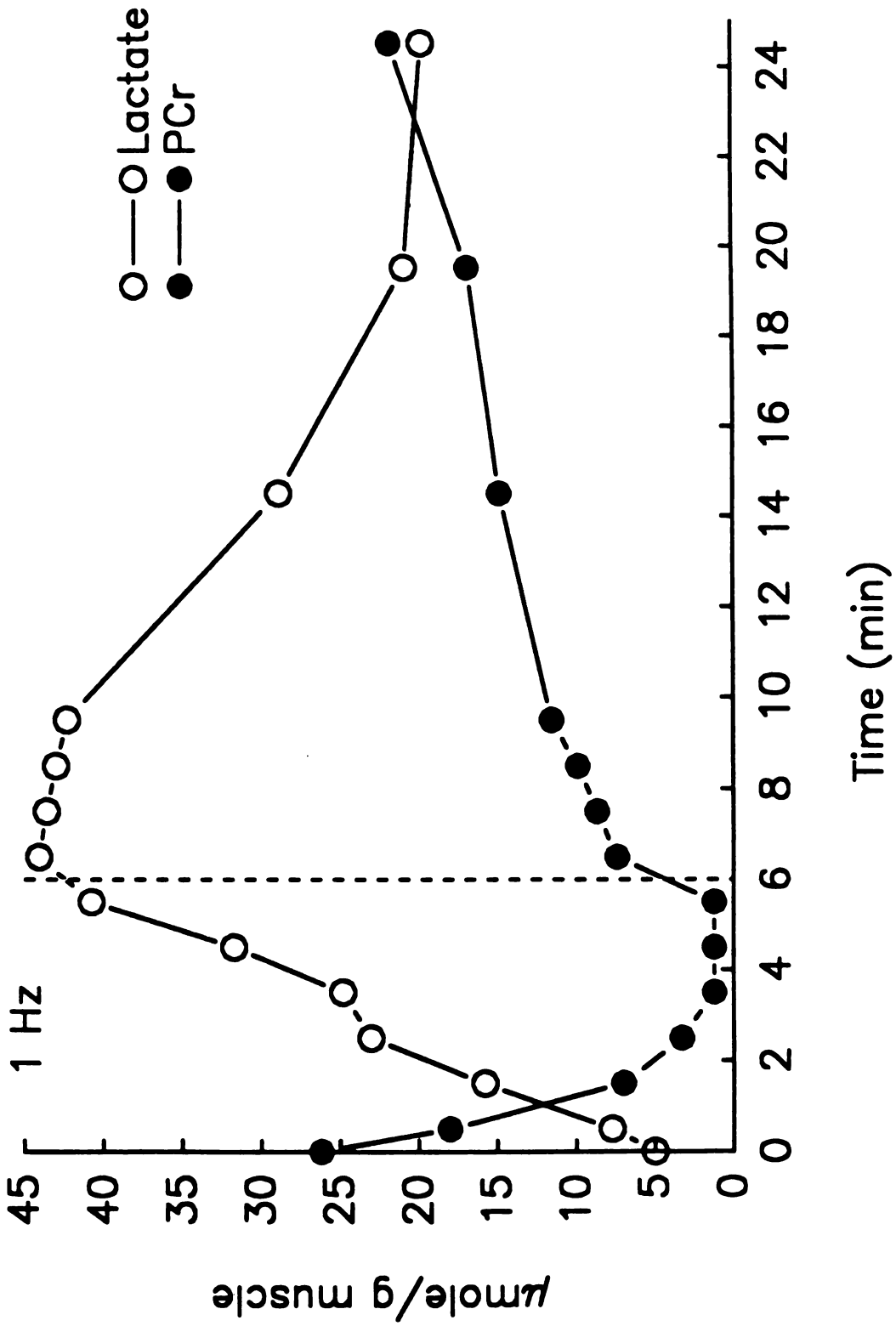
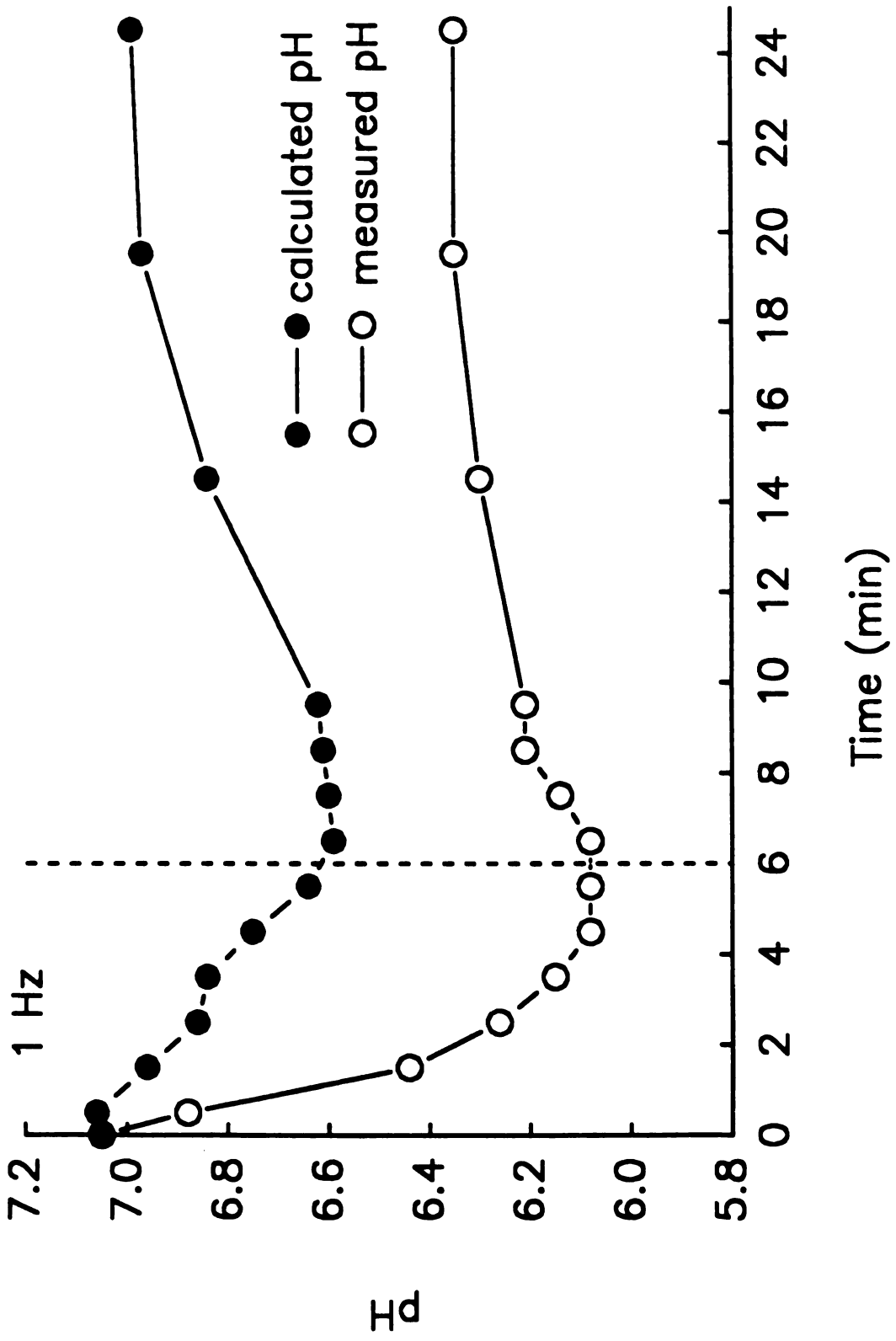
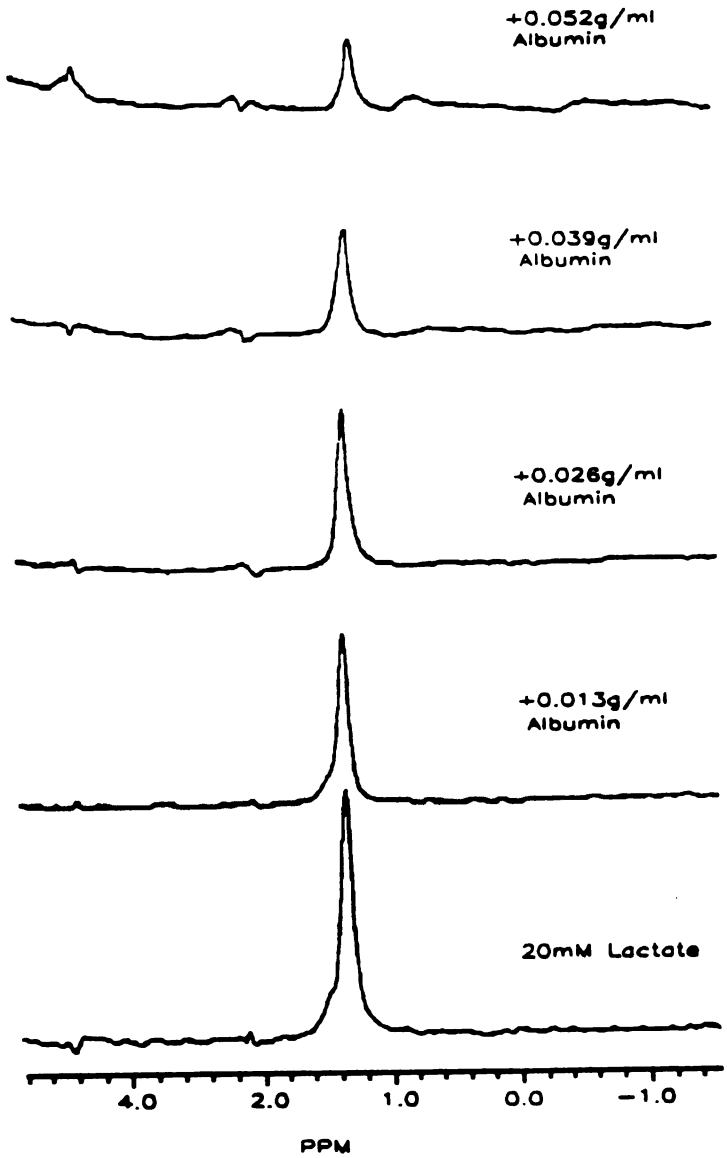

O—O Lactate
●—● PCr
1 Hz
μmole/g muscle
Time (min)
0
5
10
15
20
25
30
35
40
45
0
2
4
6
8
10
12
14
16
18
20
22
24

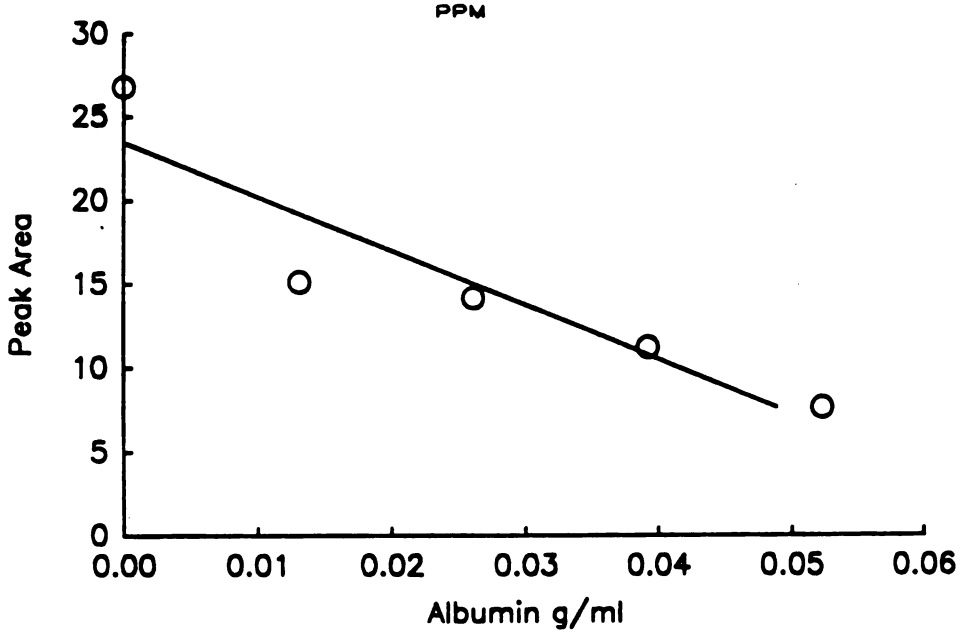
Figure 10. Intracellular pH calculated from the chemical shift of P_i in ^{31}P-NMR spectra (open symbols). Closed symbols represent the change in pH expected from a 1:1 production of H^+ by lactate accumulation measured with ^1H-NMR using the SSECHO sequence.


pH
7.2
7.0
6.8
6.6
6.4
6.2
6.0
5.8
1 Hz
calculated pH
measured pH
0
2
4
6
8
10
12
14
16
18
20
22
24
Time (min)

Spectra were collected from solutions of lactic acid, before (Figure 11A, bottom spectrum) and after the addition of 3.5% albumin (Figure 11A). In Figure 11B the peak areas from 11A are plotted against the amount of albumin present in the solution. Addition of albumin resulted in attenuation of the lactate signal.

Figure 11 A. SSECHO ^{1}H-NMR spectra of physiological saline solution containing 20mM Lactic acid (bottom spectrum) and increasing amounts of albumin. Peak at $\approx$1.3 PPM arises from the methyl protons of lactate. B. Plot of integrated peak areas from spectra in A ($r=0.92$).


+0.052g/ml
Albumin
+0.039g/ml
Albumin
+0.026g/ml
Albumin
+0.013g/ml
Albumin
20mM Lactate
4.0
2.0
1.0
0.0
−1.0
PPM


Peak Area
30
25
20
15
10
5
0
0.00
0.01
0.02
0.03
0.04
0.05
0.06
Albumin g/ml

DISCUSSION

Taken together, interleaved collection of ^{1}H and ^{31}P-NMR spectra from contracting skeletal muscle provide information on changes in the concentrations of PCr, P_i, and lactate as well as pH_i. The protocol employed in this study resulted in accumulation of lactate and significant intracellular acidosis. However, the observed lactate accumulation does not appear to be sufficient to account for the all of the change in pH_i (Figure 10). Hope et al. reported similar results in lamb brain during hypoxia and ischemia (57). These authors used spin echo collections to remove the contribution of lipid protons in the -CH_3 region. pH was measured by ^{31}P-NMR. Hypoxia-ischemia resulted in greater acidification than could be accounted for by the observed lactate accumulation.

Chang et al. conducted parallel *in vivo* and *in vitro* ^{1}H-NMR and enzymatic determinations of lactate accumulation in hypoxia and ischemic rat brains (20). Their results indicated that some of the *in vivo* lactate was not detectable.

We found that addition of albumin to a physiological salt solution containing lactate eliminated the -CH_3 lactate resonance from subsequent ^{1}H-NMR collections. This result leads us to speculate that some fraction of the lactate generated during contractions may be bound to intracellular proteins thus rendering it undetectable under our collection conditions.

Much of the utility of NMR in biological research stems from this method's relative insensitivity to nuclei in immobile compounds. However, in this instance the binding of some fraction of lactate anion to intracellular proteins would invalidate the assumption of a 1:1 relationship between NMR visible lactate and H^+ production. This would lead to an underestimation of pH_i calculated from ^{1}H-NMR measures of lactic acid accumulation. As result of

this limitation, traditional biochemical methods will have to be employed to measure lactate accumulation. Such investigations will entail the rapid freeze clamping, extraction and subsequent chemical assay of lactate concentrations.

A reasonable protocol might include the establishment of steady state lactate and P_i levels as measured by NMR. Muscles could then be rapidly removed from the magnet and frozen. The chemical assay values could then be compared to NMR measurements of these same metabolites in the extracts. Comparison of the *in vivo* and extract [P_i] would provide an indication of the degree of hydrolysis that took place during the freezing, and extraction procedures. It is possible that some known relationship between the ^{1}H-NMR visible lactate fraction and the chemically measured value might be uncovered. If so this would allow the use of ^{1}H-NMR collections with a mathematical correction to give an accurate *in vivo* concentration. Until these options are explored lactate concentrations measured by ^{1}H-NMR must be considered unreliable.

Chapter 3

Increased H^+ and $H_2PO_4^-$ do not cause fatigue in mammalian skeletal muscle.

INTRODUCTION

Repeated contraction of skeletal muscle at power outputs which cannot be sustained by aerobic metabolism results in a progressive decrease in peak isometric force (23,30,116). This decrease in force, or fatigue, is associated with decreased rates of ATP utilization (30).

Muscle fatigue at high power outputs might be of adaptive value, because it would assure the preservation of some energy reserve for maintenance of other cell functions (29). In this context, the idea that muscle force is directly regulated by specific metabolites linked to cellular energetics is attractive, and for many years attempts have been made to link muscle fatigue to changes in various metabolites (32).

Studies of skinned muscle fibers suggest that changes in hydrogen ion concentration ($[H^+]$) may modulate muscle force *in vivo*. Acidic pH is reported to decrease the calcium sensitivity of force activation (33,36), and the peak force and the maximum velocity of shortening during maximum calcium activation (21,25,82). The conductance of the ryanodine-sensitive channels responsible for calcium release from the sarcoplasmic reticulum is also strongly dependent on pH (77).

Considering these diverse effects, one might expect force in intact muscle to be profoundly depressed by intracellular acidosis. However, studies of intact muscles (30,80,89) and isolated fibers (72,124) have not established a consistent correlation between pH and force.

In the few studies which used hypercapnia to decrease pH, independent of contractile activity, force did significantly decline (79,95,102). However, the metabolic state of the superfused muscles in these studies was either not examined, or was clearly compromised (95,102).

Increased $[P_i]$ also decreases peak force in mammalian skinned fiber preparations (21,26,65). Intact skeletal muscle is known to accumulate 25-35 mmol/g

of inorganic phosphate (P_i) during sustained contraction (30,86,115). There have been several reports of a correlation between increased intracellular [P_i] and decreased force (5,30,35) in intact muscles.

In intact muscle studies the major effect of P_i on force is seen when concentrations increase above 20mM, below that level only slight depression of force is noted (29,30). In contrast, skinned fiber preparations are sensitive to increasing [P_i] up to ≈ 15mM at which point force levels off (5,26,65).

Dawson (29) and Wilkie (94) have suggested that the effects of both pH and P_i reflect direct inhibition of cross-bridge formation by the diprotonated form of P_i. While this proposal was supported by the results of Nosek, et al (91), other skinned fiber studies have not found a significant interaction between the effects of pH and P_i on force (21,26,65). Several NMR studies of human muscle have found a good correlation between diprotonated phosphate ($H_2PO_4^-$) and force during a fatiguing series of voluntary contractions in normal subjects (16,17,89,127).

The purpose of this study was to examine the relationships between intracellular pH and/or diprotonated phosphate vs. force in perfused cat fast- and slow-twitch muscles. Intracellular pH was altered both by hypercapnia, and by fatiguing series of contractions. Results indicate that increases in H^+ and $H_2PO_4^{1-}$ can be produced with no decrease in peak tetanic tension development.

METHODS

Muscle preparation. Adult male cats were sedated with Ketamine-HCl (11mg/kg, ip) and anesthetized with pentobarbital (30 mg/kg, ip), with subsequent iv doses of pentobarbital administered as indicated. Biceps brachii or soleus muscles were vascularly isolated and dissected free as described previously (86). The artery was cannulated (PE50) and perfused at 0.2-0.4ml/min/g (perfusion pressure 80-100 Torr)

with a 20% suspension of sheep red blood cells in bicarbonate buffered Krebs-Henseleit solution containing 3.5% bovine serum albumin, 5mM glucose, 0.15mM Na pyruvate, 30μg/ml papaverine HCl, 10μg/ml gentamycin sulfate, and 10mM Na phenylphosphonate (PPA), a phosphorus NMR detectable indicator of extracellular pH (86). Perfusate was initially equilibrated with 5%CO_2/95%O_2 at 37°C in a silastic tube oxygenator.

The perfused muscles were wrapped in Parafilm and mounted in a 1.5 cm diameter Helmholtz configuration coil within a custom-made 7.4 cm diameter NMR probe (see Appendix). The distal tendon was clamped to a fixed support under the coil and the proximal tendon (or in the case of the soleus, the excised head of the tibia (86)) was clamped to a custom-made cantilever beam force transducer mounted on an adjustable frame at the top of the probe. Probe temperature was monitored by a thermistor and maintained at 37$\pm$2°C by circulating water through copper tubing in the probe. Muscles were stimulated directly via platinum wire electrodes wound around the tendons. Muscle length was adjusted to produce maximum isometric twitch tension in response to a single supramaximal pulse (50-70V, 1 ms). Addition of 50μM succinylcholine, a neuromuscular blocker, to the perfusate had no effect on peak twitch or tetanic force.

NMR methods. The probe was inserted in the 7.5 cm bore, 9.4T magnet of a Bruker AM400 spectrometer, and tuned to phosphorus (162 MHz) or proton (400 MHz) resonance via two independent switch-selectable circuits. The magnet was shimmed on the proton signal of muscle water to a linewidth of 40-60 Hz. The 90° pulse width for phosphorus was estimated for each muscle at the start of the experiment. Phosphorus NMR spectra (sweep width 10,000Hz, 2K data, 15 sec pulse interval, 4 or 8 scans, corresponding to 1 or 2 min averages, for biceps and soleus, respectively) were continuously acquired throughout the experiment. FID's were zero-filled to 4K and multiplied by an exponential corresponding to 25Hz linebroadening prior to Fourier

transformation. Peaks were integrated by an iterative Lorentzian fitting routine (87). Intracellular metabolite concentrations were estimated from peak integrals by assuming P_i+PCr concentrations of 38.1 and 26.7 mM for biceps and soleus muscles, respectively (86). Intracellular (pH_i) and extracellular pH (pH_e) were estimated from the chemical shifts (δ, ppm) of inorganic phosphate and phenylphosphonate, respectively, as follows (86):

$$pH_i = 6.75 + \log (0.89-\delta)/(\delta-3.19)$$

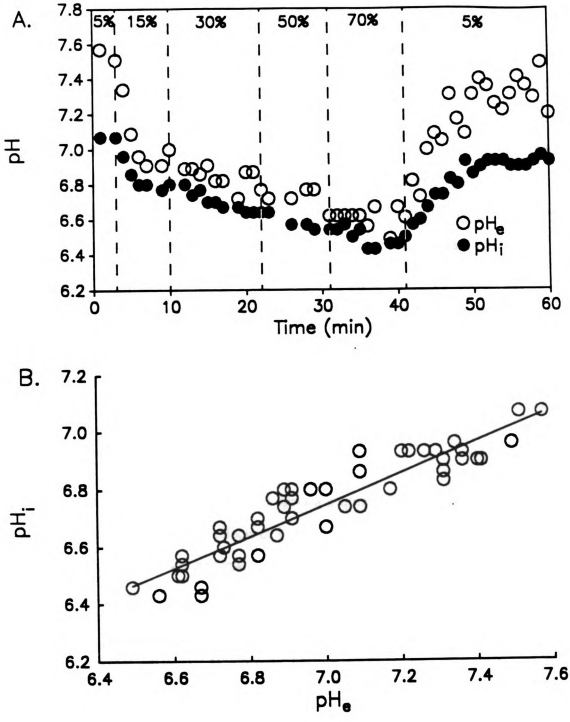
$$pH_e = 7.02 + \log (13.9-\delta)/(\delta-11.8)$$

The PCr peak (2.52 ppm) was used as a reference. Diprotonated phosphate content was calculated from P_i and intracellular pH assuming pK=6.75, and neglecting the small contributions of PO_4^{3-} and H_3PO_4.

Experimental protocol. Preliminary experiments demonstrated that graded increases in CO_2 content in the perfusate gas resulted in graded decreases in both intracellular and extracellular pH (Figure 12A). There was a linear relationship between extracellular and intracellular pH during hypercapnia (Figure 12B), but no significant decrease in PCr or P_i content during perfusion at CO_2 contents up to 70%. Based on these results, 70%CO_2 was chosen for the study.

Each muscle was perfused under control conditions (37°C, 5%CO_2 /95%O_2) for a minimum of 1 hour to establish metabolic and mechanical stability. The perfusate gas was then switched to 70%CO_2/30%O_2 and phosphorus spectra monitored until extracellular and intracellular pH approached a new steady-state, typically within 30 min. Mechanical response to several test twitches and tetani (0.5 sec @ 100 Hz for biceps, 1.5 sec @ 20Hz for soleus) applied a minimum of 5 min apart were then recorded.

Figure 12. A. The effect of perfusate equilibration with increasing percentages of CO_2 (balance O_2) on extracellular (open symbols) and intracellular (closed symbols) pH in an unstimulated biceps muscle. Figure 12B shows the relationship between extra- and intracellular pH in this muscle.


A.
5%
15%
30%
50%
70%
5%
pH
Time (min)
pHe
pHi
B.
pHi
pHe

In order to increase P_i content, the muscle was then repetitively stimulated at moderate rates, e.g., 0.1 Hz for biceps and 1 Hz for soleus, for 3-5 min. These relatively mild twitch stimulation regimens were selected on the basis of preliminary experiments which showed that they resulted in significant PCr hydrolysis and Pi accumulation without any additional metabolic acidification. Phosphorus spectra were continuously monitored during this hypercapnic stimulation period and the stimulation was stopped when PCr level was reduced by over 50% relative to prestimulation level. Additional test tetani were recorded after the hypercapnic stimulation. The perfusate gas was then returned to $5\%CO_2/95\%O_2$ and phosphorus spectra monitored until intracellular pH approached the initial $5\%CO_2$ level. Following several additional test contractions, the muscles were repetitively stimulated at 2 (biceps) or 6 (soleus) tetani/min for 10-15 min. Flow to the soleus muscles was then stopped and these muscles were stimulated again at 6 tetani/min until peak tetanic force had decreased by over 50%.

Values of $p<0.05$ were considered significant for regression coefficients and comparisons of means.

RESULTS

Figure 13 shows sample phosphorus spectra acquired from representative muscles during the protocol. The effects of hypercapnic perfusion on pH and phosphate metabolite levels are summarized in Table 4. The observed relationship between intra- and extracellular pH was similar to that measured with recessed-tip pH-sensitive microelectrodes (3). Intra- and extracellular pH decreased by over 0.5 pH units in both muscle types during hypercapnic perfusion. There was no significant change in PCr or P_i contents in either muscle. Figure 14 shows sample isometric force records from normal and hypercapnic perfusion. There was no significant effect of hypercapnia on peak tetanic force in either muscle type (Table 5). However,

hypercapnia resulted in significant increases in both the rise and relaxation times of tetani in the soleus, and of relaxation time in the biceps. The peak twitch/tetanus ratio was significantly reduced in biceps, and a similar trend was evident in the soleus.

Figures 15 (biceps) and 16 (soleus) show the time course of changes in PCr, P_i, $H_2PO_4^{1-}$, ATP, pH, and peak tetanic force during the final periods of repetitive stimulation during normocapnic perfusion. In biceps muscles there was a steady decrease in PCr, intracellular pH, and force throughout the stimulation period. In contrast, PCr and force rapidly approached steady-states in the perfused soleus muscle, despite the 6-fold greater tetanus train rate and 18-fold greater duty cycle of contraction applied to this muscle. This result is consistent with the higher aerobic capacity and lower myosin ATPase activity in slow vs. fast-twitch muscle (5). However, during ischemic stimulation PCr and force rapidly declined in soleus muscles, and pH decreased to levels similar to that achieved during hypercapnia.

Considering only the data from the final repetitive stimulation periods, there were significant correlations between both intracellular pH and diprotonated phosphate levels vs. peak tetanic force in both muscle types (Figures 17,18 open symbols). For example, in biceps and soleus muscles the correlation coefficients for force vs. pH were 0.95 and 0.91, respectively. However in both cases the relationships were very different during hypercapnia.

Figure 13. Representative ^{31}P-NMR spectra of biceps (left) and soleus (right) muscles during the experimental protocols. Each spectrum is an average of 4 (biceps) or 8 (soleus) scans with 15s pulse interval. PPA designates the phenylphosphonate peak. The vertical lines under P_i and PPA indicate the chemical shifts of these peaks at rest with 5% CO_2.

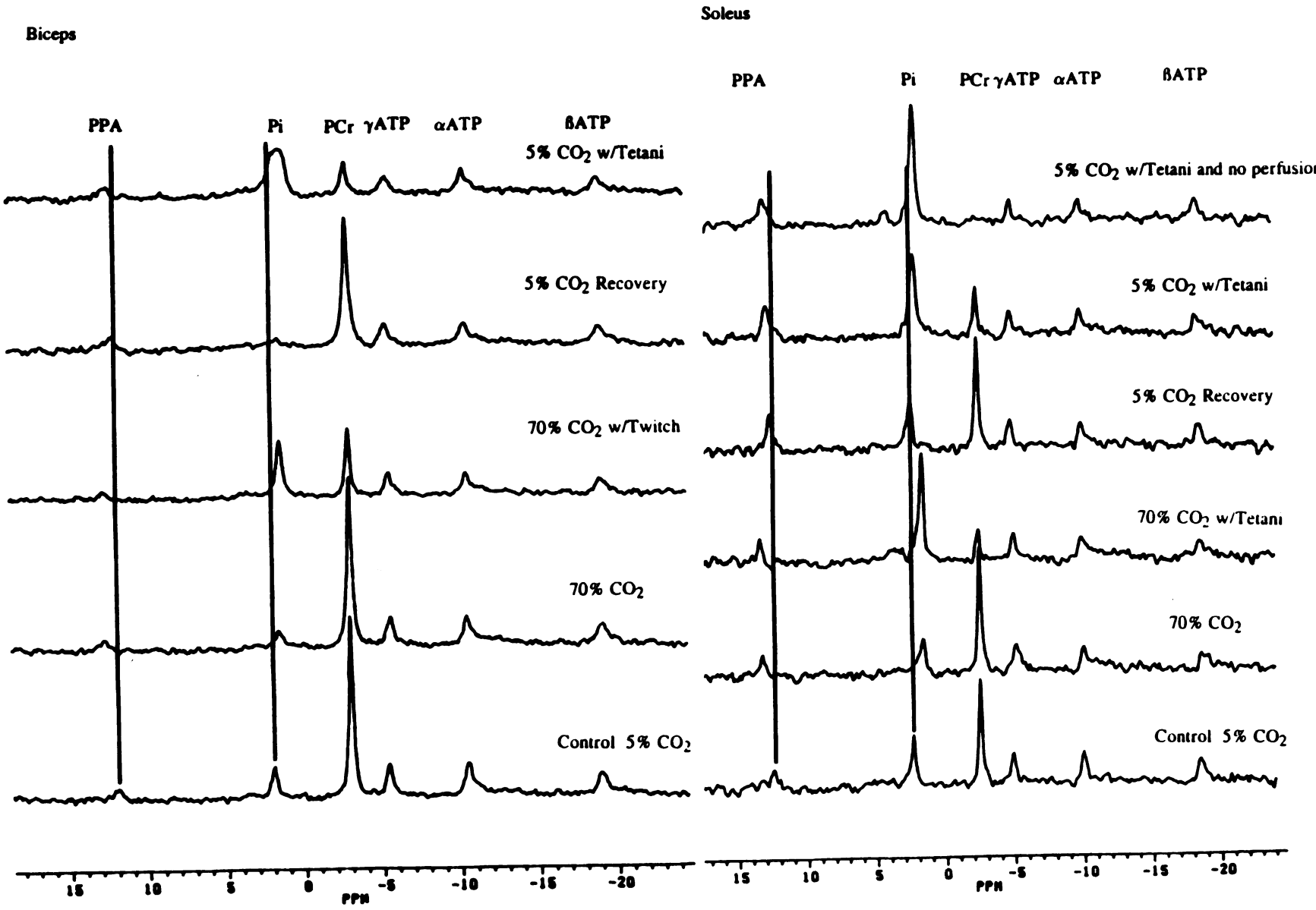

Biceps
PPA
Pi
PCr
γATP
αATP
ßATP
5% CO_2 w/Tetani
5% CO_2 Recovery
70% CO_2 w/Twitch
70% CO_2
Control 5% CO_2
15
10
5
0
-5
-10
-15
-20
PPM
Soleus
PPA
Pi
PCr
γATP
αATP
ßATP
5% CO_2 w/Tetani and no perfusion
5% CO_2 w/Tetani
5% CO_2 Recovery
70% CO_2 w/Tetani
70% CO_2
Control 5% CO_2
15
10
5
0
-5
-10
-15
-20
PPM

TABLE 4.

pH and Metabolite concentrations (x±SE, n=4).

	Biceps		Soleus	
	Normocapnia	Hypercapnia	Normocapnia	Hypercapnia
pH_e	7.42±0.05	6.73±0.07 *	7.42±0.01	6.73±0.05 *
pH_i	7.08±0.02	6.53±0.02 *	7.05±0.02	6.38±0.02 *
P_i (mM)	7.6±1.0	4.7±0.1	10.7±0.4	10.6±0.3
PCr(mM)	30.5±1.0	33.4±0.1	16.0±0.4	16.1±0.3
ATP(mM)	9.2±0.6	9.4±1.0	6.1±0.5	5.8±0.08

(* significant difference between normo- and hypercapnia by paired Student's t-test, $p<0.05$)

Figure 14. Sample force records of test tetani during 5% and 70% CO_2 perfusion in soleus (left) and biceps (right) muscle. Small deflections in the soleus record are stimulus artifact.

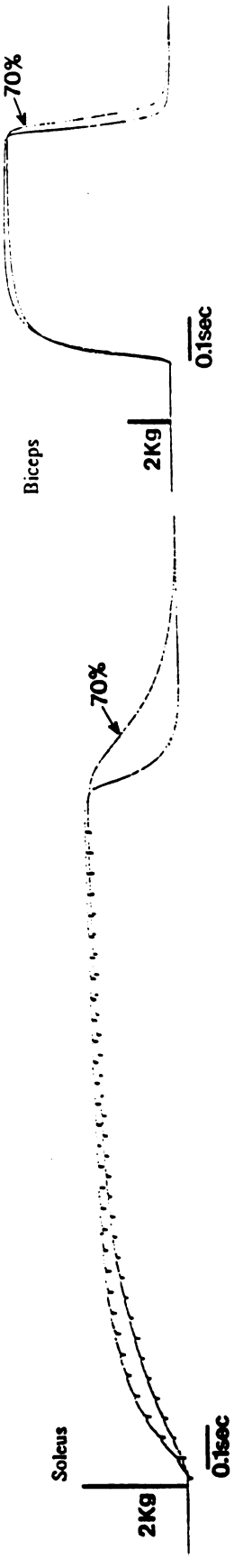

70%
Biceps
2Kg
0.1sec
70%
Soleus
2Kg
0.1sec

TABLE 5

Contraction characteristics (x±SE, n=4)

	Biceps		Soleus	
Final muscle weight (g)	10.4±1.1		6.0±0.2	
	Normo-	Hypercapnia	Normo-	Hypercapnia
Tetanic:				
Rise Time (10-90%, ms)	110±40	120±30	430±80	760±20 *
Relaxation Time (90-10%,ms)	50±10	70±20 *	200±50	540±200 *
Peak Force (g/g muscle)	789±66	793±54	369±39	384±37
Twitch:			(soleus n=3)	
Time to Peak Force (ms)	15±2	26±3 *	111±7	119±12
Time to Half Relax (ms)	21±2	26±2 *	110±11	141±21
Twitch/Tetanus Force Ratio	0.25±.01	0.17±.01 *	0.27±.01	0.14±.03

(* significant difference between normo- and hypercapnia by paired Student's t-test, $p<0.05$)

Figure 15. Time course of force and metabolite changes during final repetitive tetanic stimulation (2 tetani/min) with 5% CO_2 perfusion in cat biceps muscles ($x \pm SE$, $n=4$).

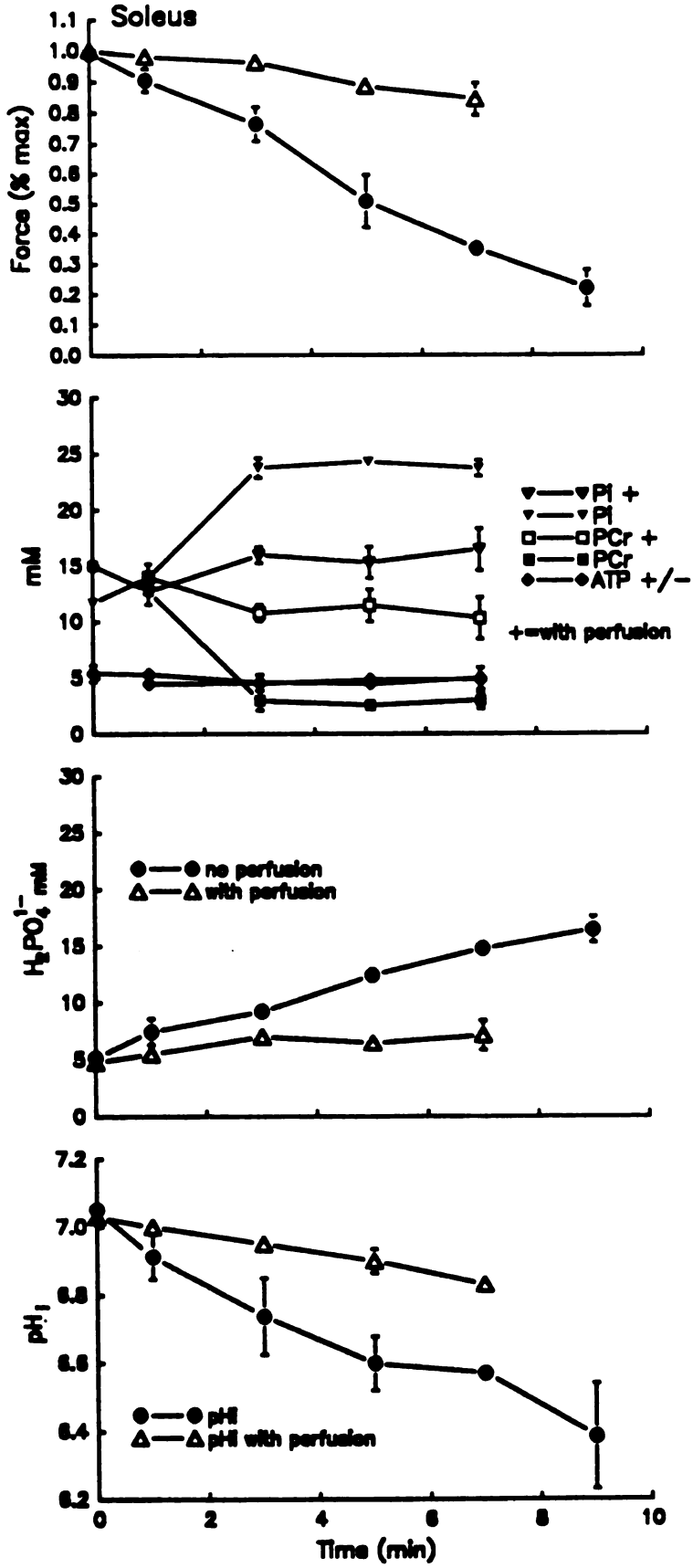

Soleus
Force (% max)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
mM
30
25
20
15
10
5
0
Pi +
Pi
PCr +
PCr
ATP +/-
+=with perfusion
$H_2PO_4^{1-}$ mM
no perfusion
with perfusion
pH_i
7.2
7.0
6.8
6.6
6.4
6.2
pHi
pHi with perfusion
0
2
4
6
8
10
Time (min)

Figure 16. Time course of force and metabolite changes during final repetitive tetanic stimulation (6 tetani/min) with 5% CO_2 perfusion (open symbols) and no perfusion (closed symbols) in cat soleus muscles ($x \pm SE$, $n=4$).

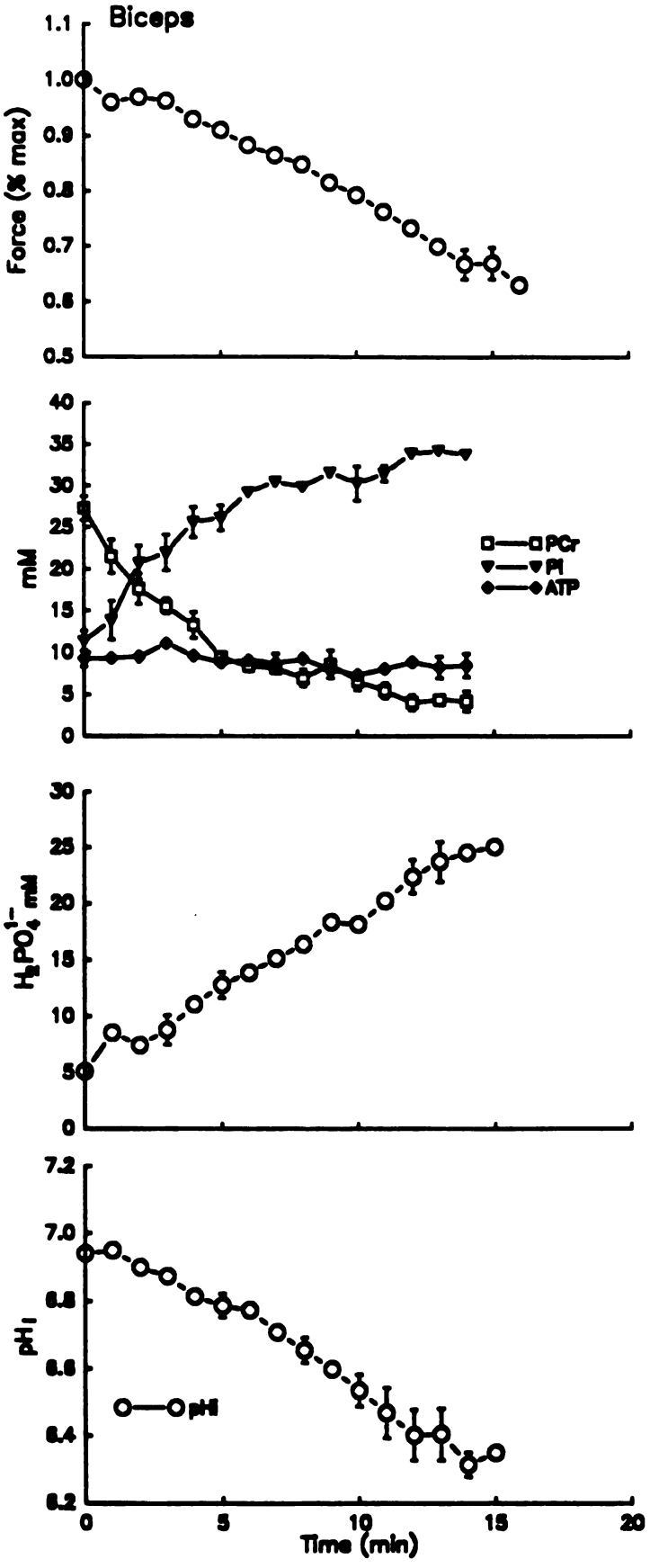

Biceps
Force (% max)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
mM
40
35
30
25
20
15
10
5
0
PCr
Pi
ATP
$H_2PO_4^{1-}$, mM
30
25
20
15
10
5
0
pH_i
7.2
7.0
6.8
6.6
6.4
6.2
pHi
0
5
10
15
20
Time (min)

Figure 17. Relationship between peak tetanic force and intracellular pH in cat biceps (A) and soleus (B) during 5% CO_2 (open symbols) and 70% (closed symbols) perfusion. Pooled results from 4 muscles of each type.

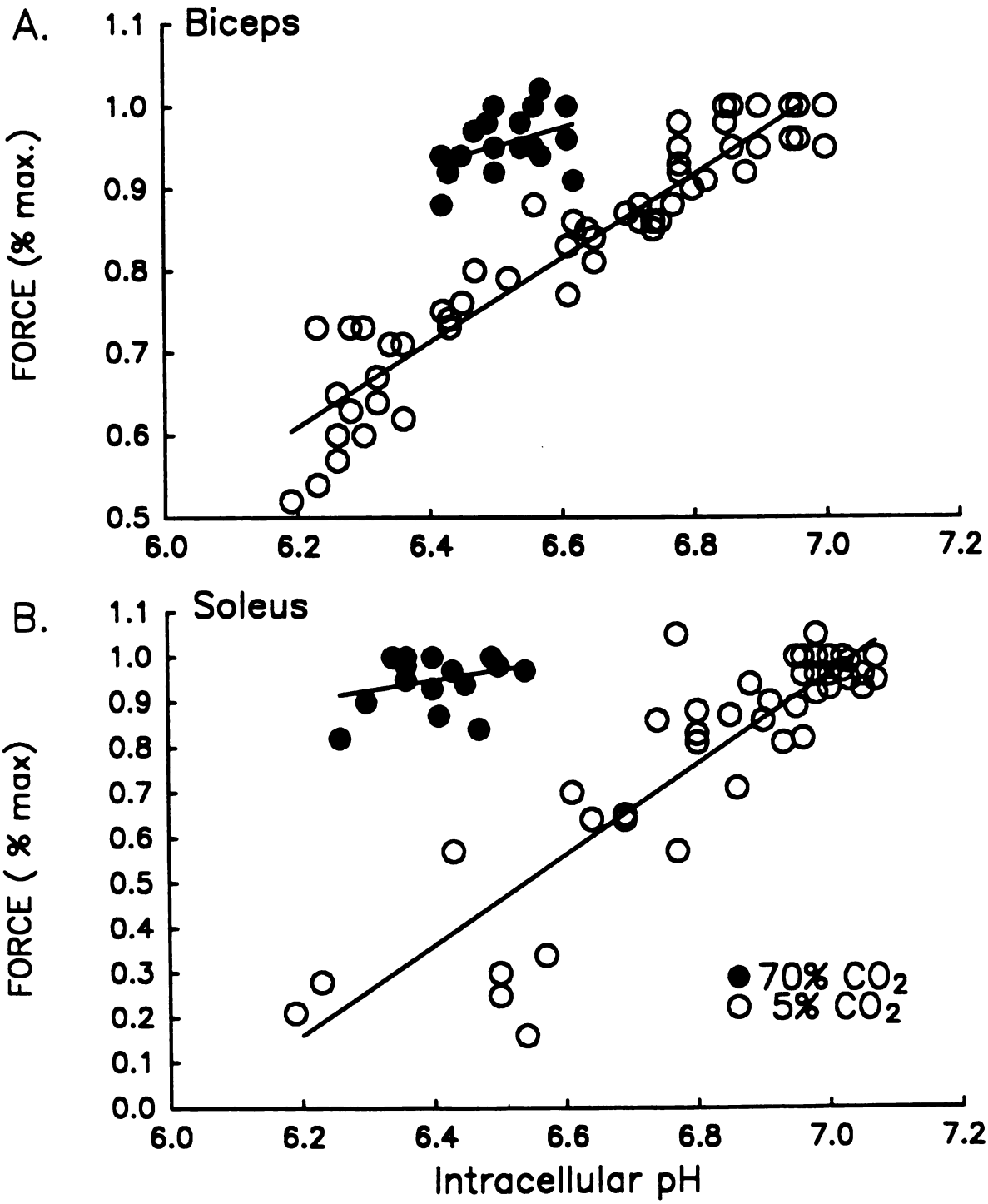
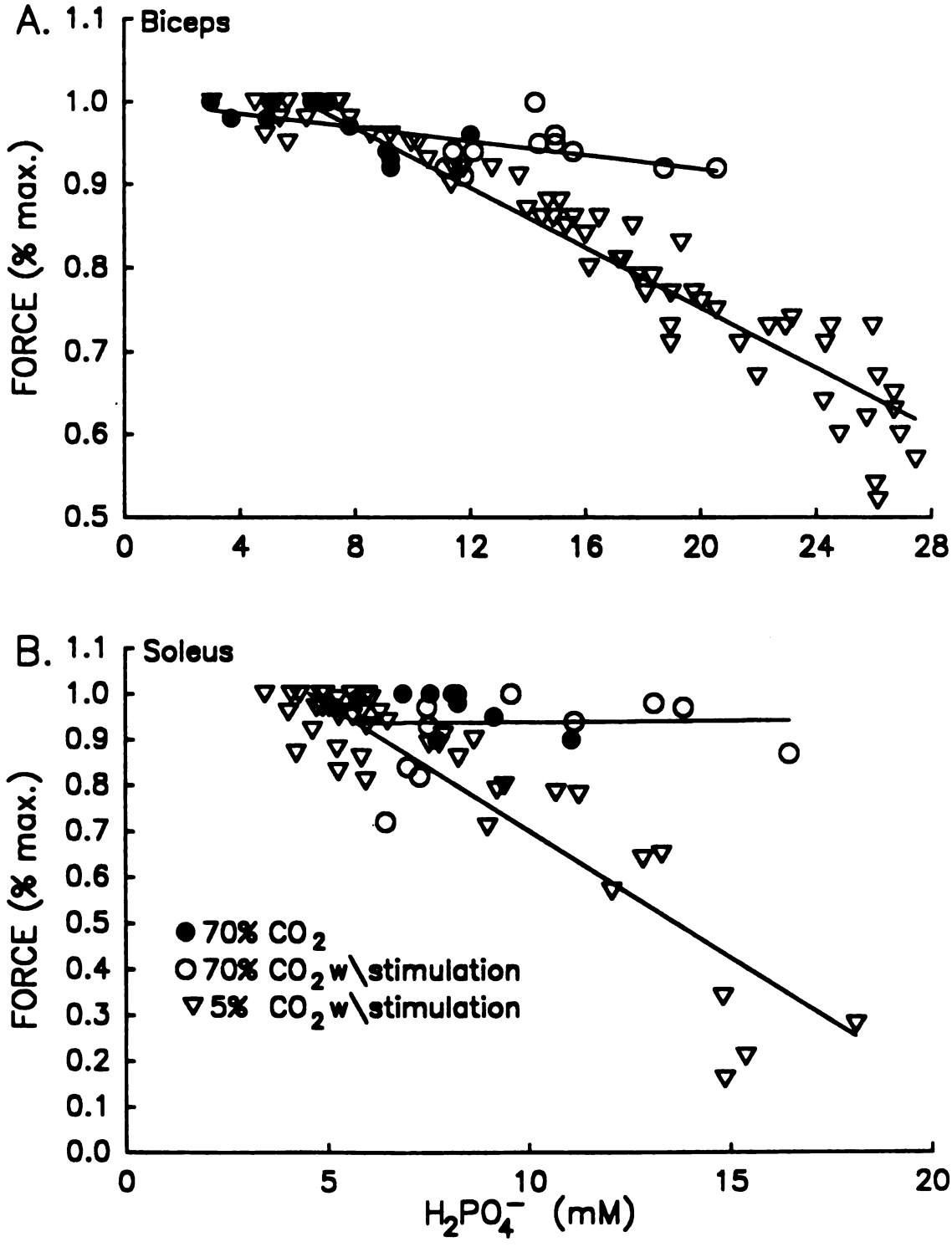

A.
Biceps
FORCE (% max.)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
6.0
6.2
6.4
6.6
6.8
7.0
7.2
B.
Soleus
FORCE (% max)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
6.0
6.2
6.4
6.6
6.8
7.0
7.2
Intracellular pH
70% CO2
5% CO2

Figure 18. Relationship between peak tetanic force and $[H_2PO_4^{1-}]$ in cat biceps (A) and soleus (B) during 5% CO_2 (triangles) or 70% (circles) perfusion. Filled circles are results from test tetani recorded before P_i was increased by stimulation. Open circles are results following stimulation to increase P_i. Pooled results from 4 muscles of each type.


A.
Biceps
FORCE (% max.)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0
4
8
12
16
20
24
28
B.
Soleus
FORCE (% max.)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
● 70% CO2
○ 70% CO2 w\stimulation
▽ 5% CO2 w\stimulation
0
5
10
15
20
H2PO4− (mM)

There was no significant correlation between intracellular pH or diprotonated phosphate and peak tetanic force during hypercapnia in either muscle. In both muscles, the relation between P_i content and force was biphasic (Figure 19), with little decrease in force up to 20 mM, and a precipitous drop in force above 25 mM. Hypercapnia had no obvious effect on this relationship. Finally, there was a significant correlation between tetanus relaxation time and intracellular pH during both normal and hypercapnic perfusion (Figure 20).

Figure 19. Relationship between peak tetanic force and P_i in cat biceps (A) and soleus (B) during 5% CO_2 (open symbols) or 70% CO_2 (closed symbols) perfusion. Pooled results from 4 muscles of each type.

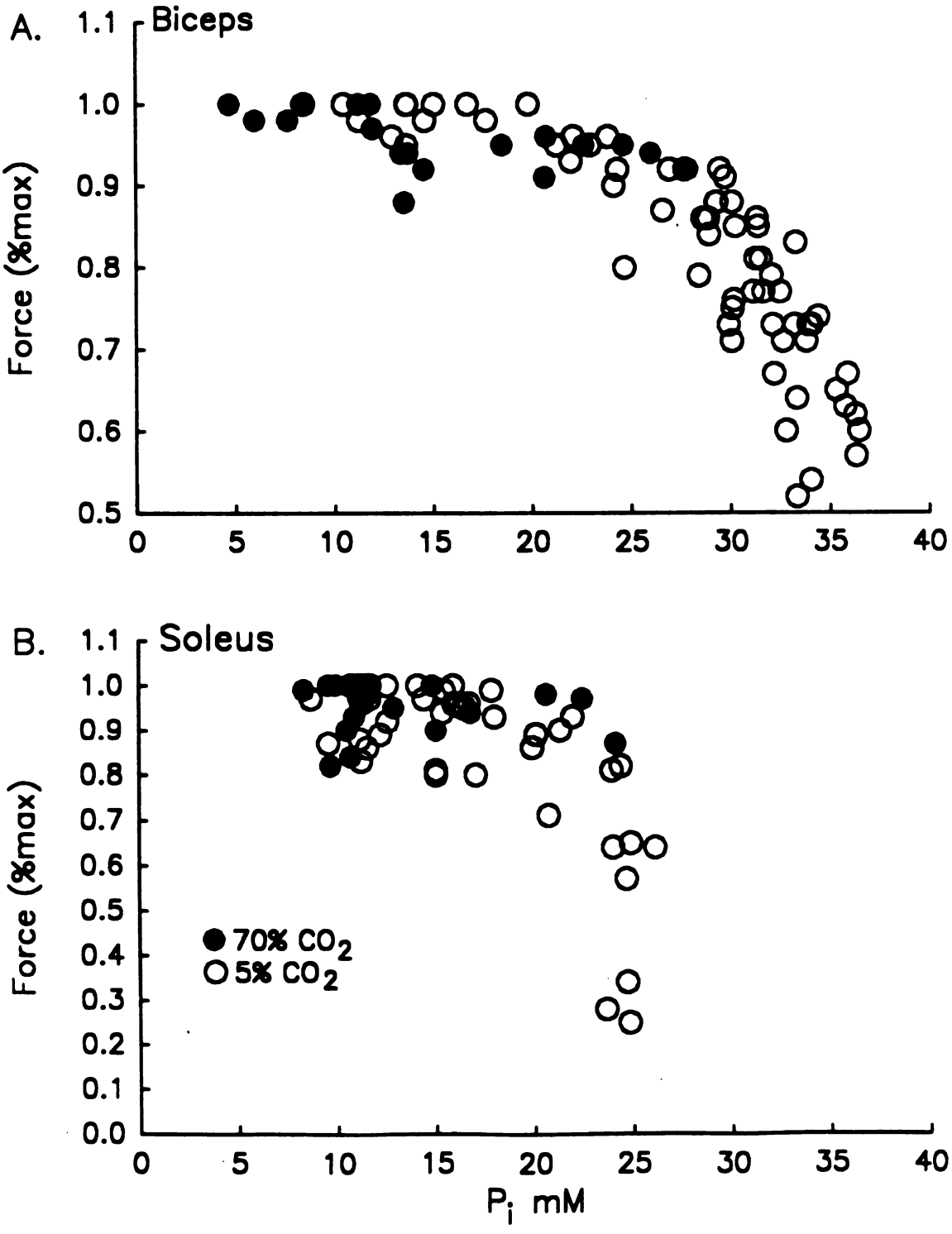
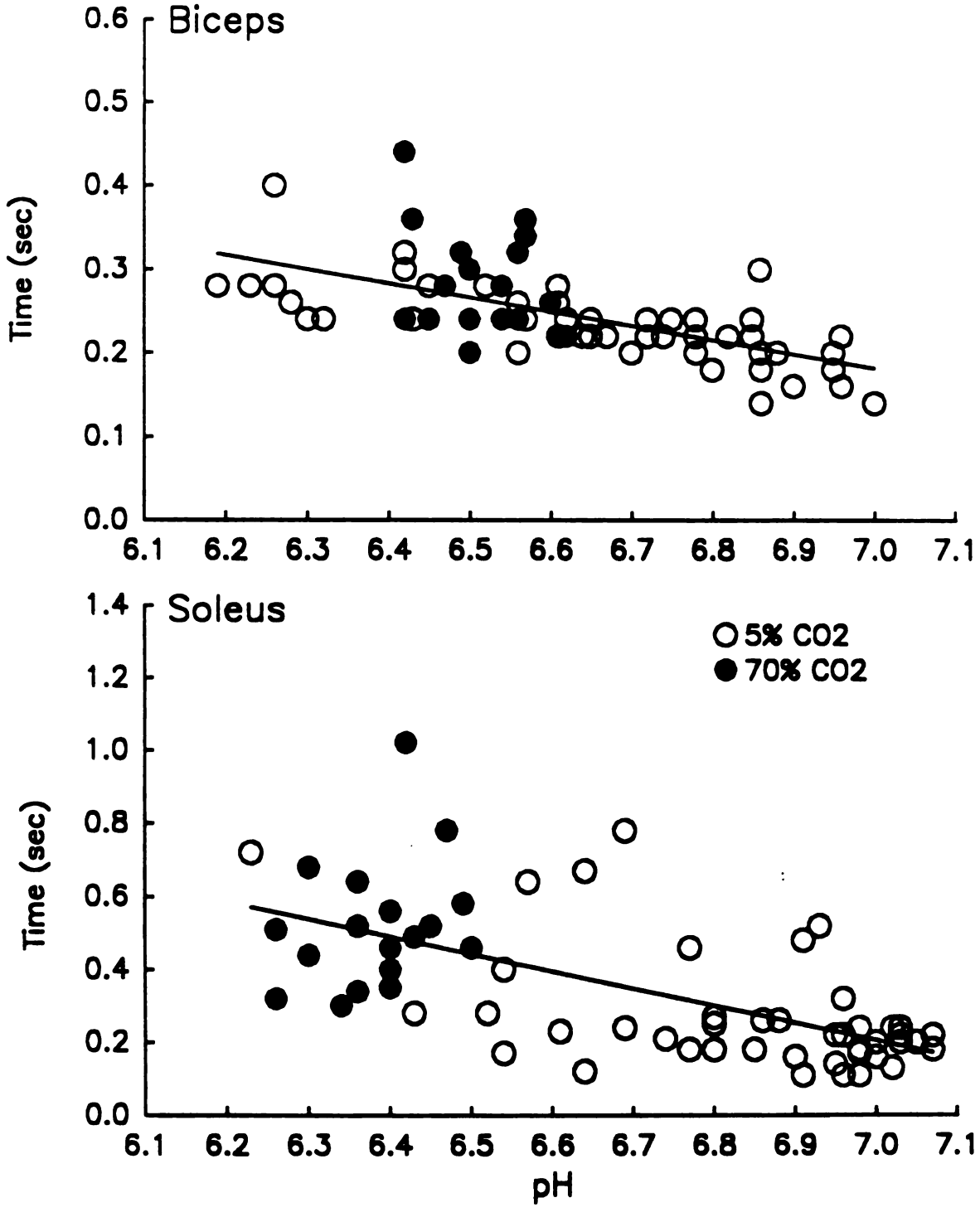

A.
Biceps
Force (%max)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0
5
10
15
20
25
30
35
40
B.
Soleus
Force (%max)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
70% CO2
5% CO2
0
5
10
15
20
25
30
35
40
Pi mM

Figure 20. Relationships between pH and time to relax from 90 to 10% of peak tetanic force in cat biceps (A, $r=0.63$, slope$=-0.17$, $p<0.05$) and soleus (B, $r=0.63$, slope$=-0.48$, $p<0.05$) muscles during 5% CO_2 (open symbols) and 70% CO_2 (closed symbols) perfusion. Pooled results from 4 muscles of each type.


Biceps
Time (sec)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
6.1
6.2
6.3
6.4
6.5
6.6
6.7
6.8
6.9
7.0
7.1
Soleus
5% CO2
70% CO2
Time (sec)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
pH

DISCUSSION

The results of this study demonstrate that neither pH nor diprotonated phosphate levels consistently correlate with peak tetanic force in either fast- or slow-twitch muscles. There were significant correlations between either pH_i or $[H_2PO_4^{1-}]$ and force only when these parameters were changed by contractile activity. Independent manipulation of pH_i and $[H_2PO_4^{1-}]$ had no effect on peak force. The simplest interpretation of these results is that decreased peak tetanic force, or fatigue, during repetitive stimulation is not directly due to either increased hydrogen ion or $H_2PO_4^{1-}$, but to some other effect of contractile activity.

An alternative explanation of these results might be that the intracellular acidification induced by metabolic activity during repetitive contraction is somehow fundamentally different from that induced by hypercapnia. It might be argued that there are subcellular compartments in which pH is modified differently by metabolic activity than by hypercapnia. The data from this study provides no evidence for such compartmentation. The P_i observed by NMR, which is used to estimate pH, is almost certainly in free solution within the same compartment as the myofibrillar apparatus (5,86). In both muscle types a single, narrow, P_i peak was observed when pH_i was lowered by hypercapnia. This peak increased without splitting during stimulation. Significant accumulation of P_i in a compartment with higher pH would have produced a second or split peak.

Measured functional responses also argue against compartmentation of H^+. Reductions in pH due to hypercapnia did modify the rise and relaxation times of contractions in both muscle types. This correlation of pH and relaxation time has been reported in other studies (16,30). Thus, the pH change induced by hypercapnia did impact aspects of the activation process which are

presumably cytosolic. The changes observed are consistent with reported inhibition of calcium release (77) and sequestration mechanisms (60), and decreased maximum velocity of shortening (21), by acidosis.

Another conceivable explanation for the differing effect of hypercapnic vs. metabolic acidosis is that lactate rather than hydrogen ion is the cause of the decrease in force during repetitive stimulation. However, Chase and Kushmerick have shown that lactate per se has no effect on force in skinned fibers (21).

The results in both the cat biceps and soleus are consistent with reports that P_i accumulation contributes to a decline in peak force (5,21,30,65). Further testing of the role of P_i must await development of a method to independently manipulate P_i levels in intact muscle.

Our results are difficult to reconcile with the consistent relationship between pH and peak force seen in skinned fiber studies. (21,25). Ignoring the additional effect of acidosis on calcium sensitivity (33,36), a pH change from 7 to 6.4 might be expected to decrease force by at least 20% in slow-twitch fibers, and 30% in fast twitch fibers (21,25).

In skinned fiber preparations, force development is most sensitive to increases in P_i from 1 to 15mM (26,65). In the current study there was no relationship between force and P_i levels below 20mM P_i. This result is similar to that seen in previous studies of intact muscles (29,30). Assuming that cat muscle is not uniquely resistant to acidosis and intracellular phosphate, our results suggest that the contractile behavior of intact muscle cannot be directly extrapolated from studies of skinned fibers.

In contrast to our results, hypercapnia caused significant reductions in force in superfused frog (95) and rat muscles (102). However, in the latter case hypercapnia was also associated with decreased PCr content, an effect attributed

to a shift in the equilibrium of the creatine kinase reaction (102). In the current study hypercapnia had no significant effect on PCr levels provided that perfusate flow was maintained sufficient for oxygen delivery to the tissue. The decreases in both PCr and force observed in superfused muscles during hypercapnia may reflect reduced oxygen availability rather than an pH effect.

Ours is not the first study to show a dissociation between acidosis and fatigue. The inverse of our results, i.e., fatigue in the absence of acidosis, has been reported in several studies of patients with McArdle's disease (myophosphorylase deficiency) (17,73,74,98). Ross et al found no acidosis in a McArdle's patient after 45 sec of ischemic exercise to exhaustion (98). Control subjects sustained the exercise for several minutes, despite a decrease in intracellular pH from 7.1 to 6.4. This clearly indicates that intracellular acidosis is not an obligatory factor in muscle fatigue. The more rapid PCr hydrolysis and greater P_i accumulation in exercised muscle of McArdles vs. control subjects suggests that P_i accumulation is a factor in fatigue in these patients (73).

The current results do not imply that pH plays no role in the more general phenomenon of fatigue during exercise. Acidosis was associated with changes in contraction times and twitch/tetanus ratio, which could have a profound effect on muscle performance in an intact organism.

The current study was limited to short bursts of intense contractile activity during hypercapnic acidosis. Prolonged repetitive stimulation may result in decreased Ca^{2+} release uncovering an inhibitory effect of acidosis on activation. In addition, decreased pH may inhibit glycolysis (110) or otherwise decrease substrate supply for aerobic metabolism. This could indirectly limit force production during a more prolonged series of contractions. Acidosis may also delay recovery of force after a fatiguing series of contractions (114).

In summary, the results of this study show that peak force development in intact cat muscle is relatively insensitive to decreased pH and increased diprotonated P_i induced by hypercapnia. This indicates that neither acidosis nor increased concentration of $H_2PO_4^-$ represents an necessary condition for muscle fatigue. The correlations between pH or diprotonated phosphate and peak force observed in this and other studies of intact muscle are apparently coincidental to some other effect of repetitive contraction.

Summary and Conclusions

Summary

Changes in the intracellular concentrations of lactic acid, H^+, P_i, $[H_2PO_4^{1-}]$ have been suggested as the causal factors in cellular level fatigue in contracting skeletal muscle. The experiments described in this dissertation were designed to characterize both changes in these metabolites and related processes which modulate the effects of those changes.

In chapter 1 a working method of measuring intracellular buffer capacities was established. An important concept developed in this chapter was the dynamic character of this parameter (ß). Studies employing calculations based on a single ß value spanning a period of muscle contraction will not accurately reflect the intracellular pH. Under these conditions the intrinsic ß and the current $[P_i]$ must be used to calculate the effective ß and pH. The intrinsic ß values for the muscles used in this study were identified for the entire physiologically relevant range. These values can be used in future studies for metabolic calculations.

The results reported in chapter 2 present a cautionary note for future attempts at quantifying intracellular lactate using ^{1}H-NMR. Although the equipment and pulse sequences used allow measurement of lactate, results suggest that some fraction of this compound may be bound and therefore NMR invisible. There have been many studies reporting ^{1}H-NMR measurements in brain (47,57,121). If the same phenomenon occurs in this tissue the results of these studies will be quantitatively in error .

The studies reported in chapter 3 were designed to assess the role of changes in pH (i.e., ΔH^+) and diprotonated-P_i in the depression of maximum force associated with fatigue. Changes in these two metabolites figure prevalently in the current theories of cellular level fatigue. The results presented here show that manipulation of pH_i and $[H_2PO_4^{1-}]$ independent of

contraction does not produce a direct relationship with maximum force as seen in previous studies. This suggests that the often reported correlation between these metabolite changes and decreased maximum force production does not indicate a cause and effect relationship. However, acidosis was associated with changes in the characteristics of contractions (e.g., relaxation time) which might effect the development of force in the intact animal.

Conclusions

There is an extensive body of literature relating metabolic events to the development of fatigue. However, much of this research has been conducted either *in vitro* or *in situ* with indirect data collection. It is possible that some of the variability and contradiction found in the fatigue literature results from previously unavoidable methodological limitation.

The application of scientific method implies the ability to control or at least measure as many system parameters as possible. However, this requirement must be tempered by the need to observe processes under conditions resembling those found in the native (i.e., *in situ*) state. The conceptual impetus for the studies presented in this dissertation was heavily dependent on the availability of a relatively new technology, NMR. Experiments using this technology were designed to assess the effects of changes in metabolites *in vivo* under controlled conditions closely resembling those found *in situ*.

The combination of NMR technology and the isolated perfused muscle preparation described in this dissertation has proven to be an effective means of studying muscle metabolism. The experimental design for each study was formulated to avoid perceived methodological limitations of earlier studies. The isolated perfused muscle preparation is a reasonable compromise between poorly controlled experiments in intact animals and highly controlled experiments in

less physiological preparations such as skinned fibers. The functional unit of a muscle, the muscle cell, remains intact and in an environment which closely resembles that *in situ*. In contrast to many investigations, the energetic and functional state of the muscles in these studies were subject to frequent assessment. Variability was avoided by collecting data under control, experimental and recovery conditions in the same muscle and the temporal resolution of the observations was well suited to the time course of the metabolic and functional events.

The experiments reported here uncovered results which may have been obscured by methodological limitations in previously published studies. The results of these experiments add significantly to our understanding of the intracellular response to an acid load and the way in which muscle function is effected by it. Future metabolic studies will benefit from the refinements in calculation of buffer capacity presented in chapter 1. The results presented in chapter 2 will help to avoid erroneous conclusions based on similar research methods. The observed dissociation of changes in pH and $[H_2PO_4^{1-}]$ from force (chapter 3) will require revision of fatigue doctrine. This revision should provoke further investigation of possible metabolic agents responsible for muscle fatigue.

Appendix

In order to conduct experiments using isolated perfused cat muscles an NMR probe was designed and constructed. Important design considerations included:

Use of Non-ferric (i.e., nonmagnetic) materials.

Both ^{1}H and ^{31}P frequency sensitivity.

Circuit tuning and matching while in magnet.

Accommodation of perfusion lines.

Muscle mounting and length adjustment.

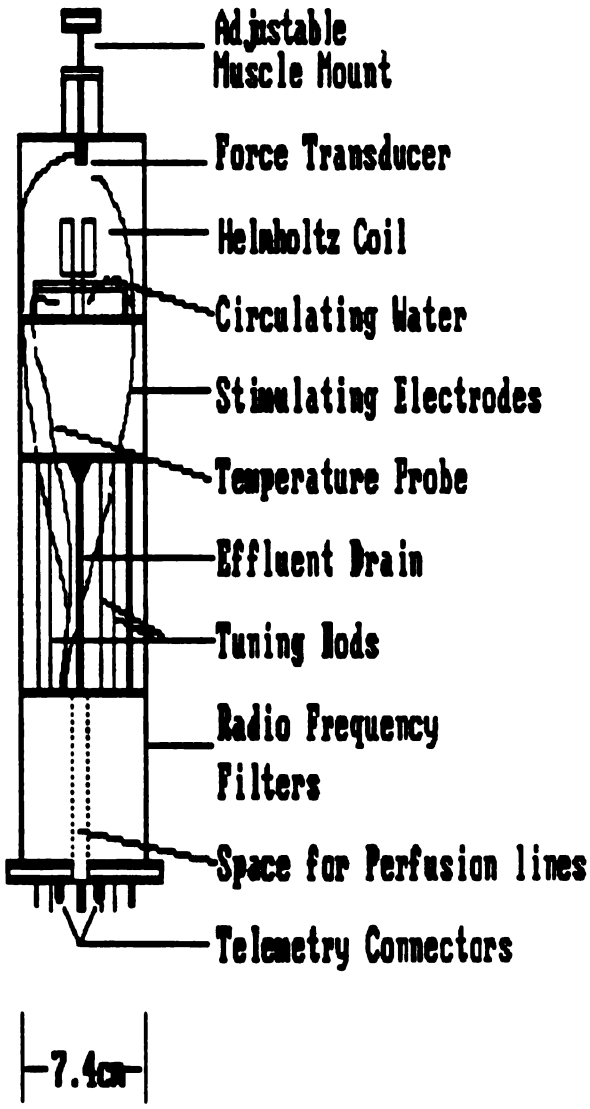
Facilities for force measurement.

Muscle stimulation electrodes.

Temperature measurement and control.

The shell of the NMR probe was constructed from an aluminum tube cut in half and provided with a base plate and surfaces for mounting the probe contents. The bottom 1/4 of the probe consisted of a service module housing the connectors for telemetry and input lines (Figure 21). All external connections were passed through radio frequency (rf) chokes (cut off frequency = 100MHz) in the service module. The rf chokes prevented unwanted signals from reaching the NMR coil circuitry. Provision was made for incoming perfusion lines and for the collection of venous out flow from the muscle.

Figure 21. NMR probe for isolated perfused muscle experiments.


Adjustable
Muscle Mount
Force Transducer
Helmholtz Coil
Circulating Water
Stimulating Electrodes
Temperature Probe
Effluent Drain
Tuning Rods
Radio Frequency
Filters
Space for Perfusion lines
Telemetry Connectors
7.4cm

Various coil circuit designs were evaluated with respect to concurrent ^{1}H and ^{31}P frequency sensitivity. Dual coils and single circuit dual tuned coils failed to provide the necessary performance (27,43,76,104,109). The final design consisted of a single coil with supporting circuitry for tuning to both 400 MHz (^{1}H) and 162 MHz (^{31}P) (Figure 22). The two circuits were isolated by a mechanical switch.

An Helmholtz configuration coil was constructed from 16 gauge, coated, copper wire. Equation 1 was used to determine the optimal coil dimensions (Figure 23).

(1) $$\text{Size (mm)} \times 2\pi / 3 = X.$$

Where each horizontal leg = X, the front and rear gaps = X/2 and the vertical legs = X × 1.1. A diameter of 20mm was used to ensure space for accommodation of cat biceps muscles.

The electronic characteristics of each coil were determined from the relationship between frequency, inductance and capacitance:

(2) $$f = 1/ 2\pi L^{1/2} \times C^{1/2}$$

Where: L= coil inductance, C= total capacitance, f=frequency.

Equation 3 is the actual working equation derived from equation 2 . This equation was used to determine L and intrinsic C (C_x) of the coil.

(3) $$C_x = (1/ (2\pi F)^2) / L - C_a.$$

Where: C_a = known (i.e., added) capacitance and F = observed frequency. Intrinsic capacitance and inductance the coil was determined by measuring F at $C_a = 0$ and at some C_a and solving for the apparent C_x in each case. The difference between these two C_x values divided by the known C_a provided L. Equation 3 was then solved for the actual C_x.

Figure 22. Diagram of NMR coil and tuning circuits. The ^{1}H and ^{31}P circuits are isolated by a mechanical switch.

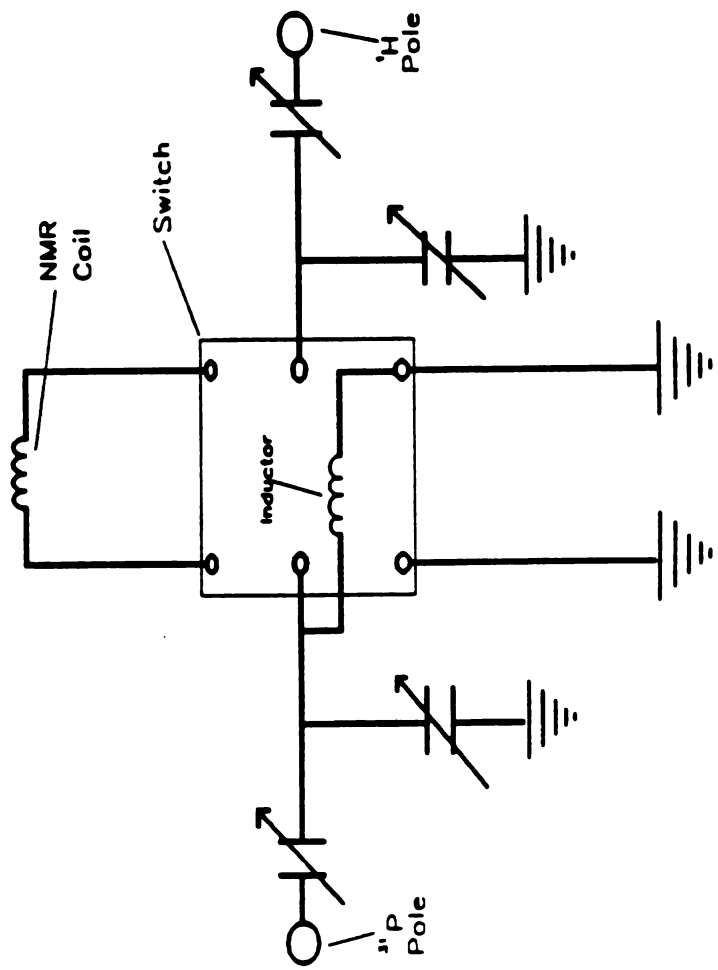
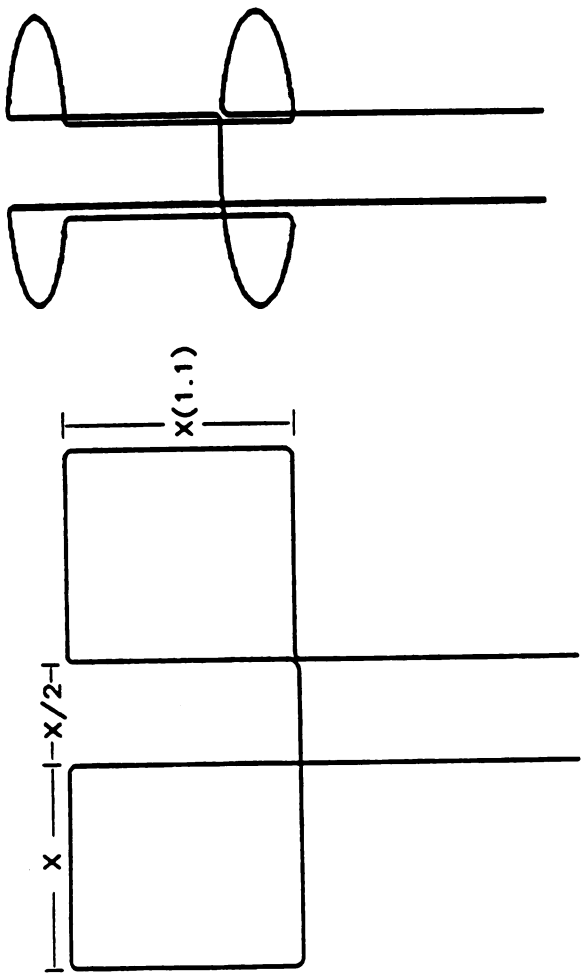

'H Pole
Switch
NMR Coil
Inductor
'P Pole

Figure 23. A. Design template for Helmholtz NMR coil. B. Final coil configuration.


x(1.1)
x/2
x

The coil circuitry was mounted in a removable module in the probe shell. All variable capacitors of the two coil circuits were mounted facing the bottom of the probe. These capacitors were mated to spring loaded screw drivers which extended through the bottom of the probe. These extensions allowed frequency tuning and matching of both circuits while the probe was in the magnet. A separate extension allowed operation of the frequency selection switch while the probe was in the magnet.

A fixed muscle mount was attached to the coil module below the NMR coil. The upper muscle mount was attached to a threaded rod which allowed for changes in length with the muscle in place (Figure 24). The variable mount could be adjusted from outside the magnet.

Force was measured as the stress on a cantilever beam made of a non-magnetic polymer (Delrin, DuPont). Microfoil strain gauges (SR-4, BLH Electronics) were attached to opposite faces of the beam (Figure 24). These strain gauges made up two of the four legs of a Wheatstone bridge (Figure 25). The bridge was balanced (i.e., no current movement) with zero stress on the beam. Stress on either face of the beam caused a change in resistance in the two strain gauges. This change in resistance resulted in current movement which was amplified and recorded on a strip chart recorder. This arrangement represents a functional force transducer. The transducer was calibrated by hanging various weights from the muscle mount and recording the output. The Delrin based transducer used in the cat muscle experiments had a linear response from 1 to 10 Kg (Figure 26).

Figure 24. Detail of variable length muscle mount with microfoil strain gauges for force measurement.

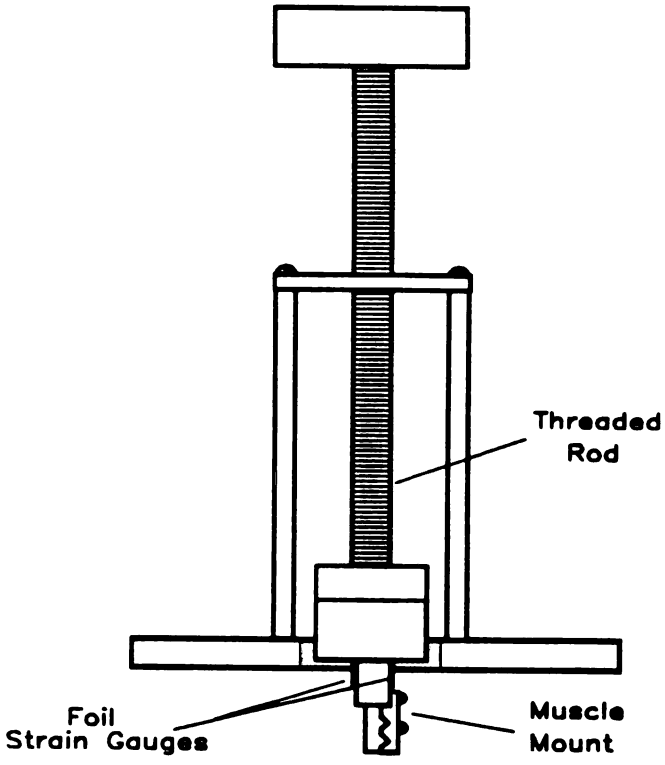

Threaded
Rod
Foil
Strain Gauges
Muscle
Mount

Figure 25. Diagram of Wheatstone bridge with microfoil strain gauges as two legs.

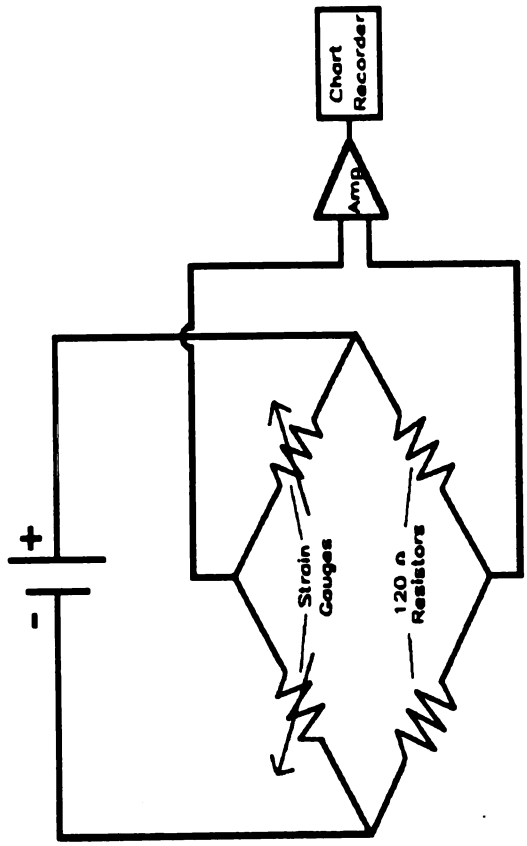
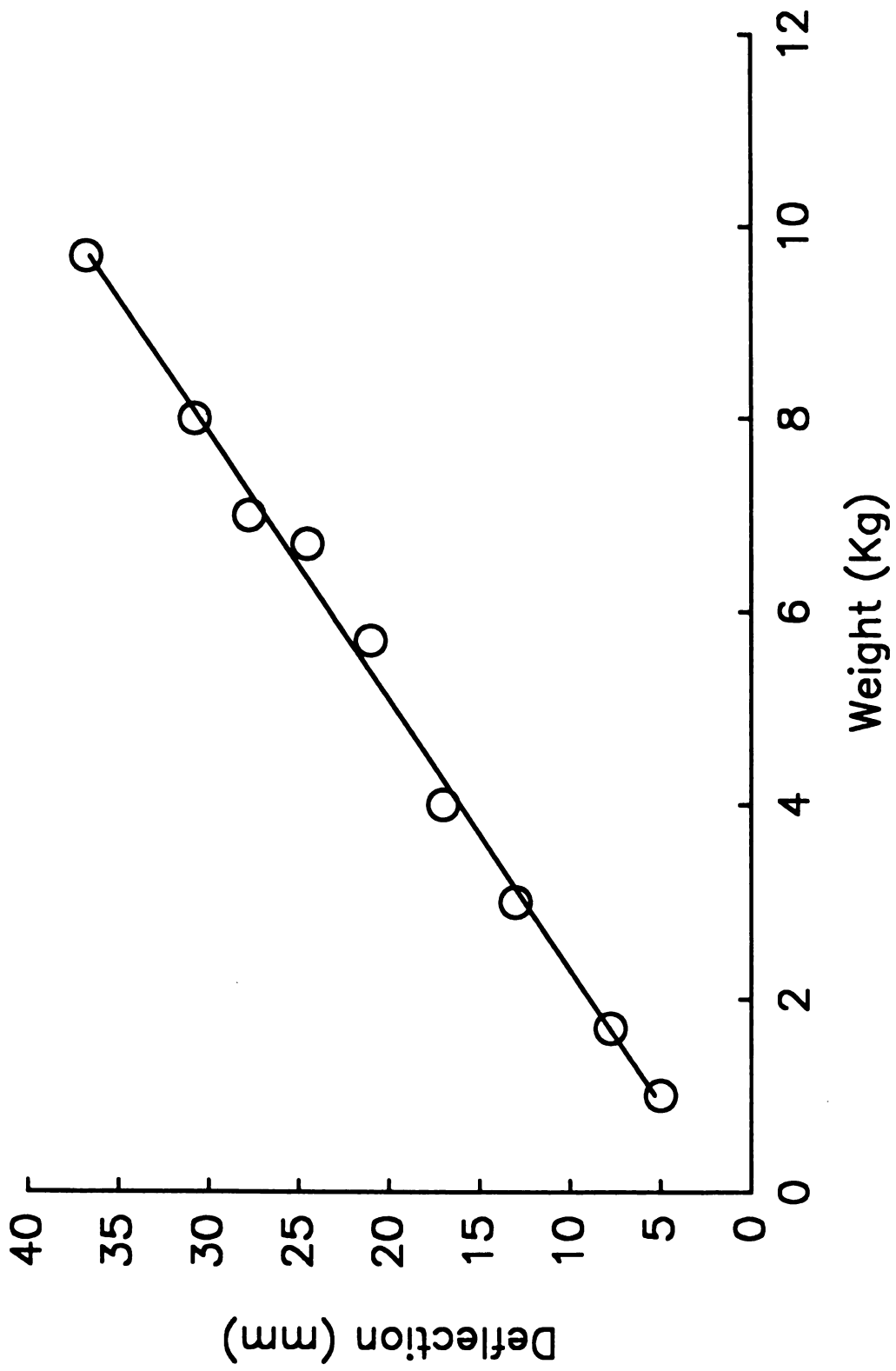


Chart Recorder
Amp
+
-
Strain Gauges
120 Ω Resistors

Stimulating electrodes consisted of 2cm lengths of platinum wire attached to copper wire.

Temperature measurement was accomplished by placing a thermistor (YSI) against the muscle. Temperature was controlled by circulating heated water through a copper tube in the muscle compartment of the probe (Figure 21).

Figure 26. Calibration plot for Delrin force transducer. Pen deflection on the chart recorder vs. weight attached to muscle mount ($r=0.997$).


Deflection (mm)
40
35
30
25
20
15
10
5
0
0
2
4
6
8
10
12
Weight (Kg)

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