ANAEROBIC ENERGY SUPPLY DURING MAXIMUM-INTENSITY SHORT-TERM VOLUNTARY SUSTAINED EXERCISE IN MAN.

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ABSTRACT

The purpose was to directly assess the relative contributions of the anaerobic energy releasing pathways to ATP provision during very brief (2-14s) maximal isometric contractions in human skeletal muscle, and to examine the recovery processes following such exercise. Eleven males performed unilateral sustained maximal isometric contractions of the knee-extensor muscles. Ten needle biopsy samples [2 pre-exercise (PreEx), 4 exercise (Ex), and 4 recovery (Rec) samples] were obtained from each subject over 3 separate testing sessions. Circulation was occluded at the upper thigh prior to all biopsies. Ex samples were taken immediately following 2-14s of maximal voluntary contraction. Contraction times were randomly pre-determined such that 6 biopsies were obtained following <u>each</u> of 2, 4, 6, 8, 10, 12, and 14s of contraction. Rec samples were collected 10, 20, 30, 40, 60, 120, and 180s following 14s maximum contractions. Significant changes occurred for both phosphocreatine (PCr) and lactate concentration within the initial 2s ($n=7$, $p \leq 0.05$) of contraction. In 14s, PCr concentration decreased to 53.3% of the PreEx value at a rate of 2.53 ± 0.18 $mmol \cdot kg^{-1}dm \cdot s^{-1}$, while muscle lactate increased to approximately 8 times the initial value, at a steady rate of 1.52 ± 0.10 $mmol \cdot kg^{-1}dm \cdot s^{-1}$.

Following contraction, PCr returned to the PreEx value within 60s of recovery (n=4, $p>0.05$), but muscle lactate concentration was still significantly elevated above resting after 180s of recovery (n=4, $p\leq0.05$). Total ATP production over 14s was 69.03±4.47 $mmol \cdot kg^{-1}dm$, with an average turnover rate of 4.93±0.32 $mmol \cdot kg^{-1}dm \cdot s^{-1}$. The initial ATP turnover rate was 7.26±1.94 $mmol \cdot kg^{-1}dm \cdot s^{-1}$ with 67% of the ATP being derived from PCr degradation and 33% from glycogenolysis. As contraction continued, however, there was an almost equal contribution to ATP provision from the 2 anaerobic energy-delivery pathways, and (with the exception of the first 2 seconds) the proportion changed very little over time.

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DEDICATION

TO MY PARENTS

FOR YEARS OF CONFIDENCE AND SUPPORT.

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CHAPTER I INTRODUCTION

1.1 OVERVIEW

Adenosine triphosphate (ATP) is the immediate energy source for contraction by skeletal muscle. The concentration of ATP in resting human muscle, however, is very small (approximately 25 $mmol \cdot kg^{-1}$ dry weight (Tesch et al., 1989)), and decreases minimally during exercise (Fitts, 1994). During the most intense muscular activity, the decrease in ATP is 30-40% (Hultman et al., 1990)). Therefore, tightly controlled mechanisms must exist for the constant regeneration of ATP as contraction continues.

Three distinct metabolic processes form the basis of ATP resynthesis during exercise. These include two anaerobic energy delivery pathways (phosphocreatine (PCr) splitting and glycogenolysis), and one aerobic pathway (oxidative phosphorylation), each of which is characterized by the maximum rate at which it can produce ATP (power), and by the total amount of ATP it can produce (capacity). Anaerobically derived ATP can be provided at much higher rates than can aerobically supplied ATP, but the capacity for anaerobic ATP provision is extremely limited. Consequently, the intensity of the exercise and the associated rate of energy demand will determine fuel selection and, thus, the relationship between anaerobic and aerobic energy utilization.

Figure 1.1 Traditional schematic representation of the relationships of energy-supplying biochemical processes in human skeletal muscle. The duration of exercise is given on a logarithmic scale, and the energy outputs are calculated on the basis of results performed by elite athletes in different activities.
From Howald et al. (1978).

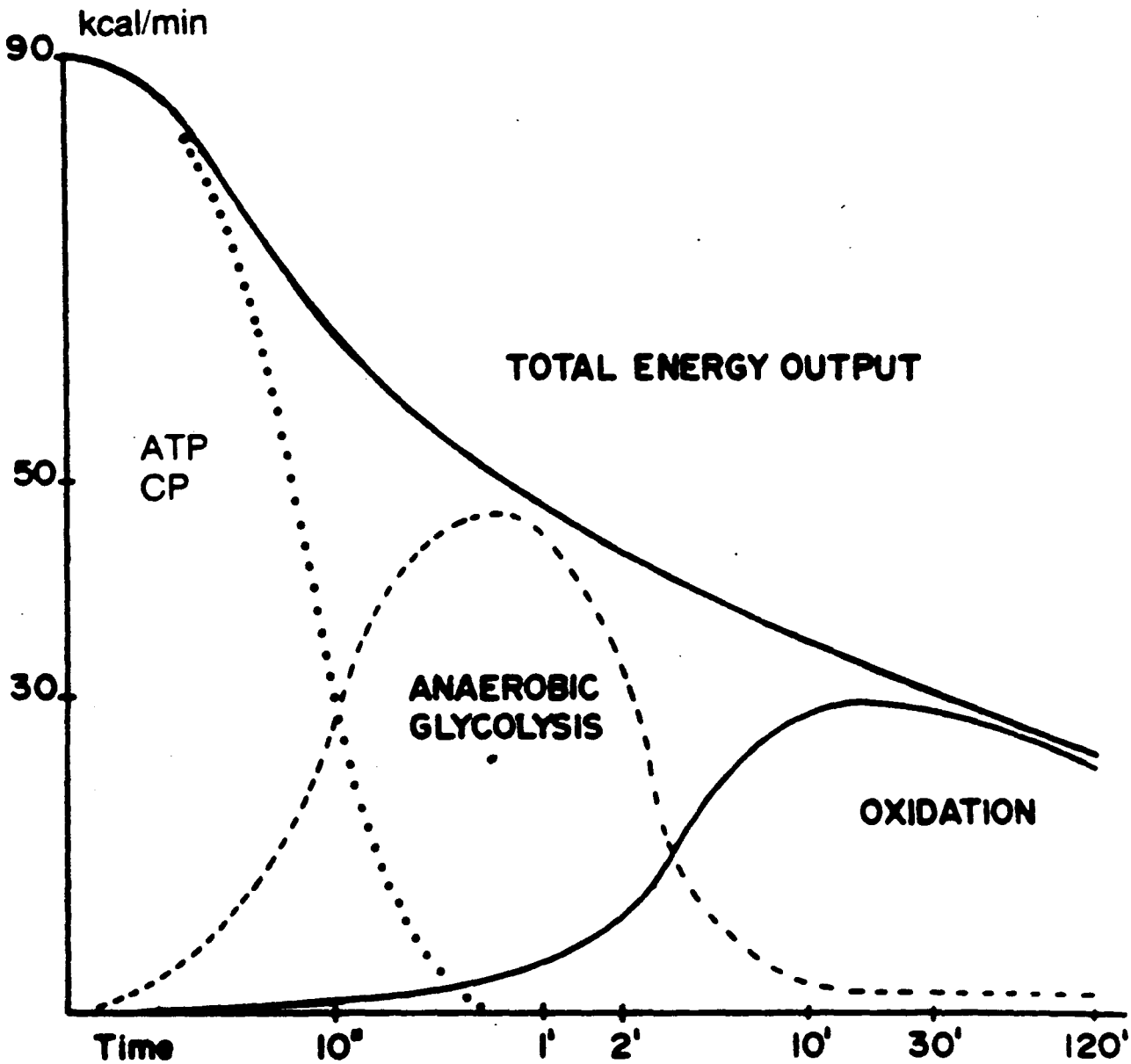

kcal/min
90
50
30
TOTAL ENERGY OUTPUT
ATP
CP
ANAEROBIC
GLYCOLYSIS
OXIDATION
Time
10"
1'
2'
10'
30'
120'

Fig. 1.1 (Howald et al., 1978) illustrates a schematic representation of what is generally thought to be the proportion of ATP supply from each pathway, dependant upon the duration and intensity of exercise. It is well established that aerobic sources provide the majority of energy during rest and during prolonged exercise. Energy provision during short term maximal exercise is, however, less understood. Until recently it was assumed that, during such high-intensity short-term exercise, energy delivery by the separate anaerobic pathways was linked sequentially. PCr degradation was thought to be the main source for ATP regeneration over the first 10-15 seconds, followed by anaerobic glycogenolysis over the next 90 seconds or so (Margaria et al., 1969). Research has since indicated that at no time will any one energy pathway act in isolation. Rather, all three processes (including oxidative metabolism) will proceed concomitantly from the onset of exercise.

Although energy provision during submaximal sustained exercise has been extensively investigated and documented, maximum energy delivery over 10 seconds or less is often indicative of success or failure in competitive sport. Methodological problems associated with quantifying non-steady state metabolism have limited the number of investigations of energy kinetics within this very short time span. It appears, however, that even over such a brief period,

anaerobic glycogenolysis may be the dominant pathway and that PCr may simply function as an "energy buffer" and thus be of considerably less importance. A systematic investigation of the relative involvement of PCr splitting and glycogenolysis during maximal voluntary energy expenditure over durations ranging from 2-14 seconds, is thus warranted.

Current knowledge regarding anaerobic metabolism during intense short-term muscular activity is reviewed in this chapter. The pathways of anaerobic metabolism are examined in terms of the history of their investigation, their capacity to provide ATP, the rates at which they can resynthesize ATP, and the speed by which they are activated. Issues regarding the regulation and control of these pathways are discussed, as well as potential mechanisms for fatigue during brief maximal exercise. Finally, the processes of recovery following anaerobic activity are outlined.

1.2 MUSCLE ENERGY SYSTEMS

1.2.1 HISTORICAL INTRODUCTION

Force development in skeletal muscle requires the conversion of chemically stored energy into mechanical energy. Although it has long been recognized that energy for external work is provided mainly through the oxidation of carbohydrates and fats, it was not until the 20th century that physiologists

began to understand the precise mechanical and metabolic processes required to produce muscular contraction.

In 1907, Fletcher and Hopkins first demonstrated that muscular work was associated with the formation of lactate. Muscle lactate production was found to increase in the absence of oxygen, and to disappear when oxygen was administered. Following this came the chemical investigations of Otto Meyerhof and coworkers in Germany (1920's), who determined that lactic acid formation from glycogen was (1) connected with the release of energy, and (2) was proportional to the amount of work performed under anaerobic conditions (Keul et al., 1972). Concurrent research by A.V. Hill in England related heat production in isolated amphibian muscle to mechanical work to determine that the direct energy for contraction was nonoxidative (Hill and Lupton, 1923).

Based on these observations, Hill and Meyerhof postulated the first theory of muscular contraction. Together they hypothesized that the fundamental chemical reaction leading to muscular contraction was the anaerobic breakdown of glycogen or glucose to lactic acid, followed by oxidative recovery during which glycogen was restored through combustion of approximately one-fifth of the produced lactic acid. Without lactic acid, no muscular contraction was supposed to be possible (Hill et al., 1924). During prolonged exercise, a steady state was achieved where the lactic acid production

rate was matched by its aerobic removal. Increased oxygen uptake which occurred following exercise (the O_2 debt) was explained as the slow return to resting conditions from this steady state of equilibrium (Asmussen, 1971). The Hill-Meyerhof theory was almost universally accepted by physiologists at that time. Evidence to challenge the theory came in 1925 when Embden detected phosphate liberated into the Ringer solution surrounding isolated contracting muscle. Shortly thereafter, improved techniques for extracting phosphagens from muscle tissue allowed Fiske and Subbarow (1927, 1929) and Eggleton and Eggleton (1927) to independently discover the compound phosphocreatine (PCr). PCr was reported to decrease with intense stimulation of frog muscle, and to become depleted with continued stimulation and occlusion of blood flow. Resynthesis of PCr occurred rapidly following contraction in the presence of O_2. It was suggested (Fiske and Subbarow, 1929) that perhaps the initial alkaline pH shift which accompanied PCr splitting served to buffer the muscle against lactic acid.

The energetics of PCr hydrolysis (the first substance found to carry the "high energy" phosphate bond) were quantified by Meyerhof and Lohmann [(1928a,b), as cited in Keul et al., 1972] who found that the degradation of one mole of PCr liberated 12 kcal. These discoveries questioned the potential for a direct association between lactic acid

formation and the contractile process. In 1930, the lactic acid theory lost credibility when Lundsgaard (Sacks and Sacks, 1933) "poisoned" a muscle with monoiodoacetate (preventing lactate production) and observed that contraction still occurred. The poisoned muscle was capable of only a limited number of contractions, the contraction medium became alkaline rather than acidic, and the concentration of PCr decreased proportionately to the amount of work done. These results led scientists to believe that perhaps PCr (rather than lactate) was the direct energy source and stimulus for muscular contraction. Lactate formation from glycogen was considered necessary to provide energy for the anaerobic resynthesis of PCr.

Since it was well known that PCr concentration in the muscle was low, lactic acid formation from glycogen was still considered the principal source of anaerobic energy responsible for muscular contraction. It was believed that the complete combustion of glycogen had to go through lactic acid as a necessary intermediate product. Furthermore, the production of muscle lactate, and the resynthesis to glycogen were considered the only mechanisms responsible for contraction and repayment of the O_2 debt (Margaria, 1976).

Based on the above hypothesis, it was expected that the size of the O_2 debt would be proportional to the amount of lactate produced. The 1933 study of Margaria, Edwards, and

Dill did not support this premise. Little or no lactic acid was detected in the blood following light to moderate exercise in humans, despite a consistent O_2 debt formation. Only after strenuous activity (approximately 66% $\dot{V}O_2$ max) was lactic acid detected in the blood.

Although no direct measure of muscle glycogen was available, measures of O_2 uptake and respiratory quotients indicated that glycogen and glucose were being utilized for energy production even during exercise where no plasma lactate was detected. It was therefore determined that glycogen derivatives must be directly oxidized. Lactic acid metabolism was too slow to account for the large absolute energy production that occurred in the body during exercise, and consequently lactate could no longer be considered as a necessary intermediate of glycogen degradation. Aerobic metabolism became considered the dominant pathway for energy delivery, while lactic acid production seemed to take place only as an emergency during strenuous exercise (Margaria et al., 1933).

The O_2 debt which was accumulated during low level exercise was attributed to the cleavage of PCr. The repayment of the O_2 debt was distinctly divided into slow and fast phases. The fast phase occurred with exercise of all intensities and appeared to account for the oxidative regeneration of PCr, while the slow phase only followed

exercise of greater intensity and appeared responsible for oxidative removal of muscle lactate acid (Margaria et al., 1933).

Other lines of research further complicated the understanding of muscle metabolism with the discovery of adenosine triphosphate (ATP) (Fiske and Subbarow, 1929). Hydrolysis of ATP to ADP (adenosine diphosphate) and inorganic phosphate (P_i) was, like PCr degradation, found to yield 12 kcal per mole [(Meyerhof and Lohmann, 1932), as cited in Keul et al., 1972]. It was later revealed that the cleavage of PCr in muscle tissue could not take place without the presence of ADP to take up the P_i [(Lohmann, 1934), as cited in Keul et al., 1972]. This meant that PCr could not deliver energy to the contractile mechanism until ATP had been split into ADP and P_i. Evidence now pointed to ATP as the immediate source of energy for muscular contraction (Asmussen, 1971).

The hypothesis that ATP was the direct energy donor was not immediately accepted since, at the time, no experimental evidence existed to indicate that ATP actually broke down during contraction. A 1933 study (Sacks and Sacks, 1933) had indicated that muscle stimulation to tetanus led to little or no decrease in ATP content. It was therefore suggested that ATP had no direct role in the chemical changes leading to muscular work. Englehardt and Ljubimowa (1939) later reported that myosin (the main structural protein of

muscle) was also an enzyme (ATPase) which allowed the hydrolysis of ATP to ADP, and Svent-Gyorgyi (1953, cited in Keul et al., 1972) found that isolated myofibrils contracted only upon addition of ATP. These findings lent support to the notion that ATP hydrolysis was directly linked to muscular contraction, but it was not until 1962 that ATP was conclusively established as the immediate source of energy for muscular contraction. Cain and Davies (1962) chemically inhibited the enzyme responsible for PCr hydrolysis (creatine phosphokinase) in isolated amphibian tissue, and determined that the number of full normal contractions which could be performed was reduced from 30 to about 3. PCr concentration in the fibers did not change, but the ATP level was found to be significantly reduced.

1.2.1 (i) Summary

With the experiment of Cain and Davies (1962), ATP was confirmed as the direct energy donor for muscular contraction. It became known that the aerobic and anaerobic pathways were integrated in such a way as to continually provide ATP during exercise of any intensity, and that aerobic metabolism dominated energy delivery during low to moderate exercise, while anaerobic glycogenolysis became a more important energy source with increased exercise intensity. PCr hydrolysis occurred at the onset of all activities in order to buffer the

change in ATP/ADP ratio while the circulation adjusted to the increased O_2 demand.

Specific biochemical processes and enzyme kinetics involved in glycogenolysis, Kreb's cycle, and the respiratory chain, were uncovered through other lines of research. Additionally, new techniques including the needle biopsy, nuclear magnetic resonance (NMR), and radioisotopic tracer technology allowed increased knowledge of the rates and capacities of each energy delivery pathway. This information is reviewed in the next few sections, with a specific focus on the integration of the anaerobic pathways.

1.2.2 MUSCLE ATP SUPPLY

1.2.2 (i) ATP hydrolysis

During muscular work energy is consumed by several chemical processes. Aside from the ATP hydrolysis which occurs by actomyosin ATPase to activate the actin/myosin cross-bridge interaction, significant amounts of energy are also used by mechanisms that control contraction (ie., Ca^{++} sarcoplasmic reticulum (SR) pumping) and maintain an appropriate environment for neural activation (Na^+/K^+ ATPase).

Net ATP hydrolysis can most simply be delineated as:

$ATP + H20 == ADP + P_i + H^+$ (Stryer, 1988)

Intense exercise results in a marked acceleration of the activities of the ATPases that catalyze this reaction. This, in turn, necessitates a rapid increase in the processes of ATP resynthesis, in order to maintain the energy charge of the cell. Evidence suggests that during brief high-intensity exercise 80-90% of the ATP resynthesized will be derived from the anaerobic processes of PCr degradation and glycogenolysis to lactate (Hultman et al., 1991).

1.2.2 (ii) Phosphocreatine (PCr)

PCr degradation resynthesizes ATP at the highest rate (Hultman et al., 1990). In the presence of PCr, ADP is rephosphorylated via the reversible creatine phosphokinase (CPK) reaction:

$$H^+ + ADP + PCr \overset{CPK}{\rightleftharpoons} Cr + ATP$$ (Newsholme and Leech, 1983)

As energy demand increases, and ATP is hydrolyzed to its breakdown products, the CPK reaction will be displaced to the right - favouring ATP resynthesis. The maximum activity of CPK is greater than that of myosin ATPase (Maughan and Greenhaff, 1991); therefore, as long as PCr is available, the concentration of ATP should remain high. That is, in the presence of PCr, ATP consumption should not exceed ATP production. As previously mentioned, however, the muscle concentration of PCr is limited [resting concentration 85

mmol·kg^{-1} dry weight (Stathis et al., 1994)] and is rapidly depleted. Energy provision from anaerobic glycogenolysis is thus also of paramount importance during high-intensity exercise.

1.2.2 (iii) Anaerobic glycogenolysis / glycolysis

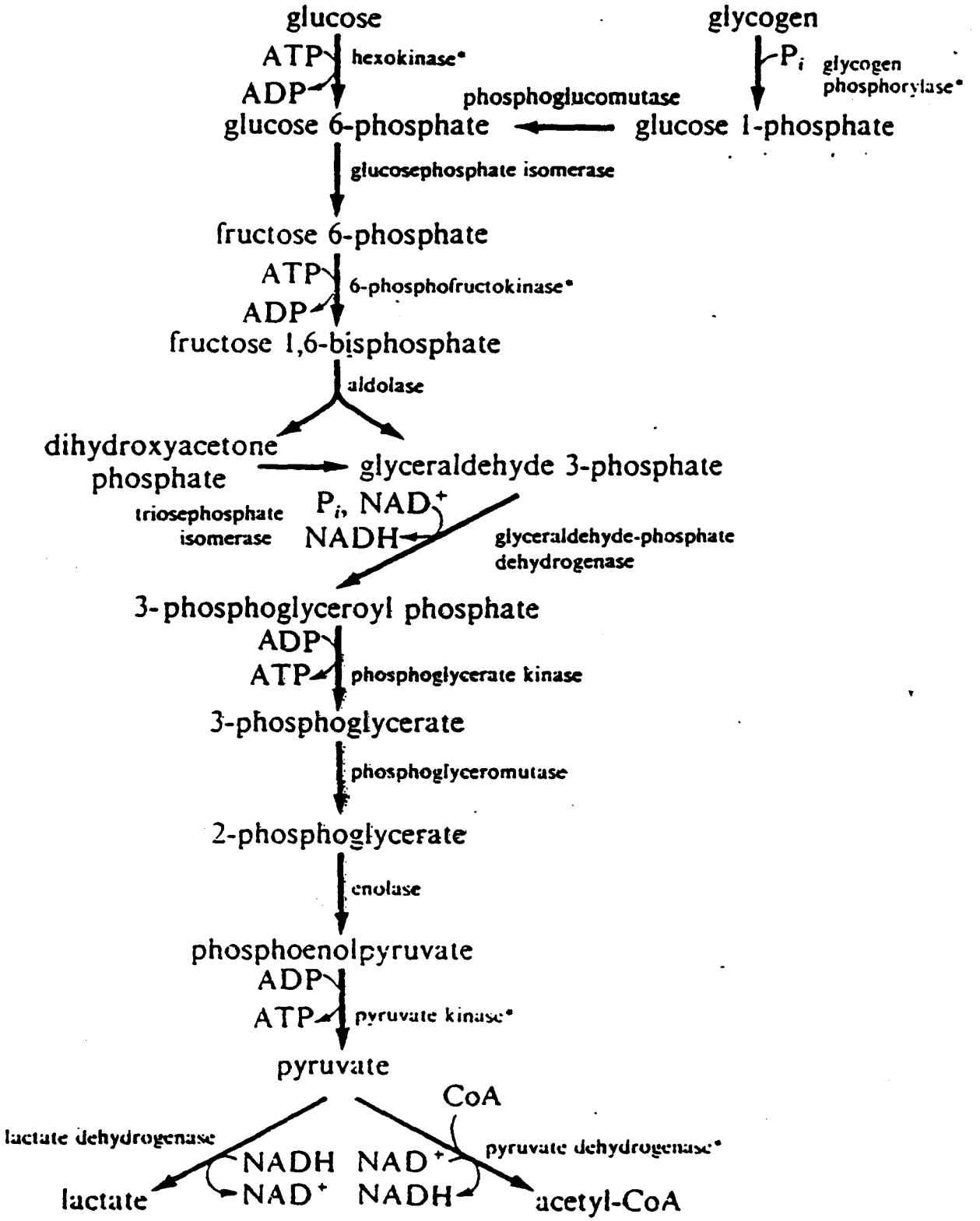
Glycolysis is the sequence of reactions that converts glucose to pyruvate with the concomitant production of ATP (Stryer, 1988). This process is rapidly initiated at the onset of muscular activity, with the net reaction being:

$$\text{Glucose} + 2\text{ADP} + 2\text{P}_i \longrightarrow 2\text{pyruvate} + 2\text{ATP} + 4\text{H}^+ \quad \text{(Stryer, 1988)}$$

Two moles of ATP are thus generated in the conversion of glucose into 2 moles of pyruvate. The series of tightly controlled enzymatic reactions of glycolysis is illustrated in Fig. 1.2.

The degradation of glycogen to pyruvate (glycogenolysis) is a similar process but includes the initial step of glycogen phosphorylation to G-1-P. This reaction is catalyzed by glycogen phosphorylase, with G-1-P then following the same pathway as glucose (Fig. 1.2). The cleavage of glycogen is energetically advantageous because the released sugar is phosphorylated. A hydrolytic cleavage would yield

Figure 1.2 Schematic representation of the glycolytic pathway in skeletal muscle.
From: Newsholme and Leech (1983), p.180.
* Indicates enzymes that are not reversible under physiological conditions.


glucose
ATP
ADP
hexokinase*
glycogen
P_i
glycogen phosphorylase*
phosphoglucomutase
glucose 6-phosphate
glucose 1-phosphate
glucosephosphate isomerase
fructose 6-phosphate
ATP
ADP
6-phosphofructokinase*
fructose 1,6-bisphosphate
aldolase
dihydroxyacetone phosphate
glyceraldehyde 3-phosphate
triosephosphate isomerase
P_i, NAD^+
NADH
glyceraldehyde-phosphate dehydrogenase
3-phosphoglyceroyl phosphate
ADP
ATP
phosphoglycerate kinase
3-phosphoglycerate
phosphoglyceromutase
2-phosphoglycerate
enolase
phosphoenolpyruvate
ADP
ATP
pyruvate kinase*
pyruvate
CoA
lactate dehydrogenase
NADH
NAD^+
NAD^+
NADH
pyruvate dehydrogenase*
lactate
acetyl-CoA

glucose, which would have to be phosphorylated at the expense of ATP in order to enter the glycolytic pathway (Stryer, 1988). The conversion of glycogen to G-1-P to pyruvate consequently yields 3 molecules of ATP, a 50% increase in ATP production over that of glucose degradation. During high-intensity short-term activity, muscle glycogen will be predominantly selected as a fuel source, since free glucose in the circulation cannot be taken up into the muscle cell at a fast enough rate to meet the energy demand (Spriet, 1992). It is for this purpose (and for one of clarity) that the processes of glycogenolysis and glycolysis will be singly referred to as glycogenolysis for the remainder of the paper. Regulation and control of glycogenolysis and PCr degradation are discussed in section 1.4.

1.2.2 (iv) Lactate production

Under aerobic conditions, pyruvate enters the mitochondria where it is completely oxidized to CO_2 and H_2O. If the O_2 supply is insufficient, however, or if the glycogenolytic rate exceeds that of pyruvate oxidation, pyruvate is converted to lactate. The reduction of pyruvate by NADH to form lactate is catalyzed by lactate dehydrogenase (LDH) as indicated in the reaction:

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \overset{\text{LDH}}{=\!=} \text{lactate} + \text{NAD}^+$$ (Stryer, 1988)

It is the regeneration of NAD^+ that sustains continued operation of glycogenolysis under anaerobic conditions. Without NAD^+, glycogenolysis could not proceed beyond the glyceraldehyde-3-P step (Fig. 1.2), and no ATP would be generated (Stryer, 1988).

1.2.2 (v) Oxidative phosphorylation

Oxidative phosphorylation of carbohydrates, fats, and amino acids provides much more ATP than do the anaerobic processes. The specific biochemistry of oxidation will not be discussed here since the primary focus is upon short duration exhaustive exercise, where the contribution of oxidation to energy provision will be minimal.

1.3 ANAEROBIC ATP UTILIZATION:

1.3.1 OVERVIEW

Early research suggested that during intense exercise, energy provision from the anaerobic pathways was linked sequentially (Margaria et al., 1964, 1969). Glycogenolysis was not expected to be a primary source of energy until PCr was greatly depleted. However, more recent investigations of high-intensity exercise have demonstrated that PCr degradation and glycogenolysis may proceed concomitantly from the onset of exercise (Boobis et al., 1982; Hultman and Sjöholm, 1983a,b;

Jacobs et al., 1983; and Jones et al., 1985). Consequently, the relative energy contributions of the anaerobic pathways during a brief burst of maximal activity will be greatly influenced by both the maximal rate each pathway is capable of producing ATP, and the capacity of each pathway to continue producing ATP. These two factors, in turn, will be influenced by the intensity and the duration of the activity.

1.3.2 MAXIMUM RATE OF ATP SUPPLY

When maximum voluntary exercise is extended beyond 10s in duration, power output and force generation will begin to decline, as will the rates of glycogenolytic and PCr derived ATP (Söderlund et al., 1992). ATP resynthesis rates peak within the first few seconds of exercise and are thus difficult to quantify.

^{31}P-Nuclear Magnetic resonance (NMR) permits non-invasive measurement of PCr concentration, but present technology requires 8-12s for an NMR scan (Blei et al., 1993; Yoshida and Watari, 1993). Since metabolite concentrations are averaged over the entire length of the scan, second to second changes which may occur during brief bursts of exercise are thus difficult to monitor. Furthermore, one cannot directly quantify lactate production with this methodology. Evidence of the maximum rates of ATP production is therefore limited to the few invasive studies where muscle metabolites

have been measured following exhaustive exercise of less than 30s in duration.

1.3.2 (i) Voluntary exercise

The first direct measures of ATP utilization in man came shortly after the reintroduction of the needle biopsy technique by Bergström (1962). In 1967, Hultman et al. investigated metabolite responses during ten minutes of cycle exercise at a constant workload (900 $kpm \cdot min^{-1}$). Two subjects had biopsy samples taken following the first 15s of exercise and PCr values were found to be significantly decreased.

Further research from the same laboratory suggested that (in 2 subjects) ATP resynthesis during 6.6s of a maximum isometric contraction was equally derived from PCr and glycogenolysis (Bergström et al., 1971; Hultman et al., 1991). More recent investigations reported similarly that PCr and glycogen degradation contributed equally to ATP production during 6s of maximal cycling exercise (Boobis et al., 1982; Gaitanos et al., 1993). The total ATP turnover rates estimated for 6s of maximal cycling (10.4 and 14.9 $mmol \cdot kg^{-1}dm \cdot s^{-1}$, respectively) were, however, much larger than the rate calculated for a 6.6s isometric contraction (7.0 $mmol \cdot kg^{-1}dm \cdot s^{-1}$; Hultman et al., 1991). The differences are most likely due to the mechanical efficiency of the mode of

exercise (Kavanagh and Jacobs, 1988) as well as to the active muscle mass of the subjects investigated.

Table 1.1 summarizes estimated ATP production rates from PCr and glycogenolysis reported in several investigations of brief maximal voluntary exercise in humans (<30s).

1.3.2 (ii) Isometric exercise and electrical stimulation

Estimates of maximum ATP production rates based on studies involving voluntary dynamic exercise or submaximal isometric exercise should be interpreted with caution. Although these types of exercise are much more common during regular sporting activities, lactate escapes from the muscle to the interstitial space and makes it difficult to accurately assess the contribution of glycogenolysis to overall ATP production.

Isometric exercise offers several advantages over dynamic exercise in that during high intensity isometric efforts, there is little or no blood flow to the muscle (Mitchell et al., 1980). Consequently, reduced amounts of O_2 and substrates will be taken up from the circulating blood, and loss of metabolites from the muscle should be at a minimum. To ensure no blood flow occurs in the brief time span between relaxation from isometric contraction and muscle biopsy sampling, the circulation to the exercising muscle is often occluded prior to exercise (Hultman and Sjöholm, 1983b).

Table 1.1 Rates of anaerobic ATP provision from phosphocreatine and glycogenolysis during intense muscular contraction. Modified from: Spriet, (1992).

Reference	Type of exercise	Duration (s)	ATP provision ($mmol \cdot kg^{-1}dm \cdot s^{-1}$)	
			PCr	Glycogenolysis
Boobis et al. (1982)	Cycling	0-6	6.0	4.8
		0-30	1.9	4.0
Jacobs et al. (1983)	(M) Cycling	0-10	-	6.0
	(F) Cycling	0-10	-	2.9
	(M) Cycling	0-30	-	3.4
	(F) Cycling	0-30	-	2.1
Jones et al. (1985)	Isok. cycling (60 rpm)	0-10	5.1	8.0
		0-30	1.4	5.8
	(140 rpm)	0-10	4.4	9.3
		0-30	0.7	6.5
Boobis et al. (1983)	Cycling	0-30	2.0	4.4
McCartney et al. (1986)	Isok. cycling (100 rpm)	0-30	1.4	5.9
Cheetham et al. (1986)	Running	0-30	1.9	3.8
Jacobs et al. (1982)	Cycling	0-30	1.3	2.6
Nevill et al. (1989)	Running	0-30	1.9	4.1

To our knowledge, very few studies to date have examined ATP utilization during maximal voluntary isometric exercise (Alborg et al., 1972; Bergström et al., 1971). Rather, most studies of isometric contraction have made use of electrical stimulation techniques which elicit a constant contraction stimulus to the muscle for a predetermined length of time, independent of volitional effort (Hultman et al., 1990). This method is particularly advantageous to elicit uniform muscular responses during submaximal activity, since voluntary contraction will result in varied spatial and temporal recruitment of the motoneuron pool (Hannerz, 1974). During maximal exercise, activation of the entire motor unit pool during each contraction would be expected voluntarily as well with stimulation (Belanger and McComas, 1981). Furthermore, high-frequency stimulation has been associated with failure of electrical propagation at the muscle fiber membrane (Bigland-Ritchie et al., 1979) due to nonphysiologically high motor unit firing rates. During sustained voluntary contractions, however, mean firing rates have been reported to decline progressively and are accompanied by concomitant reductions in muscle relaxation rates and lowered tetanic fusion frequencies (Bigland-Ritchie et al., 1983). Muscles can therefore remain fully activated by the CNS despite reduced excitation rates (Bigland-Ritchie et al., 1983).

Hultman and Sjöholm (1983a,b) occluded the circulation and stimulated the vastus lateralis muscle at 50 Hz (near maximal contraction) for a period of 1.26s and estimated from needle biopsies that 80% of the total ATP turnover was derived from PCr breakdown, while 20% was produced through degradation of glycogen to lactate. (n=?) (1983b). Further stimulation (1.26s-2.56s) demonstrated an increase in the contribution of glycogenolysis to ATP production (50%). A subsequent study (Hultman et al., 1990, as cited in Hultman and Greenhaff, 1991) used a similar but intermittent stimulation protocol (0.8s on/ 0.8s off, 50 Hz) and biopsies were taken at rest, after 5, 10, 20, and 30s (n=?). The estimated rate of ATP resynthesis from PCr declined from 5.3 $mmol \cdot kg^{-1}dm \cdot s^{-1}$ during the first 5s to 0.2 $mmol \cdot kg^{-1}dm \cdot s^{-1}$ between the 20th and 30th second of contraction, while the energy provision from glycogenolysis was constant at 4.5 $mmol \cdot kg^{-1}dm \cdot s^{-1}$ during the initial 20s, and declined to 2.1 $mmol \cdot kg^{-1}dm \cdot s^{-1}$ for the final 10s (20-30s).

The results of these stimulation studies are summarized in Table 1.2 and illustrate the decline in the maximum rate of ATP production which occurs during intense exercise. Interestingly, these data indicate that the rate of PCr utilization begins to decline after only 1.26s of contraction, while the corresponding rate from glycogenolysis does not peak until closer to 3s of contraction. This

Table 1.2 Rates of anaerobic ATP resynthesis from phosphocreatine degradation and glycogenolysis during intense muscular contraction in man. Values were calculated from muscle metabolite changes measured in muscle biopsy samples obtained during intense intermittent electrically stimulated (50 Hz) isometric contraction.
From: Hultman and Greenhaff, (1991).

Duration (s)	ATP provision ($mmol \cdot kg^{-1}dm \cdot s^{-1}$)	
	PCr	Glycogenolysis
0-1.3	9.0	2.0
0-2.6	7.5	4.3
0-5	5.3	4.4
0-10	4.2	4.5
10-20	2.2	4.5
20-30	0.2	2.1

suggests that the rapid initial utilization of PCr buffers the momentary lag in energy provision from glycogenolysis (Hultman and Greenhaff, 1991).

1.3.3 CAPACITY

Tables 1.1 and 1.2 both indicate that as high intensity exercise increases in duration, glycogenolysis becomes the dominant energy delivery pathway. This is because the PCr store is limited and depletes rapidly (Boobis, 1987). Evidence to support this comes from Hirvonen et al., (1987) who found that 88 to 100% of the total PCr broken down during an 11s sprint running exercise was utilized in only 5.5s. That is, after 5.5s of exercise, little or no further PCr breakdown occurred. As mentioned previously, the resting muscle tissue content of PCr is approximately 85.4 $mmol \cdot kg^{-1}dm$ and numerous studies have reported significant depletion of the PCr store during supramaximal exercise of short duration (Boobis et al., 1982; Gaitanos et al., 1993; Jones et al., 1985). This depletion of the PCr store, particularly in type II fibers (Söderlund et al., 1992) has been cited as a potential cause for fatigue in brief maximal exercise (Katz et al., 1986; Tesch et al., 1989). In contrast, anaerobic glycogenolysis has a much higher capacity than PCr degradation (Sahlin, 1986) and is unlikely to be significantly taxed in exercise less than 30s in duration.

1.4 REGULATION AND CONTROL OF THE ANAEROBIC PATHWAYS

1.4.1 PHOSPHOCREATINE DEGRADATION

Utilization of PCr is regulated by changes in energy demand. Because the CPK reaction is near equilibrium, small changes in substrate concentration will trigger a relatively large flux through the pathway (Newsholme and Leech, 1983). Accordingly, when the muscle is in a resting state and ATP concentration is relatively high, the reaction proceeds with net synthesis of PCr, whereas at times of high metabolic activity, when ATP concentration is low, the equilibrium shifts to yield net synthesis of ATP.

1.4.2 REGULATION OF GLYCOGENOLYSIS / GLYCOLYSIS

Similar to PCr degradation, the rate of carbohydrate metabolism during exercise is related to the intensity of the activity (Hultman and Sjöholm, 1983). Regulation and control of the glycogenolytic - glycolytic pathway will depend primarily upon the key non-equilibrium enzymes glycogen phosphorylase, and phosphofructokinase (PFK) (Chasiotis et al., 1983).

1.4.2 (i) Phosphofructokinase (PFK) regulation

PFK is one of the most intensely studied regulatory enzymes. It functions to catalyze the phosphorylation of

fructose-6-phosphate (F-6-P) to fructose-1,6-bisphosphate (F-1,6-BP) (Fig. 2), and it is the main rate-limiting enzyme in the glycolytic pathway (Newsholme and Leech, 1983). It is allosterically inhibited by high levels of ATP (Kemp and Foe, 1983), and a rise in H^+ concentration has been shown *in vitro* to increase this inhibition by facilitating ATP binding (Bock and Frieden, 1976). It is for this reason that H^+-mediated inhibition of PFK has been frequently cited as a potential fatigue agent during intense exercise (For review, see Jones and Heigenhauser, 1992). However, other *in vitro* experiments have revealed that the inhibitory action of ATP is reversed by a combination of glucose-1,6-bisphosphate (G-1-P), adenosine monophosphate (AMP), ammonia (NH_4^+), and inorganic phosphate (P_i) (Dobson et al., 1986), and fructose-2,6,-bisphosphate (F-2,6,-BP) has been suggested to be the most potent activator of PFK (Hers and Van Schaftingen, 1981).

Human muscle pH values as low as 6.0 have been measured through NMR spectroscopy following voluntary exercise (Wilson et al., 1988). Since *in vitro* studies of PFK have found that it is essentially inactive below pH 6.4 (Danforth, 1965), the continued production and dissociation of lactic acid (leading to pH 6.0) would not have been expected. Furthermore, Sahlin et al. (1975) found that the rate of glycolysis was maintained constant throughout an isometric contraction to fatigue (67% MVC), in spite of a decreased

muscle pH from 7.09 to 6.56 at fatigue. Consequently it was expected that any H^+-mediated inhibitory effect on PFK was adequately overcome.

More recent electrical stimulation experiments on humans have also indicated the maintenance of PFK activity below pH values of 6.6-6.7 (Spriet et al., 1987). It thus appears, that during intense contraction, PFK activity and ATP provision are well matched to the demand for ATP despite decreases in pH (Spriet, 1991).

1.4.2 (ii) Regulation of glycogen phosphorylase

During very brief exercise (<10s), it is unlikely that muscular pH will drop to inhibitory levels. Control of the maximum rate of ATP production in this situation is therefore dependant upon the activation of the flux-generating enzyme, glycogen phosphorylase. Like PFK, control of phosphorylase activation and deactivation has been elucidated through *in vitro* analysis. Glycogenolysis regulation under physiological conditions, however, is still under investigation.

The phosphorylase enzyme exists in two interconvertible forms: an active phosphorylase *a* and a less active phosphorylase *b* (Cori and Green, 1943). Phosphorylase *b* can be allosterically activated by high concentrations of AMP; however, ATP and G-6-P compete for the AMP binding site to inhibit phosphorylase *b* (Fischer et al., 1971).

Transformation of phosphorylase *b* to *a* can be achieved by both a hormonal mechanism mediated by cyclic AMP (cAMP) as well as by an increase in cytoplasmic Ca^{++} concentration. Epinephrine triggers the activation of adenylate cyclase which in turn catalyses the formation of cAMP. cAMP activates a protein kinase which allows phosphorylase kinase to catalyze the conversion of phosphorylase *b* to *a* (Stryer, 1988). Phosphorylase kinase is also activated by an increased cytoplasmic Ca^{++} concentration and high pH (Brostrom et al., 1971). The set of controls for activation of phosphorylase thus includes endocrine signals (epinephrine), muscle activation (Ca^{++} and pH), as well as other metabolic activity (AMP and G-6-P).

At the onset of contractile activity, it was originally presumed that the rapid mobilization of glycogenolysis was mediated by a conversion of phosphorylase *b* to *a* (Gross and Mayer, 1974). This supposition was based on the premise that, in resting muscle, most of the phosphorylase (about 99%) was thought to be in the much less active *b* form (Fischer et al., 1971). It now appears, however, that a significant percentage (20-25%) of phosphorylase in resting muscle is in the *a* form under physiological conditions (Chasiotis et al., 1982), and regulation of glycogenolysis probably depends on other factors in addition to simple transformation of phosphorylase *b* to *a* (Ren et al., 1992)

Chasiotis et al. (1982) found that during isometric exercise in humans, the conversion of phosphorylase *b* to *a* proceeded only transiently after the onset of contraction, and that thereafter the *a* form converted back to the *b* form despite a continued high rate of glycogenolysis. A subsequent study (Chasiotis et al., 1983) revealed that, although infusion of epinephrine resulted in a significant increase in the proportion of phosphorylase in the *a* form (from 20%-90%), *in vivo* glycogenolytic activity remained low. It was postulated that the activity of the phosphorylase enzyme was limited by the availability of the substrate P_i (Chasiotis et al., 1983), however, Hultman and Sjöholm (1983b) have reported that glycogenolysis occurred in the very brief time span of 1.26s, despite a negligible increase in P_i. Furthermore, it was later demonstrated that even if the P_i concentration was significantly elevated, and the proportion of phosphorylase *a* was high, glycogenolysis still remained low (Ren and Hultman, 1989). The rates of glycogenolysis were found to be significantly different during varied intensities of electrical stimulation, despite similar P_i and phosphorylase *a* availability (Ren and Hultman, 1990). It was thus concluded that the rate of glycogenolysis was regulated by ATP turnover rate (specifically, transient increases in free AMP level), rather than P_i concentration or the proportion of phosphorylase *a* (Ren and Hultman, 1990).

1.4.2 (iia) Muscle fiber types

Human skeletal muscle is heterogenous with respect to muscle fiber type. Individual muscle cell fibers include slow twitch highly oxidative fibers containing many mitochondria and low myosin ATPase activity (type I); fast twitch fibers which are both highly oxidative and glycolytic (type IIa); and fast twitch glycolytic fibers which exhibit high ATPase activity and have a very limited oxidative metabolic capacity (type IIb). Studies of metabolism in single human muscle fibers have revealed that during electrically stimulated exercise, significant differences exist in the rates of glycogenolysis in slow and fast fibers (Greenhaff et al., 1991; Greenhaff et al., 1993).

During intermittent (1.6s on/1.6s off) electrical stimulation of the quadriceps muscle group (50Hz) with open circulation the glycogenolytic rate was 20 times greater in type II than in type I fibers (Greenhaff et al., 1991). Additionally, infusion of epinephrine led to an increase in the rate of glycogenolysis in type I but not type II fibers. It thus appears that during intense stimulation with circulation intact, glycogenolysis is restricted to type II fibers, and epinephrine induced increases in glycogenolysis are restricted to type I fibers (Greenhaff et al., 1991).

When the circulation to the exercising muscle group was occluded, the rate of glycogenolysis in type I fibers was

markedly increased; there was, however, no further increase in glycogenolysis in type II fibers, suggesting that the rate of glycogen breakdown in this fiber type is already maximal with circulation intact (Greenhaff et al., 1993).

These results led to the hypothesis that glycogenolysis is primarily regulated by the concentration of free AMP and IMP in the vicinity of the muscle fibers (Greenhaff et al., 1993). This hypothesis explains the marked difference in glycogenolysis between the two fiber types, since ATP degradation (resulting in increased AMP and IMP) has previously been shown to be greater in type II fibers (Söderlund et al., 1992). In addition, the increased glycogenolysis in type I fibers which occurred with occluded circulation can be explained by a reduction in O_2 availability, which would lead to increased AMP in this fiber type (Greenhaff et al., 1993). Interestingly, when the rate of glycogenolysis was measured in single fibre types following volitional exercise (treadmill sprinting), the difference in the glycogenolytic rate between fibre types was greatly reduced (Greenhaff et al., 1992).

1.4.2 (iii) Summary

It appears that the increase in glycogenolysis which occurs during exercise cannot be adequately explained solely by the transformation of phosphorylase *b* to *a*, or by

accumulation of P_i. Rather, the complex regulation will depend upon a combination of the above factors and, more importantly, the energy demand of the specific muscle fibers. The latter of which will determine the increase in ATP breakdown products - AMP and IMP, potent activators of phosphorylase.

1.5 MUSCLE FATIGUE

1.5.1 METABOLIC LIMITATIONS DURING MAXIMAL EXERCISE.

Fatigue is commonly defined as the failure to maintain the required force, leading to reduced performance of a given task (Edwards, 1983). Despite extensive research, the mechanisms which cause fatigue during exercise are unclear (Sahlin, 1992). The etiology is complex because the agents of fatigue are dependent upon the intensity, type, and duration of the task, the fiber content of the muscle, and the individual's degree of exercise training (Fitts, 1994). Fatigue during short-term, high-intensity exercise will thus be induced through different mechanisms than those which precipitate fatigue during prolonged activity. Only those factors which might be involved in the development of fatigue during brief maximal exercise will be discussed here, with specific emphasis on potential limitations of substrate supply and energy metabolism.

1.5.2 POTENTIAL SITES OF FATIGUE

Because the command chain for muscular contraction is complex, there are several potential sites of fatigue. Firstly, to initiate contraction, a signal must be sent from the brain. This impulse travels to the spinal cord, where it synapses with the peripheral nerve and is then directed towards the muscle fibers. An impairment in these processes (i.e., decreased central drive) would certainly result in a reduction of force (Bigland-Ritchie and Woods, 1984); however, the bulk of evidence suggests that the primary sites of fatigue lie within the muscle itself (Edwards, 1983; Fitts, 1994). A brief discussion of the possibility of neuromuscular transmission failure during high-intensity exercise is included in Sect. 1.5.6..

When the motoneuron signal reaches the motor end plate, the action potential must be propagated along the sarcolemma and through the t-tubules. Depolarization of the t-tubules allows the message to be passed on to the sarcoplasmic reticulum (SR) and subsequently Ca^{++} is released from the terminal cisternae. Ca^{++} ions bind with troponin-tropomyosin in the actin filaments in such a way as to allow the actin and myosin cross-bridge to form. Actin activates myosin ATPase, which then splits ATP. Consequently, tension is created because the energy from ATP splitting fuels movement of the myosin cross-bridge. ATP then binds to the

myosin bridge, breaking the actin-myosin bond and allowing the cross-bridge to dissociate from actin. The thick and the thin filaments then slide across each other, and the muscle shortens.

As long as the Ca^{++} concentration remains high enough to inhibit the action of the troponin-tropomyosin system, cross-bridge activation will continue. However, when neural drive to the muscle is discontinued, the Ca^{++} ions are actively reaccumulated into the reticulum lumen. Once the Ca^{++} concentration is sufficiently decreased, troponin-tropomyosin inhibition will be restored and actin and myosin will remain dissociated. For a muscle to reach its full potential for force generation, each of these processes must be capable of maximally activating subsequent processes. Failure at any level will ultimately result in submaximal force generation (Green, 1990).

1.5.3 THE ROLE OF PCr DEPLETION IN FATIGUE

1.5.3 (i) Overview

As described in Sect. 1.2.2.(ii), the presence of PCr allows the anaerobic resynthesis of ADP to ATP via the creatine phosphokinase reaction. Because of the high maximum activity of CPK, and the close proximity of the enzyme (CPK) to reactants in the cytoplasm (Hultman and Sjöholm, 1983a), it

is through the degradation of PCr that ATP is resynthesized at the highest rate (Hultman et al., 1990).

Due to the low resting concentration of PCr in muscle, this high rate of ATP resynthesis can only be maintained for a short period of time, and depletion of PCr is often cited as a primary cause of muscular fatigue during short-term high-intensity activity (For review, see Fitts, 1994). This interpretation is based on the assumption that once PCr is depleted, ATP from glycogenolysis can not be resynthesized at a fast enough rate to meet the demand (Sahlin et al., 1987).

The depletion of PCr stores has been closely related to fatigue during both isometric contractions (Karlsson et al., 1975; Sahlin and Ren, 1989) and dynamic cycling exercise where the decrease in power output was found to parallel the decrease in contribution of PCr to ATP resynthesis (Boobis, 1987). In addition, electrical stimulation studies have shown that force can be well maintained as long as PCr is available, but decreases thereafter (Hultman and Greenhaff, 1991; Hultman et al., 1990). Finally, single fiber analyses suggest that PCr depletion specific to type II fibers may be responsible for the decline in whole muscle force production (Greenhaff et al., 1991; Greenhaff et al., 1993; Hultman and Greenhaff, 1991; Söderlund et al., 1992; Söderlund and Hultman, 1991).

In contrast, several experiments have indicated that the fall in PCr is not linearly related to the drop in force

generation (Miller et al., 1987; Jones et al., 1985; Spriet et al., 1987). PCr falls extremely rapidly for the first few seconds, while the decline in force is more gradual, suggesting that a direct cause and effect relationship is unlikely. This argument is supported by the work of Bergström and Hultman (1988) who found that intermittent electrical stimulation (1:1 work to rest ratio) given at different durations of stimulation (at the same frequency with the same total contraction time) produced dissimilar decreases in force generation (range from 90% to 35% of initial force) even though each model showed an equal decrease in PCr content (90%). Therefore, in this stimulation protocol, PCr could not be shown to be the cause of the variations in decreased force.

1.5.3 (ii) PCr and early contractile failure

Hultman and Sjöholm (1983b) demonstrated that during electrical stimulation at 50 Hz, the rate of PCr utilization peaked immediately upon onset of contraction, but began to decline after only 1.28 seconds. The corresponding rate of glycogenolysis did not peak until 3 seconds of contraction. Therefore due to the momentary lag in energy provision from glycogenolysis, most of the energy during the initial phase of intense activity was considered to have been derived from PCr breakdown (Hultman and Greenhaff, 1991). In a recent review, Maughan and Greenhaff (1991) suggested that it is the rapid

decrease in the utilization of PCr during this initial acceleration phase that is responsible for the drop in maximal velocity which occurs in elite sprinters after 50m, as well as the drop in peak power which ensues after only a few seconds of a maximal cycling effort. This hypothesis is supported by the work of Hirvonen et al. (1987) who assessed running speed in elite sprinters (40-100m) and found that a decrease in speed began when the PCr stores were depleted.

Dietary manipulation in rats also lends support to the suggestion that PCr may play a role in fatigue by resisting early contractile failure during vigorous muscular activity (Meyer et al., 1986). Gradual replacement of PCr can occur by substituting the inert analogue B-guanidinopropionatephosphate (B-GPAP) for creatine in the diet. NMR studies have revealed that upon exercise in the B-GPAP substituted rat, the small amount of PCr that is still present is immediately hydrolysed. This depletion of PCr is coincident with a precipitous drop in tension development which is far in excess of that observed in normal control muscle with ample PCr availability (Meyer et al., 1986). This implication that PCr may effectively resist early fatigue must be taken with caution, since dietary manipulation also affects total ATP stores (ATP decreased to 56% resting levels) and similar experimentation is not possible with human subjects.

1.5.3 (iii) Cr supplementation and fatigue

Recent literature has suggested that oral supplementation of creatine (Cr) leads to increases in muscle total creatine and PCr content (Harris et al., 1992). Several subsequent studies have demonstrated that Cr ingestion can significantly increase the amount of work performed during repeated bouts of maximal exercise (Balsom et al., 1993; Greenhaff et al., 1993; Harris et al., 1993). It has been hypothesized that the ergogenic effect of Cr feeding is due to both a rise in the muscle PCr stores, as well as to an enhanced rate of PCr resynthesis between bouts (Greenhaff et al., 1993). If the level of PCr in the muscle could be increased, then the required rate of ADP phosphorylation might be sustained longer during contraction, thus delaying fatigue.

The present literature on Cr loading, however, is controversial. Although Harris et al. (1992) reported that 2-5 days of Cr supplementation led to a significant (up to 20%) increase in PCr in biopsy samples of 17 normal subjects, Odland et al. (1994) found no increase in PCr content in 8 normal subjects after 3 days of Cr loading. Consequently, Cr supplementation had no effect on a single 30s bout of maximal cycle exercise (Odland et al., 1994). Similarly, studies investigating repeated exercise bouts have shown minimal changes in performance during the initial bout of exercise

(Balsom et al., 1993; Greenhaff et al., 1993). These results indicate either that Cr supplementation does not significantly increase muscle PCr (as demonstrated by Odland et al., 1994), or, the unloaded (i.e., no Cr feeding) pre-exercise PCr content is not limiting. That is, an increase in PCr before a single bout of maximal exercise may not enhance performance because there is already sufficient PCr; however, in situations where PCr concentration is lower (such as in the case of repeated bouts), an increase in PCr may exert a much greater effect.

1.5.3 (iv) Summary

In summary, it appears that the concentration of PCr plays a role in preventing fatigue during the initial stages of supramaximal exercise, but the mechanisms regulating energy provision during this very short time period are not well understood.

1.5.4 FATIGUE OF THE ANAEROBIC GLYCOGENOLYTIC PATHWAY

Anaerobic glycogenolysis involves a series of tightly controlled enzymatic reactions resulting in a relatively rapid rate of ATP resynthesis. Decreased glycogenolytic / glycolytic rate has been proposed as a cause of muscle fatigue and related to inhibition of the glycogenolyic / glycolytic enzymes by metabolic by-products and decreased pH (Hultman et

al., 1990). As exercise progresses, changes occur in the intramuscular environment which have been subsequently linked to the slowing of glycogenolysis. These changes include increased content of glycolytic hexosephosphates and accumulation of lactic acid. Because of its low pK in the physiological state, lactic acid exists primarily in its ionized form [lactate ions + H^+]; consequently, accumulation has been associated with a large fall in muscle pH.

Although brief high-intensity exercise will require the maximal rate of glycogenolysis, it is unlikely that in this very short time span (<10s), metabolites will accumulate or muscular pH will drop to inhibitory levels. Thus, the rate of glycogenolysis during this type of activity will largely be determined by the regulation of the flux-generating enzyme glycogen phosphorylase, as previously discussed in Section 1.4.2 (ii).

1.5.5 THE ROLE OF ATP CONCENTRATION IN FATIGUE

During high-intensity exercise, the rate of ATP resynthesis has often been found to decline at approximately the same rate as the force generation (Hultman et al., 1990). The question arises as to whether it is the observed decline in the rate of ATP resynthesis that inhibits force, or are other factors involved which decrease the rate of ATP

hydrolysis such that energy demand and force generation are subsequently lowered?

The latter argument is supported by the work of Bergström and Hultman (1988) who found that varied durations of intermittent stimulation (at the same frequency) led to varied rates of ATP utilization. Interestingly, the stimulation protocol which produced the least amount of force, also showed the highest rate of ATP utilization - thus indicating that the availability of energy cannot be directly related to force production.

As previously discussed (Sect. 1.2.2 (i)), ATP is required for the optimum performance of all cell ATPases. The sarcolemma ATPase functions to maintain the proper ionic gradients for Na^+ and K^+ across the cell membrane. Alterations in sarcolemmal function have been suggested to induce muscle fatigue through the prevention of cell activation (Lindinger and Sjøgaard, 1991; Sjøgaard, 1991). During high-intensity activity the Na^+-K^+ pump may be unable to keep pace with the K^+ efflux and the Na^+ influx, consequently leading to cell depolarization and reduced action potential (AP) amplitude (Lindinger and Sjøgaard, 1991). Decreased AP amplitude has, however, not been conclusively shown to compromise the muscle's ability to generate force (Metzger and Fitts, 1986), and a considerable safety factor is thought to exist regarding

the extent of depolarization necessary for full activation (Sandow, 1952).

Muscle fatigue is also associated with a prolonged relaxation time, indicating that during fatigue, the rate of resequesteration of Ca^{++} from the SR is reduced (Byrd et al., 1989). Although ATP is required for the re-uptake of Ca^{++} into the SR, considerable evidence exists to suggest that it is increased H^{+} (rather than insufficient ATP) that compromises the functional capacity of the SR pump protein (Cady et al., 1989; Inesi and Hill, 1983; Sahlin et al., 1981).

The data reviewed above suggests that cell ATP content does not directly limit force production; however, the most convincing argument comes from the fact that even during the most strenuous exercise, ATP rarely falls below 60-70% of the pre-exercise level (Thompson and Fitts, 1992), a content that should be sufficiently greater than the Km for ATPase (Nanninga and Mommaerts, 1960). Although the possibility of localized ATP depletion at the contractile site cannot be ruled out (Cheetham et al., 1986), it appears unlikely because the estimated ATP concentration required for full cross-bridge tension [100 $\mu mol \cdot kg^{-1}dm$ (Godt, 1974)] is less than 1% of biochemically measured post fatigue cell ATP. Thus, for compartmentalization to occur, approximately 99% of the whole cell ATP content must be unavailable to the cross-bridges.

Consequently, it appears unlikely that the fatigue which occurs during exercise can be accounted for by ATP depletion.

1.5.6 NEUROMUSCULAR TRANSMISSION FAILURE

It has been postulated that during high-intensity exercise, action potential dropout, or neuromuscular transmission failure, may be a causative agent in reduced force production, or fatigue (Bigland-Ritchie et al., 1979). This phenomenon is, however, generally associated with unphysiologically high stimulation frequencies (Gardiner and Olha, 1987). Furthermore, early research by Brown and Burns (1949) indicated that although neuromuscular block could occur at physiological stimulation frequencies, this block could not be held solely responsible for the concomitant decrease in tension. Similarly, Lüettgau (1965) observed that when frog muscle fibers were stimulated above 40 Hz, action potentials dropped out without any effect on mechanical force production.

It is important to realize that as fatigue develops, there is a consequent increase in the rate of relaxation, and the optimal stimulation frequency for force development will decrease (i.e., the force-frequency curve will shift to the left). Therefore, the reduced neural drive and α-motor nerve activation frequency which are frequently observed during high-intensity exercise (Bigland-Ritchie et al., 1986) are thought to function as protectors against the development of

fatigue, rather than causative agents (Bellemare and Garzaniti, 1988).

1.6 RECOVERY

1.6.1 OVERVIEW

Immediately following exercise, a number of processes are activated to restore body homeostasis. This restoration includes the replenishment of the energy and oxygen stores that were depleted, and the removal of waste products that were accumulated during exercise. Following very short duration high-intensity exercise these processes will include the resynthesis of PCr and the removal of lactic acid from the muscles and the blood; both of which processes occur in the post exercise recovery period and require energy from ATP.

1.6.2 ENERGY REQUIREMENT FOR RECOVERY

To provide the energy necessary for the processes of recovery, muscles take up increased amounts of oxygen at the completion of exercise (Margaria, 1933). The length of this elevated O_2 uptake period will be dependant upon the intensity and duration of the preceding exercise (Bahr et al., 1987) and the rate at which O_2 is taken up will not be constant, but rather will decrease exponentially with time. During the first 2 or 3 minutes of recovery, O_2 uptake decreases very

rapidly (rapid recovery phase) then more slowly until a constant rate is reached (slow recovery phase) (Margaria, 1933).

The initial rapid phase of elevated O_2 uptake has been correlated with the restoration of myoglobin bound O_2 stores and the resynthesis of the high energy phosphates (ATP and PCr) (diPrampero et al., 1973; Harris et al., 1976; Piiper and Spiller, 1970). ATP is produced through oxidative metabolism and serves to replete diminished ATP stores, as well as to resynthesize PCr via the reversible CPK reaction. PCr resynthesis has been clearly established as an O_2 dependant process since several investigations have reported that occluded circulation prevents any resynthesis of PCr following intense exercise (Harris et al., 1976; Quistorff et al., 1992; Blei et al, 1993).

The O_2 consumed during the slower recovery phase was originally assumed to be quantitatively related to the removal of lactic acid accumulated in the blood and muscles during exercise (Margaria, 1933). The 'O_2 debt' concept of Hill et al. (1924) was that lactate produced during exercise was converted back to glycogen during recovery. The extra O_2 consumed during recovery was thus expected to provide the energy necessary for this restitution of glycogen from lactate. It is now known, however, that the postexercise fate of lactate is mainly to oxidation (Brooks and Gaesser, 1980).

In addition, the elevated O_2 consumption during this slow recovery phase can now be explained by a number of physiological events including (but not limited to) elevated body temperature, the O_2 cost of ventilation and heart activity, and the effect of catecholamines (Hagberg et al., 1980; Gaesser and Brooks, 1984). Finally, direct measures of O_2 utilization at the muscle level have been compared to O_2 uptake at the mouth, and the values were found to be unrelated (Bangsbo et al., 1990).

1.6.3 RESYNTHESIS OF PCr

Literature pertaining to the resynthesis of PCr following brief maximal exercise is limited. Early research involving muscle biopsies taken from the quadriceps muscle group has indicated, however, that PCr depleted in muscle during exhaustive submaximal exercise is restored very quickly, and occurs in a biphasic manner. Hultman et al. (1967) reported that following 10 min of intense cycle ergometry, phosphagen restoration was 70% completed during the initial fast phase (20-30s) and essentially 100% complete during the second slower phase (3-5 min). Several subsequent investigations confirmed these results (Harris et al., 1976; Karlsson et al., 1975; Sahlin et al., 1979). The second slower phase of PCr recovery shows a high inverse correlation with muscle lactate, and Harris (1976) has theorized that the

equilibrium state of the CPK reaction is altered by the associated H^+, thus slowing PCr recovery. The initial rapid phase of PCr recovery is, however, not affected by pH, since during this phase pH will continue to fall due to the H^+ produced through PCr resynthesis.

Recent literat^Txe has utilized ^{31}P-NMR to follow the depletion and resynthesis of PCr. Bangsbo et al. (1993) found that a 40s isometric contraction of the calf muscle (90% MVC) led to a 71% decrease in PCr. After 1 min of recovery, PCr had increased to 88% of the pre-exercise value (Bangsbo et al., 1993). Similar findings were reported by Quistorff et al. (1992).

1.6.3 (i) Single fiber analysis

It was mentioned in section 1.5.3 that PCr depletion specific to type II fibers may be responsible for fatigue during brief maximal exercise. This is because single fiber analyses have indicated that PCr depletes more rapidly in these highly glycolytic fibers, as compared to the slow oxidative type I fibers (Tesch et al., 1989; Söderlund and Hultman, 1991; Söderlund et al., 1992). The resynthesis of PCr specific to single fibers has also been measured, and, following intense electrical stimulation (Söderlund and Hultman, 1991) or voluntary contraction of the knee extensors (Tesch et al., 1989), 1 min of oxidative recovery has been

shown to lead to a significantly higher PCr content in type I fibers compared to type II fibers. This more rapid resynthesis of PCr in type I fibers has been attributed to a higher mitochondrial density and capillary supply of the highly oxidative type I fibers (Söderlund and Hultman, 1991).

1.7 LACTATE CLEARANCE

During exercise at workloads exceeding 50-60% of $\dot{V}O_2$ max, lactic acid is formed through the anaerobic breakdown of glycogen and/or glucose, and will accumulate in the muscle and the blood. At physiological pH, lactic acid is almost completely dissociated and hydrogen ions (H^+) are therefore formed in an amount equivalent to lactate (Katz and Sahlin, 1990). Most of the H^+ produced will be buffered within the tissue and only a small fraction (<0.001%) appears as free ions which causes a decrease in pH (Sahlin, 1986).

The lactate ion is not known to have any direct adverse effect on energy metabolism, or on the contraction process (Sahlin, 1986). In contrast, a decrease in muscle pH may influence many of the processes involved in the pathway transforming chemical energy into chemical work (Fitts, 1994). These potential effects of H^+ concentration on the processes of fatigue have been extensively researched and reviewed elsewhere (Jones and Heigenhauser, 1992); therefore, they will not be discussed here. Rather, the processes of lactate / H^+

clearance during recovery following intense exercise will be addressed.

1.7.1 RATE OF LACTATE REMOVAL

Early research by Karlsson and Saltin (1971) demonstrated that following 5 1-min bouts of cycle ergometry, muscle lactate increased significantly, and approximately 1 hr was required to remove most of the accumulated lactic acid. Similar results were reported by Fox et al., (1969) following exhaustive treadmill exercise. In general it can be said that following intense exercise approximately 25 min of rest are required following maximal exercise in order to remove half of the accumulated lactic acid (Hermansen et al., 1975).

The data reviewed above are based on subjects undergoing complete rest following intense exercise. The performance of light exercise following exhaustive exercise has been shown to greatly increase the rate of lactate clearance (Bonen and Belcastro, 1976). This phenomenon was illustrated by having male subjects run a mile on 3 separate days, followed by 3 separate recovery periods: 1)rest, 2)continuous jogging at self selected pace, and 3) intermittent activity. Both of the active recovery protocols resulted in substantial increases in the rates of lactic acid removal from the blood (Bonen and Belcastro, 1976).

Unfortunately, there is little or no literature available regarding the rate of lactate removal following brief maximal exercise. Exercise of this type is often performed intermittently - rather than in single bouts, and consequently blood and muscle lactate concentrations usually remain elevated between bouts and the rates of lactate removal from muscle tissue are rarely investigated in the final recovery period.

1.7.2 LACTATE AND H+ TRANSLOCATION

The accumulation of lactate associated with intense exercise results in a decrease in pH which may impair muscle function. In an open system it is difficult to quantify the accumulation of lactate because the amount will be dependant not only on lactate production, but also on the dynamics of the lactate efflux from the muscles.

Evidence suggests that the sarcolemma membrane forms a rate-limiting barrier for the release of lactate (for review, see Mainwood and Renaud, 1985), however, investigation of lactate transport dynamics across the sarcolemma is complex because the lactate content of the muscle cannot be measured continuously, and some of the lactate produced will be metabolized by neighbouring fibres (Brooks, 1986). In addition, efflux of lactate may be dependent on blood flow and muscle capillarization.

Experiments involving isolated and perfused muscle tissue have indicated that at resting pH, passive diffusion does not appear to be the major mechanism by which lactate crosses the sarcolemma (Roth and Brooks, 1990). As pH declines, however, lactic acid diffusion probably contributes a larger component to net flux (Moon et al., 1987).

The preponderance of recent evidence suggests the major component of lactate efflux is mediated by a lactate-proton co-transport mechanism (Juel and Wibrand, 1989; Roth and Brooks, 1990;Juel, 1991). The lactate transporter has been found to be a membrane-bound protein which demonstrates saturation kinetics, stereospecificity, and inhibitor specificity (Juel and Wibrand, 1989; Roth and Brooks, 1990;Juel, 1991). At pH 7.4 carrier-mediated lactate flux has been reported to make up approximately 80% of the total transport capacity (Juel and Wibrand, 1989), while the remaining 20% is expected to consist of diffusion of undissociated lactic acid. In addition, pH recovery has been shown to be accelerated by activation of sodium ion (Na^+)/ H^+ exchange (Vighe et al., 1982). The inorganic anion exchange does not, however, appear to be involved in lactate efflux or pH recovery following muscular activity (Juel and Wibrand, 1989).

1.8 PURPOSE OF THE INVESTIGATION

From the preceding review of the processes of anaerobic muscle metabolism and their regulation and control, it is apparent that there have been surprisingly few investigations of these pathways during very brief maximal exercise; however, it is these brief bursts of activity that often determine success or failure in competitive sport. The purpose of the present investigation then, was to determine the relative contribution of the anaerobic energy delivery pathways to ATP provision during maximal voluntary exercise 2-14s in duration, and to gain a greater understanding of the specific processes of energy delivery pathway regulation and mechanisms of fatigue during such exercise. In addition, the recovery processes following exercise were investigated in order to determine if depletion and repletion kinetics were similar.

CHAPTER II

ANAEROBIC ENERGY SUPPLY DURING MAXIMUM-INTENSITY SHORT-TERM VOLUNTARY SUSTAINED EXERCISE IN MAN

2.1 INTRODUCTION

Adenosine triphosphate (ATP) is the immediate energy source for contraction by skeletal muscle. During high-intensity short-term exercise where energy demand exceeds that which can be met by oxidative delivery processes, ATP must be resynthesized anaerobically by the breakdown of glycogen to lactate and the degradation of phosphocreatine (PCr). It was originally assumed that during intense exercise, energy provision from the separate pathways was linked sequentially (Margaria et al., 1964, 1969). PCr depletion was thought to occur over the first 10s of maximal activity with further contractile activity being sustained through anaerobic glycogenolysis. Research has since indicated that PCr breakdown and glycogenolysis may proceed concomitantly from the onset of exercise (Boobis et al., 1982; Hultman and Sjöholm, 1983a,b; Jacobs et al., 1983; Jones et al., 1985).

Despite evidence of 'simultaneous mobilization' of PCr and anaerobic glycogenolysis, the time course and relative contribution of each pathway to ATP production in voluntary

maximal contraction of skeletal muscle remains to be elucidated. Although ^{31}P-Nuclear Magnetic Resonance (NMR) permits non-invasive measurement of muscle PCr concentration, present technology requires 8-12s for an NMR scan (Blei et al., 1993; Yoshida and Watari, 1993). Since metabolite concentrations are averaged over the entire length of the scan, second to second changes which may occur during maximal exercise of short duration are difficult to monitor. Furthermore, one cannot directly quantify lactate production with this methodology. Knowledge is therefore limited since few invasive studies exist where muscle metabolites have been measured following maximal voluntary exercise less than 30s in duration.

Some recent studies suggest that PCr depletion and anaerobic glycogenolysis may contribute equally to the production of ATP during the first 6s of maximal cycle ergometry (Boobis, 1987; Gaitanos et al., 1993). Additionally, large increases in muscle lactate have been observed with a 70-75% drop in PCr content following 10s of isokinetic cycling (Jones et al., 1985). Jacobs et al. (1983) reported muscle lactate values of 46.1 mmol·kg^{-1}dm (dry muscle) after 10s of maximal cycling, but resting lactate and resting and 10s PCr concentrations were not determined. A systematic evaluation of the time course for muscle lactate accumulation

in combination with PCr depletion during maximal voluntary exercise, however, has not been conducted.

Hirvonen et al. (1987) studied PCr breakdown and muscle lactate production following various durations of maximal sprint running exercise. In well trained athletes, PCr decreased from 10.3 to 3.8 $mmol \cdot kg^{-1}$ wet wt in 5.5s (40m), while muscle lactate increased to 5 $mmol \cdot kg^{-1}$ wet wt. Following 11s of exercise (100m) no further depletion of PCr occurred, yet muscle lactate continued to increase. With such designs, however, the escape of lactate from the muscle to the interstitial and plasma space makes it difficult to accurately assess the contribution of anaerobic glycogenolysis to overall ATP production.

Closed circulation prevents significant O_2 and glucose uptake and lactate efflux from the muscle. Hultman and Sjöholm (1983a,b) used this approach with electrical stimulation. Following a 1.26s period of stimulation at 50 Hz (near maximal contraction) 80% of the total ATP turnover was derived from PCr breakdown, while 20% was produced through degradation of glycogen to lactate (1983b). Further stimulation (1.26s - 2.56s) demonstrated an increase in the contribution of glycogenolysis to ATP production (50%). In a subsequent study (Hultman and Greenhaff, 1991) using a similar protocol biopsies were taken at rest, after 5, 10, 20, and 30s. The estimated rate of ATP resynthesis from PCr declined

from 5.3 mmol·kg^{-1}dm·s^{-1} during the first 5s to 0.2 mmol·kg^{-1}dm·s^{-1} between the 20th and 30th second of contraction, while the energy provision from glycogenolysis was steady at 4.5 mmol·kg^{-1}dm·s^{-1} during the initial 20s, and declined to 2.1 mmol·kg^{-1}dm·s^{-1} for the final 10s (20-30s).

The preceding results are indicative of anaerobic energy provision during intermittently electrically stimulated skeletal muscle, and not maximal voluntary exercise. The present research examined energy supply during short-term maximal voluntary exercise with occluded circulation, and muscle samples were obtained every 2s between 0 and 14s.

Literature pertaining to the resynthesis of PCr and the clearance of lactate following exercise of short duration is also limited. Early research has indicated that PCr depleted in muscle during exhaustive submaximal exercise is restored very quickly. Hultman et al. (1967) reported that phosphagen restoration was 70% completed in 30s and essentially 100% completed within 3 to 5 min following 10 min of cycle ergometry. Several subsequent investigations tend to confirm this suggestion (Harris et al., 1976; Karlsson et al., 1975; Sahlin et al., 1979). In addition, several recent studies have estimated PCr recovery at a single time point following short-duration maximal exercise (Gaitanos et al., 1993; Jansson et al., 1990; McCartney et al., 1986; Tesch et al., 1989), but no study to date has systematically

investigated the time course for PCr restoration following maximal exercise of less than 30s. The present research collected muscle samples 10, 20, 30, 40, 60, 120, and 180s after a 14s maximal isometric contraction.

The purposes of the present experiment were (1) to determine the time course (every 2s up to 14s) of PCr depletion and muscle lactate accumulation during a sustained maximal voluntary contraction of the quadriceps muscle; and, (2) to determine the time course for resynthesis of PCr and for lactate clearance during recovery from a 14s maximum contraction.

2.2 METHODS

2.2.1 SUBJECTS

Eleven physically active and 'motivated' subjects were recruited (age = 22.2 ± 1.6 yr, height = 175 ± 9.12 cm, mass = 78.2 ± 9.56 kg). Subjects were screened to ensure each was able to give a consistent maximal effort over several testing sessions. Nine of the 11 subjects were familiar with the muscle biopsy procedure and had participated in previous studies. The experimental procedures, possible risks and benefits were explained to each subject before written consent was obtained. The study received ethics approval from the Human Ethics Committee of McMaster University (Appendix I).

2.2.2 DESIGN

Exercise consisted of unilateral voluntary sustained maximal isometric extension of the quadriceps muscle group. Ten needle biopsy samples [2 pre-exercise (PRE-EX), 4 exercise (EX), and 4 recovery (REC) samples] were obtained from each subject over 3 separate testing sessions using the Bergström technique (Bergström, 1962) and modified for suction. On each occasion, the entire biopsy needle was plunged into liquid nitrogen immediately following sampling (1-2s). In each of the first 2 sessions, 1 PRE-EX sample and 2 EX samples (1 from each leg) were obtained from each subject. Subjects were randomly assigned to a pre-determined schedule of contraction times such that (in total) 6 muscle samples were collected at each time point (every 2s up to 14s). In order to ensure adequate healing of the biopsy wound such that maximal contractions could be elicited, at least 10 days separated subsequent testing sessions.

Recovery data was collected in the final session. All subjects performed a maximal contraction for 14s, and biopsies were collected 10, 20, 30, 40, 60, 120, and 180s following exercise. Similar to the EX sessions, a schedule of sampling times was implemented such that a total of 6 muscle samples were taken at each time point. Following each 14s contraction, two biopsies (rather than one, as in the EX sessions) were taken in succession from the exercised leg.

2.2.3 PROTOCOL

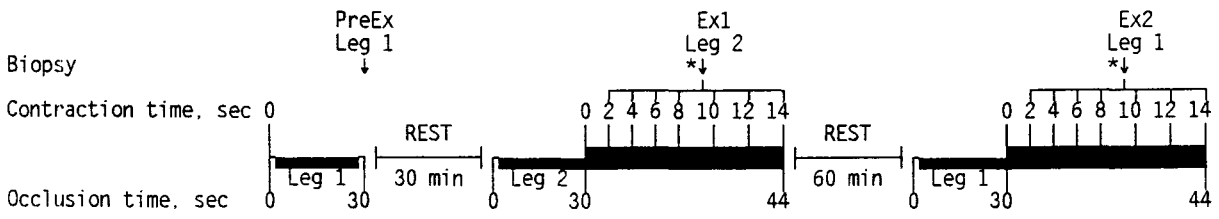
Prior to the actual testing sessions, each subject was familiarized with the trial procedure (Figure 2.1). The subjects were seated with their lower leg positioned 90° to the horizontal. The upper and lower leg were both firmly secured to the dynamometer with velcro straps. Maximal quadriceps extension torque was measured via a force plate located just above and in front of the ankle. Visual feedback of muscle force was provided on a computer screen and subjects were trained to provide a maximum effort throughout each contraction.

The sampling site selected for muscle biopsies was the lateral potion of the quadriceps muscle (vastus lateralis). Circulation was occluded 30s before the start of each contraction with a thigh blood pressure cuff inflated to 280 mm Hg in order to create an anaerobic environment, to prevent blood from reaching the muscle, and to prevent metabolites from escaping the muscle following contraction (i.e. before muscle sampling). The circulation was also occluded for 30s prior to the PRE-EX biopsies in order to provide a standard reference point for subsequent analysis.

Subjects were instructed not to perform strenuous exercise for 24 hours before each test occasion, but were encouraged to conform to their habitual diets. On each test

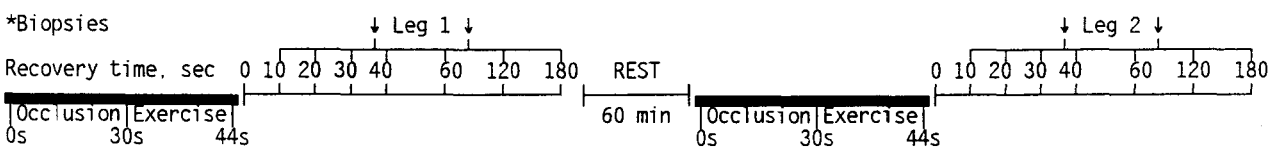
Figure 2.1a,b Schematic representation of experimental design. Each subject participated in 2 EX sessions (*top*, a), and 1 recovery session (*bottom*, b).

2.1 A Exercise sessions



* During the Ex sessions the biopsy was taken at the pre-determined time point between 2-14s

2.1 B Recovery sessions



* After each contraction, 2 biopsies were taken in succession at the pre-determined time points

day, subjects arrived at the lab after a 2 hour fast. For the first 2 sessions, each subject rested quietly for 5-10 minutes upon arrival before both legs were prepared and anaesthetized (lidocaine 2% without epinephrine) for biopsy sampling. The circulation to the PRE-EX leg was then occluded for 30s, the PRE-EX sample was obtained, and the pressure cuff was released. The incision was closed with a single suture, and ice and pressure were applied. Following the PRE-EX sampling procedure, the subject was positioned in the knee extension device, the EX leg was firmly strapped, and the incision for the first EX sample was made. The pressure cuff was inflated for 30s, the subject contracted maximally for the pre-determined time period, the biopsy sample was taken immediately upon relaxation, the entire needle was plunged into liquid nitrogen, and the cuff was released. The incision was closed with a suture, and the subject rested passively for a minimum of 60 min before the second EX sample was taken from the same leg as the PRE-EX sample.

Upon arrival at the REC sessions, subjects were positioned in the knee extension device, and the exercise leg was prepared for the biopsy procedure. Because 2 muscle samples were obtained in the recovery period following each contraction, both incisions were made prior to exercise. The cuff was inflated for 30s, the subject contracted maximally for 14s, occlusion was released within the first 2s of the

contraction, and 2 biopsies were collected in succession at the pre-determined time points following exercise. The biopsy wounds were stitched, and again ice and pressure were applied while the subject rested (60 min) before the opposite leg was tested.

2.2.4 ANALYSIS

2.2.4 (i) Muscle powdering procedure.

All tissue was stored in liquid nitrogen, or frozen at -70° lyophylization. Lyophylized samples were powdered according to the following procedure: The biopsy was first broken apart on a piece of tissue paperto allow the absorption of blood onto the tissue paper. Large pieces of visible connective tissue were removed with tweezers. The biopsy was then pulverized between the ends of 2 pairs of tweezers. Fractions of pulverized tissue were searched for connective tissue, which was subsequently removed by scraping the tissue fraction against the inner rough edge of a pair of tweezers. Muscle tissue flaked away, while connective tissue did not. Each sample of clean powdered tissue was carefully weighed out and divided into small eppendorf tubes for extraction of glycogen (>2 mg) and metabolites (>5 mg). The freeze-dried powdered tissue was stored dessicated at -70° until extraction. When samples were sufficiently large to allow it,

duplicate extraction and analyses were performed on separate aliquots of muscle tissue.

2.2.4 (ii) Metabolite extraction procedure.

All EX muscle tissue was analyzed for concentrations of ATP, ADP, PCr, total creatine (TCr), glucose-6-phosphate (G-6-P), glyceraldehyde-3-P (G-3-P), and lactate. REC tissue was analyzed for ATP, PCr, TCr, and lactate. Pre-weighed, freeze-dried samples were brought to room temperature in a dessicated container. Metabolites were then extracted with 0.5 M perchloric acid (PCA) (1 mM EDTA), and later neutralized with 2.2 M potassium bicarbonate ($KHCO_3$). Immediately following extraction, samples were assayed enzymatically (Harris et al., 1974; Appendix VII) on a Beckman spectrophotometer for the concentrations of tissue lactate, G-3-P, G-6-P, ATP, and PCr. Extracted samples were stored at -70°, and later analyzed enzymatically for Cr and ADP. All metabolites, except lactate (due to its relatively high extracelluar presence), have been adjusted to the highest total creatine content for each subject. All metabolites are given as millimoles per kilogram of dry muscle ($mmol \cdot kg^{-1}dm$).

2.2.4 (iii) Glycogen extraction procedure.

Pre-weighed, freeze-dried samples were brought to room temperature in a dessicated container. Background glucose and

hexose monophosphates were destroyed by incubating the samples in 0.1 M sodium hydroxide (NaOH) at 80°C for 10 min. Following incubation, samples were cooled and neutralized with a mixture of hydrochloric acid (HCl) and citric acid. Glycogen was then degraded by incubating the samples in amyloglucosidase (AGS) for 1 hour at room temperature. Samples were assayed enzymatically for glucose content.

2.2.4 (iv) Calculations.

Given that the blood flow to the exercising muscle group was occluded prior to and during all contractions, the following calculations were made using the measured metabolite concentrations (Spriet et al., 1987).

[1] Total ATP production = ▲PCr + 1.5▲Lactate

[2] Rate of ATP turnover = (▲PCr+1.5▲Lactate) / time (s)

[3] Rate of glycogenolysis =

[▲G6P + (.33 * ▲G6P) + .5(▲Lac + ▲G3P)] / time (s)

[4] Rate of glycolysis = .5(▲Lac + ▲G3P) / time (s)

The calculation for total ATP production is expressed in $mmol \cdot kg^{-1}$ dry muscle, while all calculations of rate are expressed in $mmol \cdot kg^{-1} \cdot s^{-1}$ dry muscle . Measurable changes in ATP and ADP are usually included in the calculations for total ATP production and ATP turnover rate; however, since in the

present study the concentrations of ATP and ADP remained constant throughout the contraction protocol, these values were not included in the calculations. In addition, the accumulation of hexose monophosphates other than G-6-P (▲glucose-1-phosphate + ▲fructose-6-phosphate) is assumed equivalent to (0.33 * ▲G-6-P) (Harris et al., 1981). Finally, the accumulation of pyruvate was neglected, since it was assumed to represent <2% of the ATP production (Spriet et al., 1987).

2.2.4 (v) Statistical methods

The nature of the experimental design experiment disallowed the use of analysis of variance. Linear and exponential regression analyses were employed, however, metabolite changes, and significant differences between means were determined using the Student's paired *t*-test. The limit for statistical significance was set at $p \leq 0.05$, and values are presented as means±SE unless otherwise stated.

2.3 **RESULTS**

2.3.1 EX SESSIONS

2.3.1 (i) Torque

In the EX testing sessions, each subject performed 4 maximum voluntary contractions (MVC) of the knee extensor muscles. Two MVC's were performed in each test session (1 by each leg), and each contraction differed in its duration which was pre-determined to last between 2 and 14s. Mean peak torque produced by subjects in the left and right legs was 268.91±12.48 Nm and 272.00±14.44 Nm, respectively. The decrease in torque production during each contraction was calculated by expressing the mean torque produced during the final 2s of the contraction as a percentage of the peak torque. There was no measurable decrease in torque production during contractions lasting less than 6s, and the largest decline occurred during contractions lasting 14s where torque was reduced to 91.95±1.74% of the peak (Fig. 2.8).

2.3.1 (ii) Metabolite data

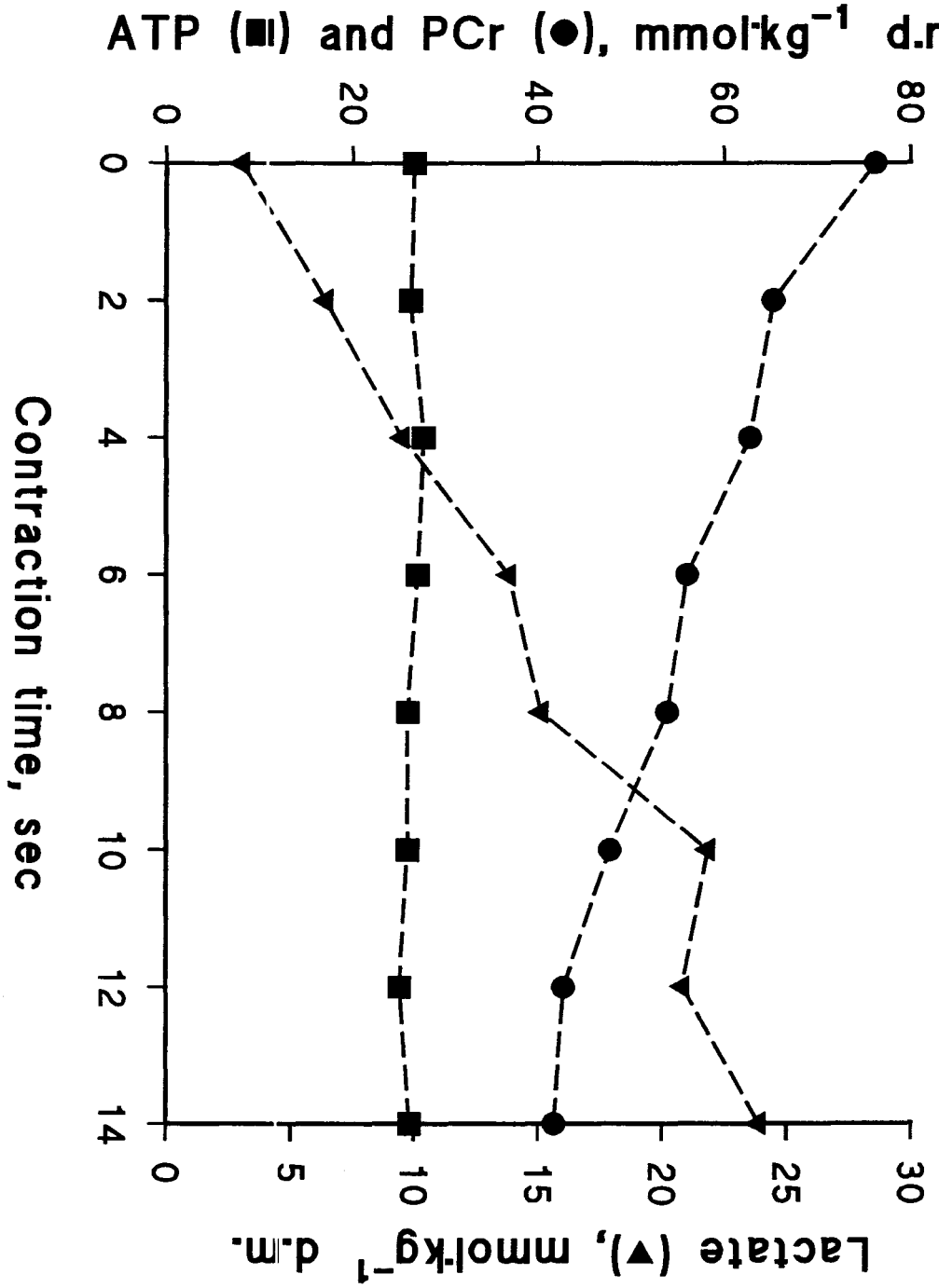
Resting metabolite concentrations (Table 2.2) were in the normal range for human skeletal muscle. Muscle ATP (Table 2.2, Fig. 2.2) and ADP (Table 2.2) remained constant

TABLE 2.2. Muscle metabolite concentrations at rest and following maximal voluntary isometric contractions with occluded circulation.

Metabolite	Rest (n=11)	Duration (s) 2.0 (n=7)	4.0 (n=7)	6.0 (n=6)	8.0 (n=6)	10.0 (n=5)	12.0 (n=6)	14.0 (n=6)
ATP	26.7±0.4	26.4±0.5	27.7±0.4	27.1±0.7	26.0±0.7	26.0±0.7	25.1±0.6	26.2±0.8
ADP	2.0±0.2	1.9±0.1	1.9±0.2	2.1±0.1	1.7±0.2	2.3±0.3	2.3±0.3	2.2±0.5
PCr	76.2±2.2	65.3±5.2	62.8±2.2	56.1±4.9	53.9±3.1	47.7±5.5	42.7±3.5	41.7±3.9
Lactate	3.0±0.3	6.4±0.7	9.5±0.9	13.8±1.3	15.1±1.2	21.8±1.0	20.8±2.1	24.0±2.2
G6P	1.0±0.2	1.9±0.3	3.2±0.5	2.1±0.6	3.9±0.3	5.7±1.2	5.3±0.7	7.4±0.7
G3P	0.5±0.2	0.6±1.1	1.3±0.7	1.7±0.4	2.6±0.2	1.8±2.3	1.8±1.1	3.1±1.0
Cr	44.3±1.5	51.8±2.2	58.2±2.7	64.3±4.3	65.8±4.3	72.9±2.3	76.6±4.2	78.0±4.9
TCr	120.5±2.8	117.1±4.4	121.0±2.6	120.3±4.8	119.7±4.7	120.6±4.4	119.3±3.9	119.7±3.9

Values are means ± SE in $mmol \cdot kg^{-1}$ dry muscle. All metabolites were determined enzymatically. ATP, adenosine triphosphate; ADP, adenosine diphosphate; PCr, phosphocreatine; G6P, glucose-6-phosphate; G3P, glycerol-3-phosphate; Cr, creatine; TCr, total creatine.

Figure 2.2 Muscle phosphocreatine (PCr), adenosine triphosphate (ATP), and lactate concentrations during maximal sustained voluntary isometric contraction of the quadriceps muscle group in man. Data points represent mean values. SE and n values are not included but appear in Table 2.2.


ATP (■) and PCr (●), mmol·kg⁻¹ d.m.
0
20
40
60
80
Contraction time, sec
0
2
4
6
8
10
12
14
Lactate (▲), mmol·kg⁻¹ d.m.
0
5
10
15
20
25
30

throughout all contractions, while PCr decreased progressively to reach a low of 53.3% (Table 2.2, Fig. 2.2) of the resting concentration following 14s of sustained contraction. Paired *t*-tests indicated that the concentration of PCr was significantly less than the resting value after 2s ($p \leq 0.05$, Fig. 2.3). Muscle lactate concentration rose significantly ($p \leq 0.05$ after 2s, Fig. 2.4) from a mean resting concentration of 3.01 ± 0.27 mmol·kg^{-1}dm to reach 23.89 ± 2.17 mmol·kg^{-1}dm after 14s (Table 2.2, Fig 2.2). Fig. 2.2 illustrates the mean absolute measurements at each time point for PCr, lactate, and ATP.

G-6-P and G-3-P were both found to exist in small concentrations in the muscle, and both increased slightly throughout the contraction protocol (Table 2.2). Changes in Cr were reciprocal to PCr changes and the total Cr content remained constant throughout the duration of contraction (Table 2.2).

Due to the complexity of the 'semi repeated-measures' design, PCr and lactate concentration changes were analyzed both in absolute and relative terms. Firstly, to analyze for absolute concentration changes in both PCr and lactate, all individual metabolite concentrations were correlated against time (scatterplot) (Fig. 2.5a,b). The pearson correlation coefficient $r^2 = 0.624$ ($p \leq 0.05$, Fig. 2.5a) for the decrease in

Figure 2.3 Phosphocreatine concentration values at each measurement time. Bars represent group means±SE, n values are given in the bars.

* $p \leq 0.05$ from PRE-EX (0s)

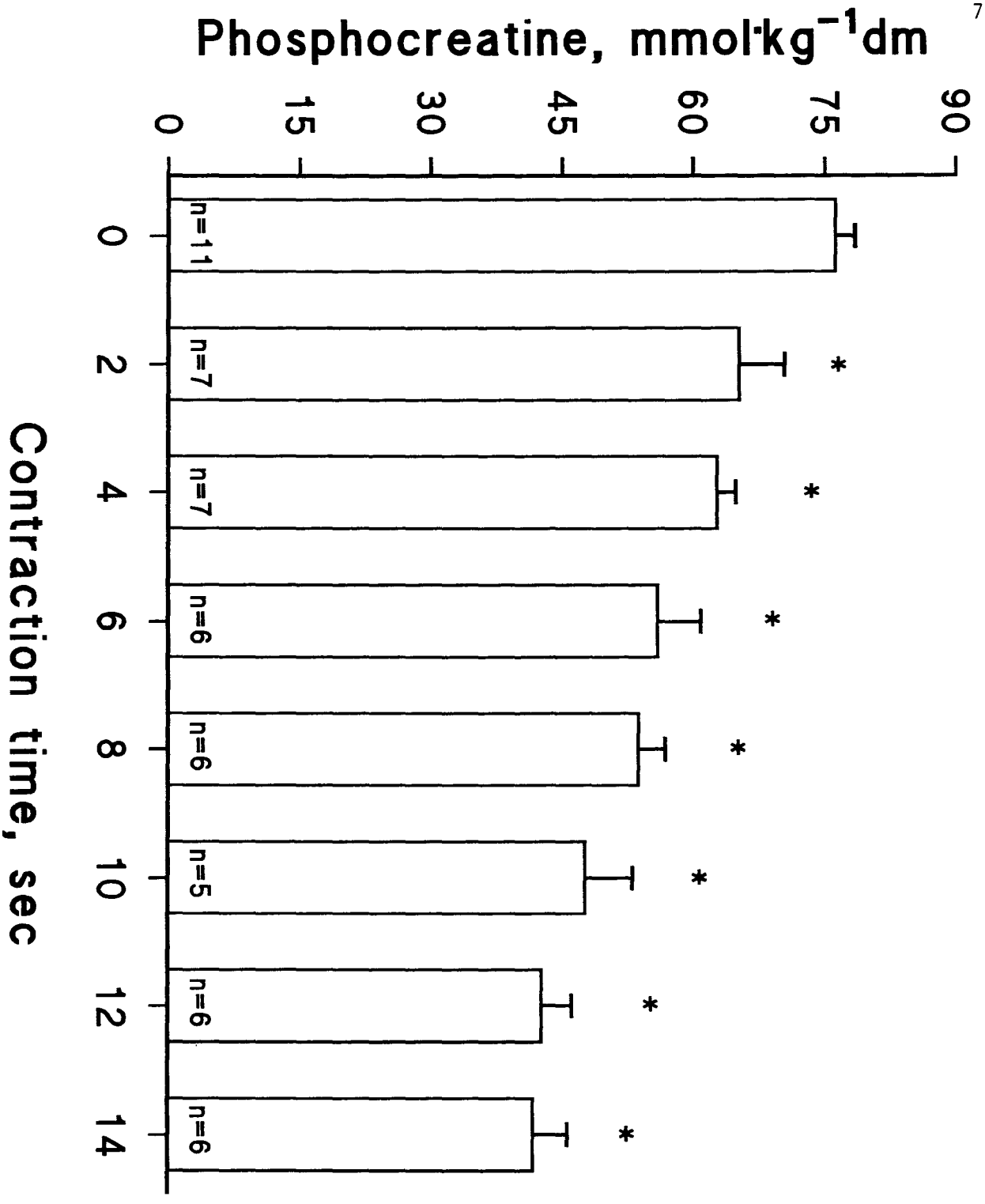

Phosphocreatine, mmol·kg⁻¹dm
0
15
30
45
60
75
90
Contraction time, sec
0
2
4
6
8
10
12
14
n=11
n=7
n=7
n=6
n=6
n=5
n=6
n=6
*
*
*
*
*
*
*

Figure 2.4 Lactate concentration values at each measurement time. Bars represent group means±SE, n values are given in the bars.

* $p \leq 0.05$ from PRE-EX (0s)

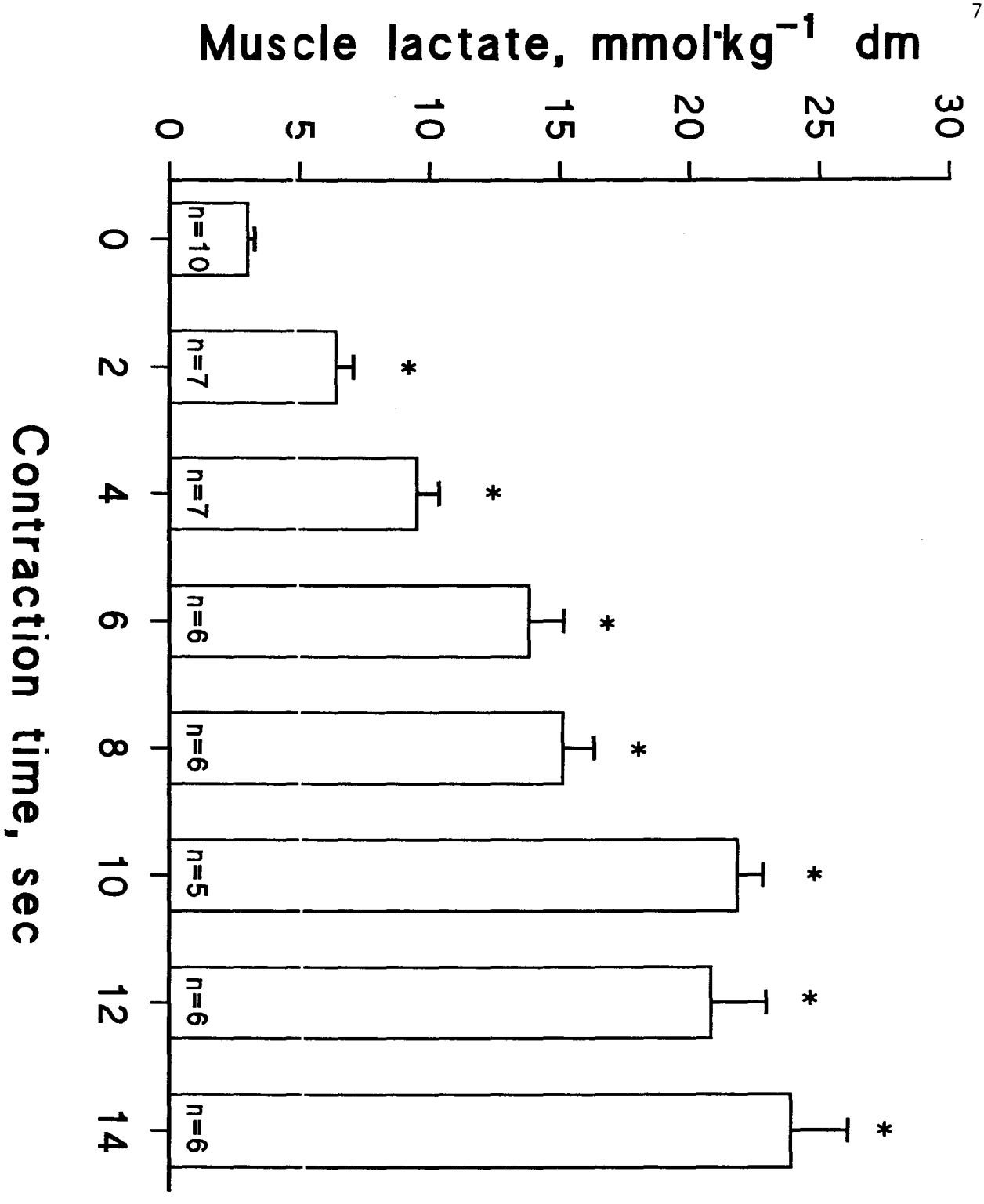

Muscle lactate, mmol·kg⁻¹ dm
0
5
10
15
20
25
30
Contraction time, sec
0
2
4
6
8
10
12
14
n=10
n=7
n=7
n=6
n=6
n=5
n=6
n=6
*
*
*
*
*
*
*

Figure 2.5a Relationship between absolute changes in phosphocreatine degradation and contraction duration during maximal voluntary sustained isometric contractions in man.

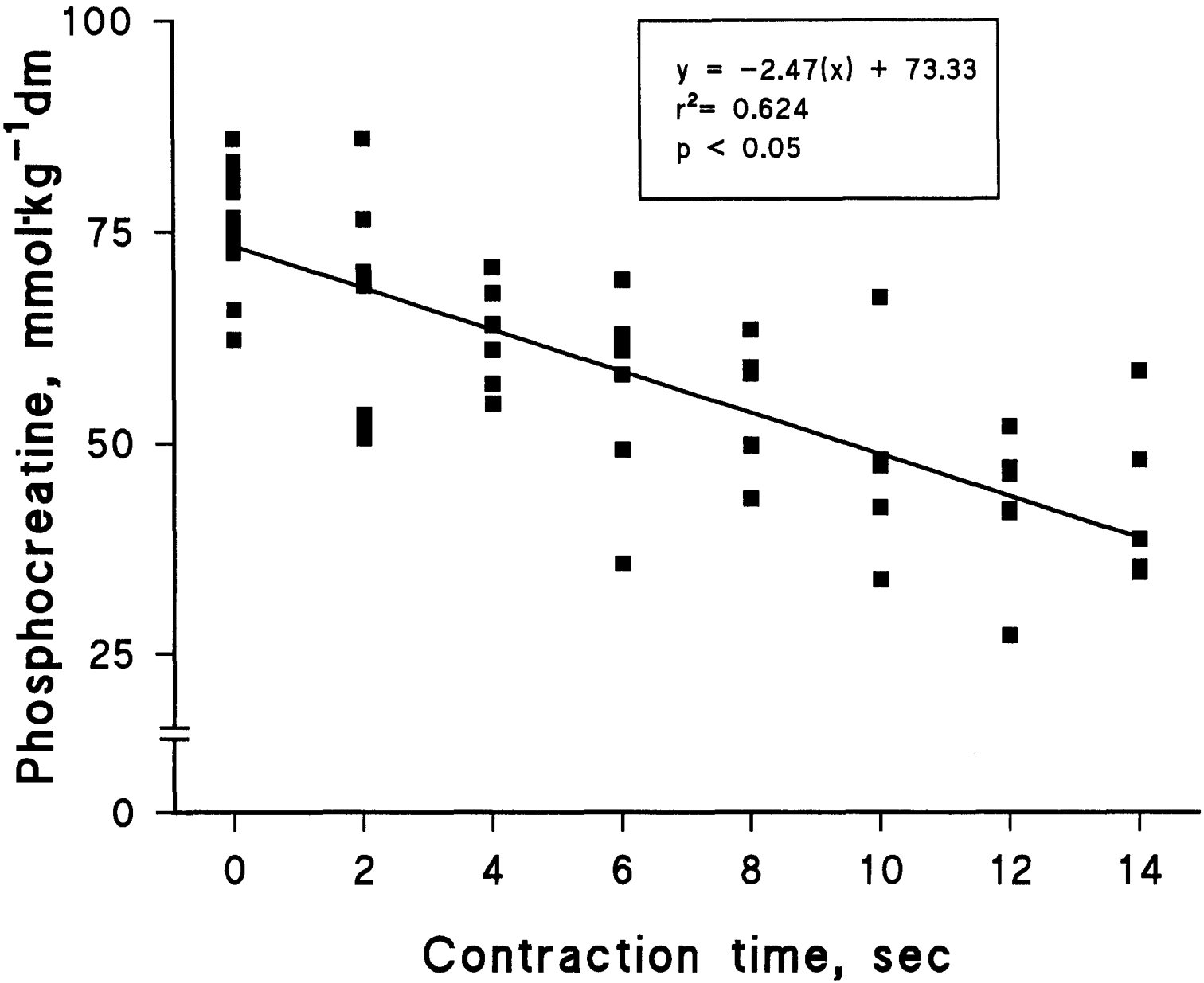
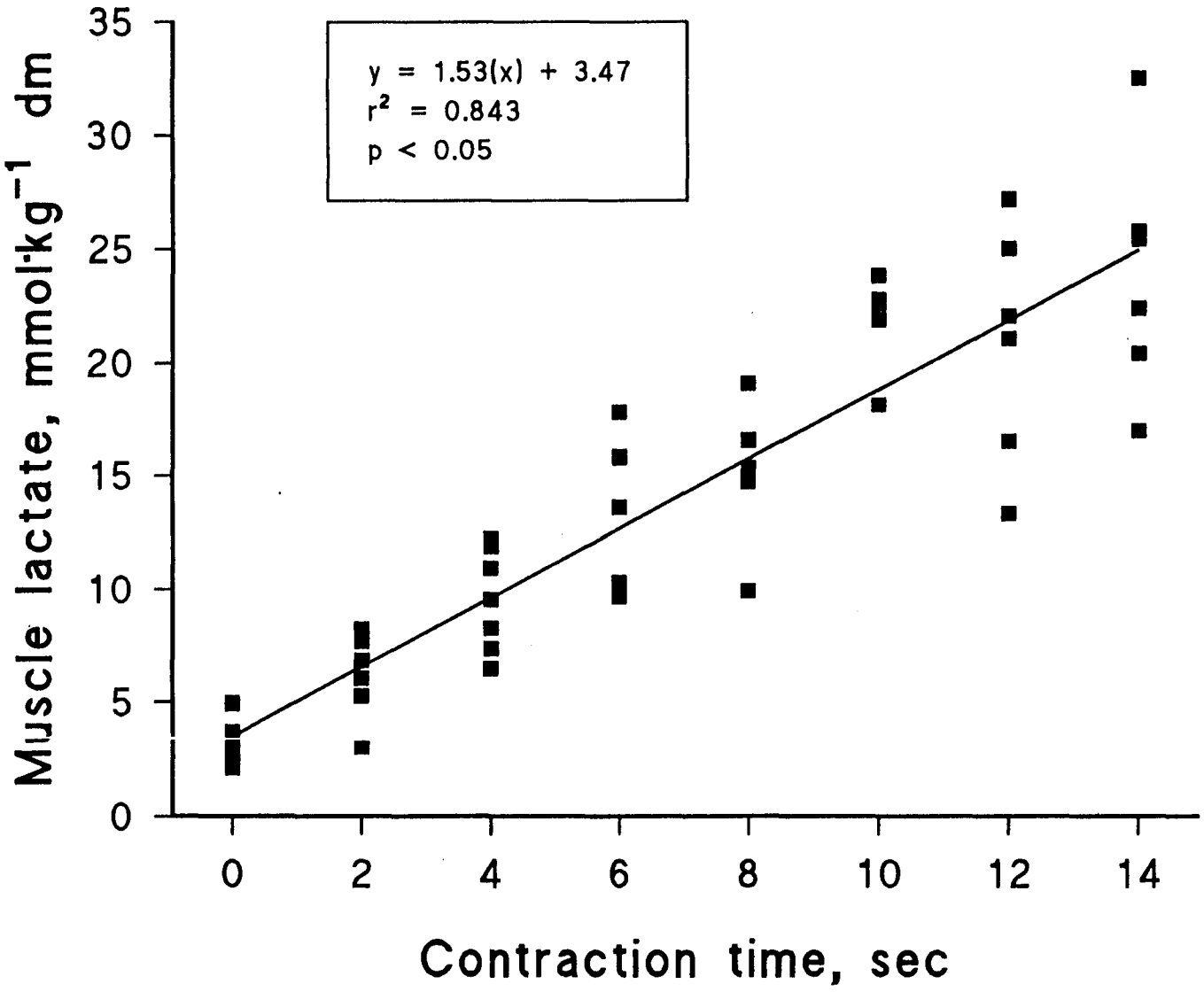

y = −2.47(x) + 73.33
r²= 0.624
p < 0.05
Phosphocreatine, mmol·kg⁻¹dm
100
75
50
25
0
0
2
4
6
8
10
12
14
Contraction time, sec

Figure 2.5b Relationship between absolute changes in lactate accumulation and contraction duration during maximal voluntary sustained isometric contractions in man.


y = 1.53(x) + 3.47
r² = 0.843
p < 0.05
Muscle lactate, mmol·kg⁻¹ dm
0
5
10
15
20
25
30
35
0
2
4
6
8
10
12
14
Contraction time, sec

PCr, and r^2=0.843 ($p \leq 0.05$, Fig. 2.5b) for the increase in lactate over time. Secondly, because each subject only participated in 4 of the 7 contraction protocols (between 2 and 14s), linear regression analyses were performed on the data from each individual subject (Appendix IV), and mean regression equations (±SD) were calculated for both the decrease in PCr and the increase in lactate over time (Fig 2.6a,b).

Finally, the relationships between relative concentration changes in PCr and lactate and contraction time were determined (Fig. 2.7a,b). To calculate the relative changes in PCr, concentrations of PCr at each time point were expressed as percentages of each individual's resting PCr concentration. These percentages were then pooled together, and the pearson correlation coefficient was calculated to r^2=.756 ($p \leq 0.05$, Fig. 2.7a). When the increase in lactate (similar to the decrease in PCr) is analyzed according to the individual relative changes, r^2=0.813 ($p \leq 0.05$, Fig. 2.7b). It is difficult to express the increase in muscle lactate concentration in relative terms, however, because the resting concentration is small, and increases in some cases to more than 1000%. In addition, this method of analysis (relative percentages) may artificially elevate the r^2 value, because there is no variance around the initial value of 100% for each individual.

Figure 2.6a Relationship between absolute changes in phosphocreatine degradation and contraction duration expressed as the mean of 11 individual regression equations (Appendix IV)

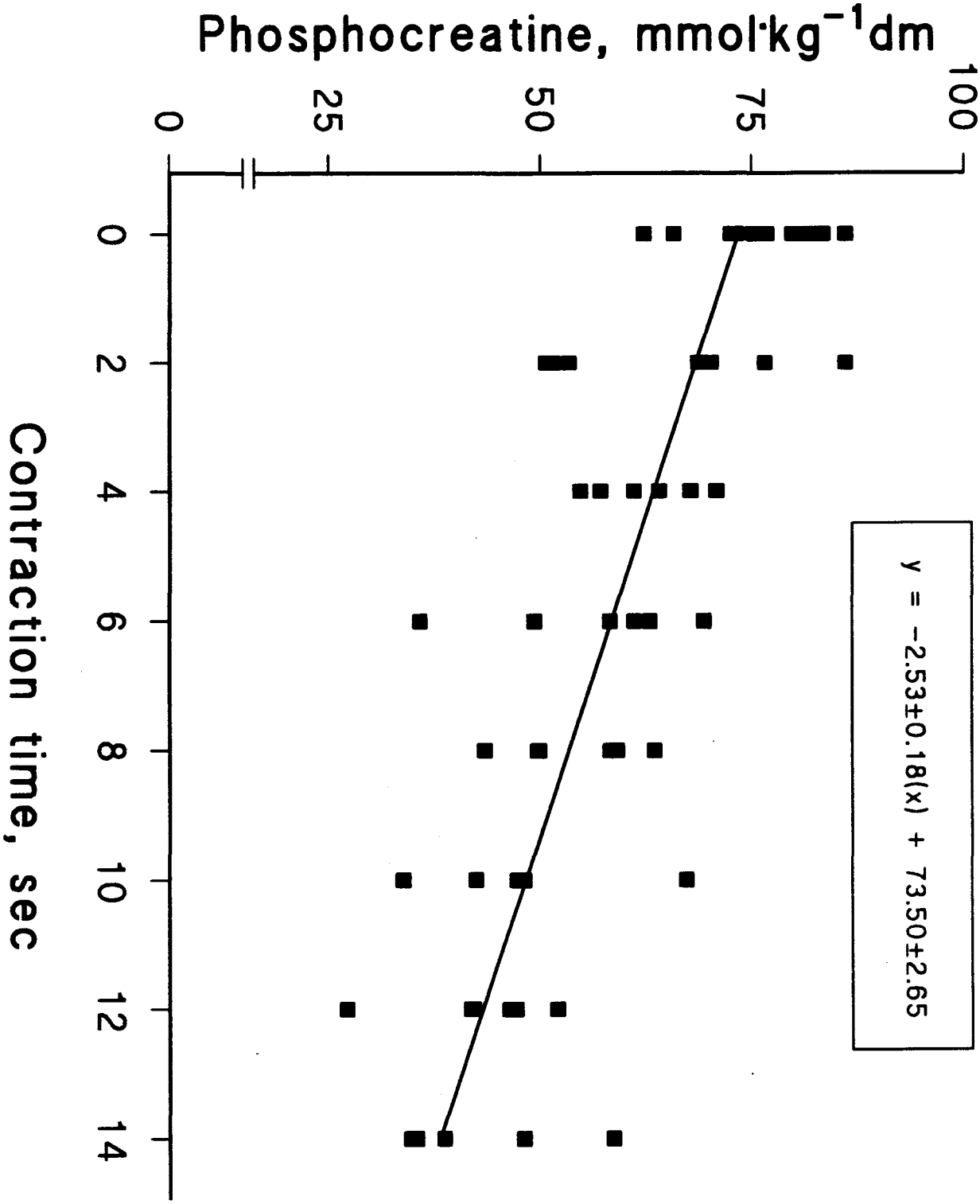

Phosphocreatine, mmol·kg⁻¹dm
0
25
50
75
100
Contraction time, sec
0
2
4
6
8
10
12
14
y = −2.53±0.18(x) + 73.50±2.65

Figure 2.6b Relationship between absolute changes in lactate accumulation and contraction duration expressed as the mean of 11 individual regression equations (Appendix IV)

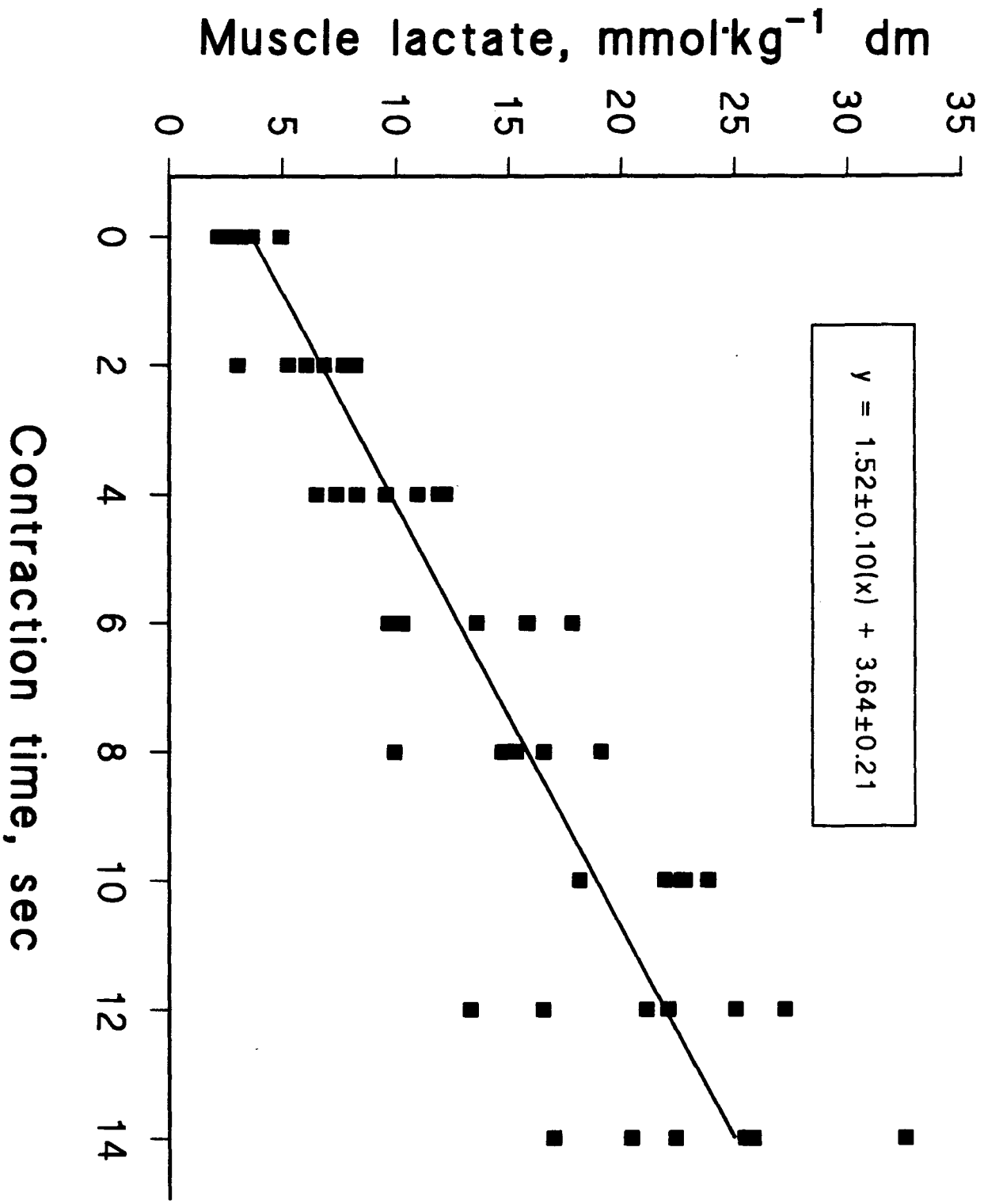

Muscle lactate, mmol·kg⁻¹ dm
0
5
10
15
20
25
30
35
Contraction time, sec
0
2
4
6
8
10
12
14
y = 1.52±0.10(x) + 3.64±0.21

Figure 2.7a Relationship between relative changes in phosphocreatine degradation and contraction duration during maximal voluntary sustained isometric contractions in man.

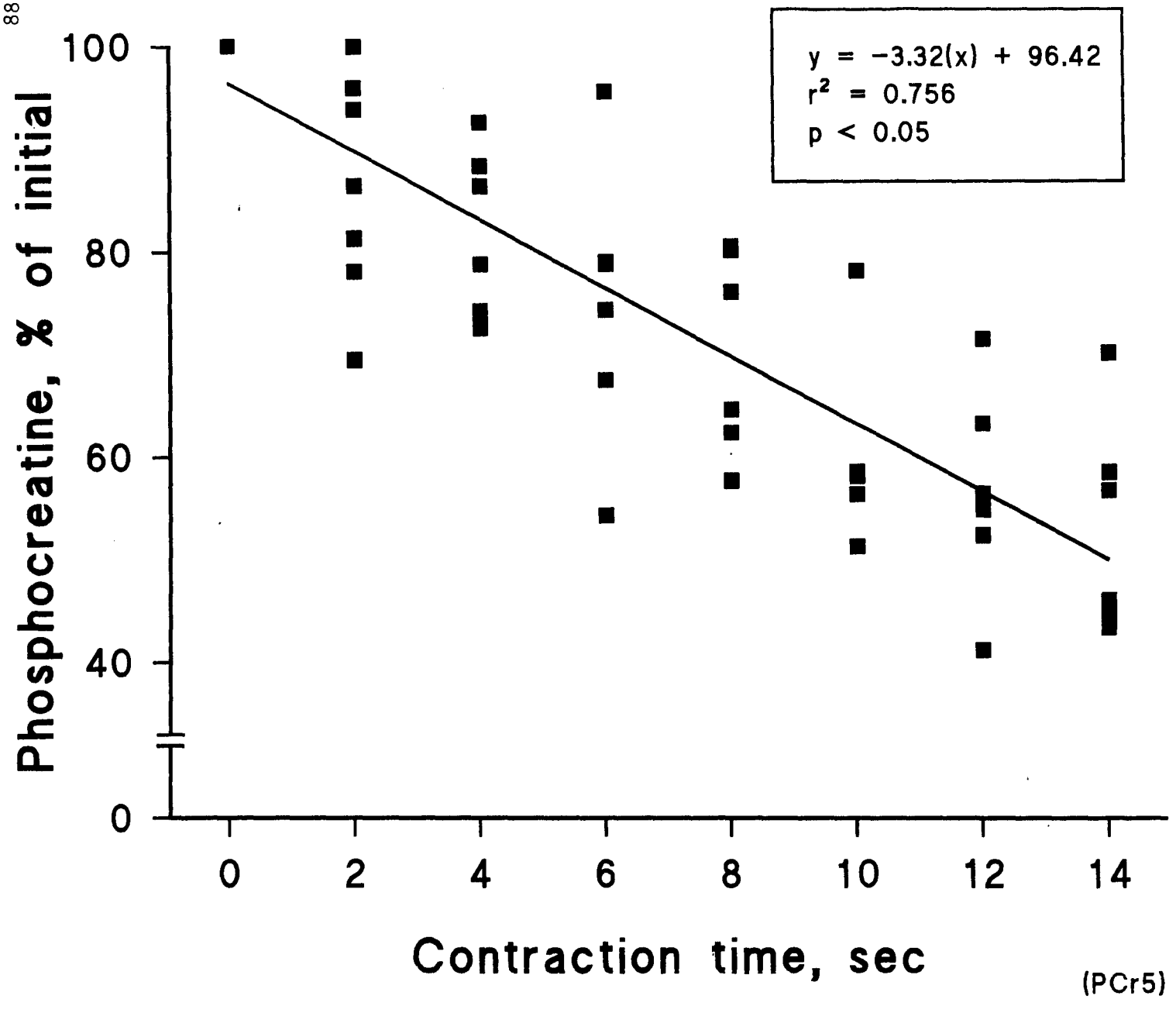
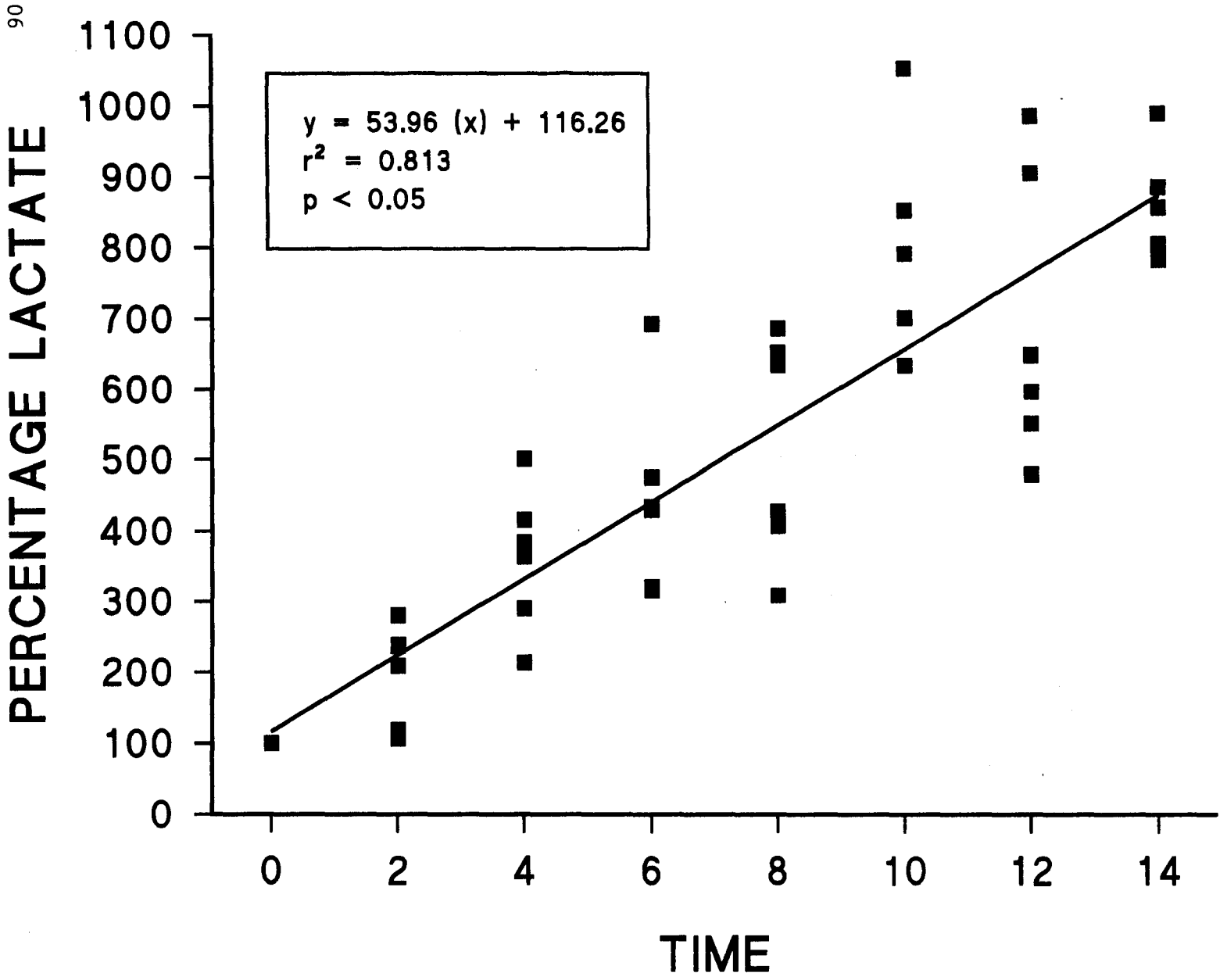

y = −3.32(x) + 96.42
r² = 0.756
p < 0.05
Phosphocreatine, % of initial
100
80
60
40
0
0
2
4
6
8
10
12
14
Contraction time, sec
(PCr5)

Figure 2.7b Relationship between relative changes in lactate accumulation and contraction duration during maximal voluntary sustained isometric contractions in man.


y = 53.96 (x) + 116.26
r² = 0.813
p < 0.05
PERCENTAGE LACTATE
1100
1000
900
800
700
600
500
400
300
200
100
0
0
2
4
6
8
10
12
14
TIME

2.3.1 (iii) ATP turnover rates

It is assumed that occlusion of the blood flow prior to and throughout each muscle contraction limited the production of ATP to anaerobic processes by providing a closed metabolic system. The total amount of ATP produced, and the corresponding rate of ATP turnover could therefore be estimated from the changes in metabolites. The mean values±SE are tabulated in Table 2.3, and the specific calculations utilized to determine these values are listed in Sect 2.2.4 (iv). Total anaerobic ATP production increased from 14.52±3.88 mmol·kg^{-1}dm at 2s to total 69.03±4.47 mmol·kg^{-1}dm after 14s. The ATP turnover rate during the first 2s was estimated to be 7.26±1.94 mmol·kg^{-1}dm·s^{-1} with 67% of the ATP being derived from PCr degradation and 33% from glycogenolysis (Fig. 2.8). As contraction continued, the ATP turnover rate decreased to reach 4.93±0.32 mmol·kg^{-1}·s^{-1} after 14s which represented 68% of the initial rate, and 53% of the ATP derived during 14s was produced through PCr degradation, while 47% came from glycogenolysis. As indicated in Table 2.3 and Fig 2.8, the rate of ATP production from PCr decreased steadily over the 14s, while the rate of ATP resynthesis from glycogenolysis remained fairly stable throughout the entire contraction period. Fig 2.8 also illustrates the mean force production over the contraction period as a percentage of the initial value.

Table 2.3 Total ATP production, and rates of ATP resynthesis from phosphocreatine (PCr) degradation and glycogenolysis during maximal voluntary isometric contractions with occluded circulation.

Duration (s)	n	ATP production ($mmol \cdot kg^{-1}$ d.m.)	ATP provision ($mmol \cdot kg^{-1}$ d.m. $\cdot s^{-1}$)		
			Total	PCr	Glycogen-olysis
0-2	7	14.52±3.88	7.26±1.94	4.87±1.50	2.39±0.55
0-4	7	25.91±2.79	6.48±0.70	3.75±0.62	2.73±0.39
0-6	6	34.54±4.92	5.76±0.82	3.11±0.68	2.64±0.31
0-8	6	41.70±4.81	5.21±0.60	2.86±0.40	2.35±0.30
0-10	5	59.87±3.59	5.99±0.36	3.04±0.29	2.95±0.21
0-12	6	59.09±5.23	4.92±0.44	2.71±0.25	2.21±0.24
0-14	6	69.03±4.47	4.93±0.32	2.62±0.28	2.31±0.23

Values are means ± SE. Calculations are given in Sect 2.2.4 (iv)

Figure 2.8 Muscle contraction force (±SD) and ATP turnover rates during maximal sustained voluntary isometric contractions of the quadriceps muscle group with occluded circulation. Energy contributions from phosphocreatine (PCr) and glycogenolysis are indicated, n values are given in the bars. ATP turnover rates are taken from Table 2.3 and represent durations 0-2s, 0-4s, 0-6s, 0-8s, 0-10s, 0-12s, and 0-14s.

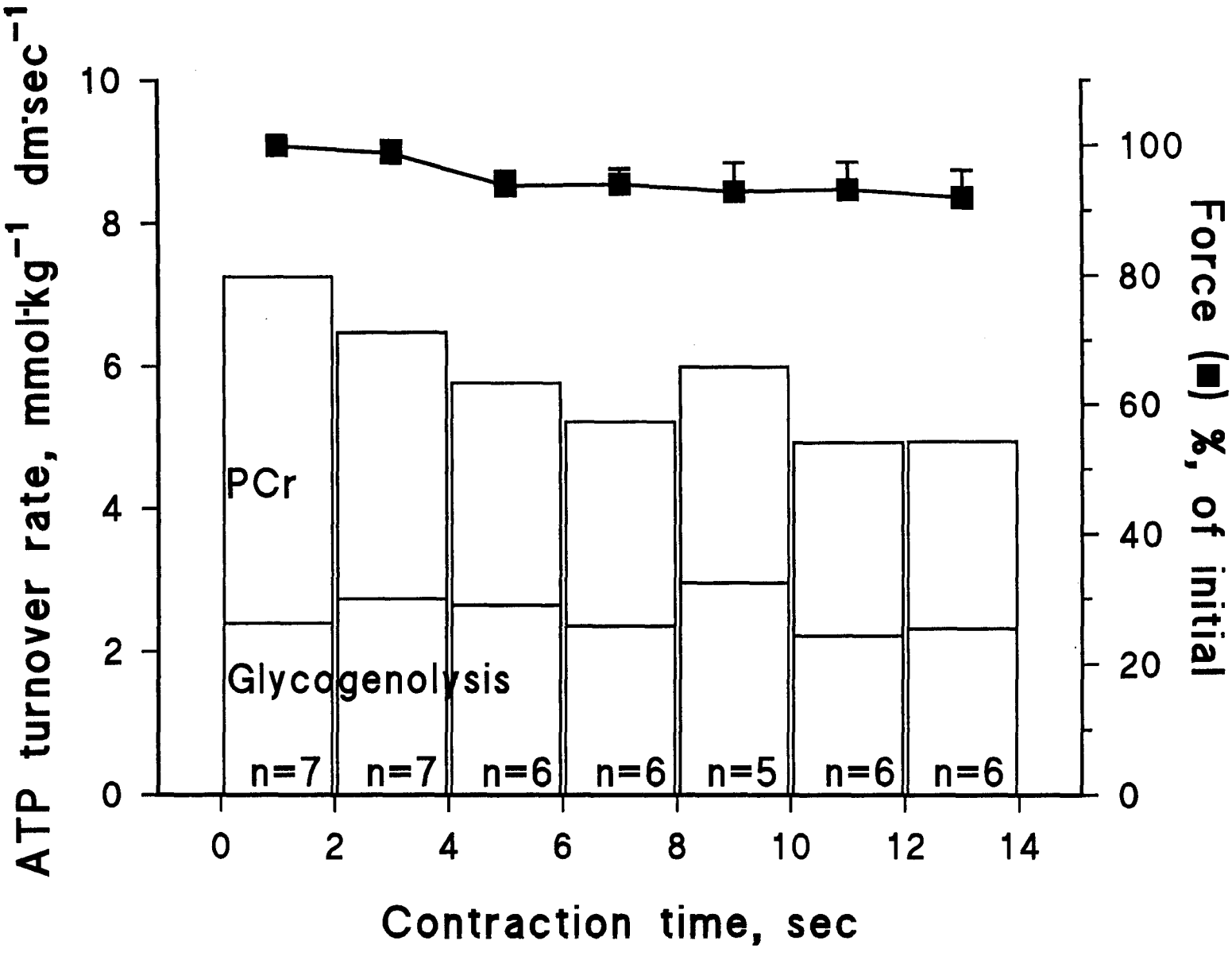

ATP turnover rate, mmol·kg⁻¹ dm·sec⁻¹
Force (■) %, of initial
Contraction time, sec
PCr
Glycogenolysis
n=7
n=7
n=6
n=6
n=5
n=6
n=6
0
2
4
6
8
10
12
14
0
2
4
6
8
10
0
20
40
60
80
100

Table 2.4. Rates of glycogenolysis and glycolysis during maximal voluntary isometric contractions with occluded circulation.

Duration (s)	($mmol \cdot kg^{-1}$ d.m. $\cdot s^{-1}$)	
	Glycogenolysis	Glycolysis
0-2	1.58±0.29	1.02±0.24
0-4	1.82±0.22	1.04±0.15
0-6	1.22±0.21	0.97±0.11
0-8	1.39±0.10	0.91±0.12
0-10	1.75±0.21	1.11±0.10
0-12	1.27±0.13	0.80±0.08
0-14	1.48±0.13	0.87±0.11

Values are means ± SE. Calculations are given in Sect 2.2.4 (iv)

2.3.2 RECOVERY

2.3.2 (i) Torque

In the REC session, each subject performed 2 MVC's, each of 14s duration (1 by each leg). Peak torque production in the left and right legs was 237.12±10.39 Nm and 242.79±11.24 Nm, respectively. During contraction, mean torque decreased to 87.75±1.85% of peak torque.

2.3.2 (ii) Metabolite data

Recovery metabolite concentrations are summarized in Table 2.5. Because all contractions for this phase of the study were 14s in duration, the metabolite concentrations at 0s of recovery were considered to be counterpart to the concentrations measured during the EX sessions after 14s of contraction. It is for this reason that 'EX14s' is included in Table 2.5. Similar to the EX data, muscle [ATP] remained constant throughout recovery, while [PCr] increased rapidly to reach 73.05±6.07 $mmol \cdot kg^{-1}dm$ after 60s and 73.50±2.07 $mmol \cdot kg^{-1}dm$ after 3 min. Muscle lactate concentration decreased to reach 9.63±2.28 $mmol \cdot kg^{-1}dm$ after 1 min and 10.56±1.97 $mmol \cdot kg^{-1}dm$ after 3 min. Although 6 muscle samples were taken at each time point of recovery, at some time points (Table 2.5) results were only available for 3, 4, or 5 samples. This is because some samples were accidentally destroyed during analysis.

TABLE 2.5. Muscle metabolite concentrations following 14s maximal voluntary contractions with occluded circulation, and during recovery following 14s MVC's with circulation occluded for 30s prior to contraction.

Metabolite	PreEx (n=11)	Ex14s (n=6)	Recovery duration (s)						
			10.0 (n=4)	20.0 (n=3)	30.0 (n=5)	40.0 (n=4)	60.0 (n=4)	120.0 (n=5)	180.0 (n=4)
ATP	26.7±0.4	26.2±0.8	24.9±1.2	25.6±0.9	25.2±0.6	26.1±0.5	25.6±1.0	26.2±0.8	26.1±0.4
PCr	76.2±2.2	41.7±3.9	49.6±4.8	50.1±6.0	55.3±5.2	62.5±4.8	73.1±6.1	71.5±5.6	73.5±2.1
Lactate	3.0±0.3	23.9±2.2	15.9±3.1	15.7±3.6	15.5±3.9	13.3±2.5	9.6±2.3	11.9±2.6	10.6±2.0

Values are means ± SE in $mmol \cdot kg^{-1}$ dry muscle. All metabolites were determined enzymatically. ATP, adenosine triphosphate; PCr, phosphocreatine.

2.3.3 (iii) PCr resynthesis

The time course for PCr resynthesis is given in Fig 2.9. The '0s' time point has been taken from the EX data and is equivalent to the PCr concentration immediately following 14s of contraction. During recovery the PCr concentration in biopsy samples rose rapidly until 60s after which it was not significantly different from the resting value ($n=4$, $p>0.05$).

2.3.3 (iv) Lactate clearance

Fig. 2.10 depicts the time course of lactate clearance during recovery. Similar to Fig. 2.10 the '0s' time point represents the muscle lactate concentration immediately following 14s of contraction. A large decrease in muscle lactate content appeared to occur during the initial 10s of recovery. Following 10s, however, there did not appear to be any further change in lactate concentration, and after 180s, the lactate content was still significantly higher than the resting value ($n=4$, $p\leq0.05$).

Figure 2.9 The time course of phosphocreatine (PCr) resynthesis during recovery from 14s of maximal sustained voluntary contraction of the quadriceps muscle group with circulation occluded for 30s prior to contraction. Values are means ± SE.

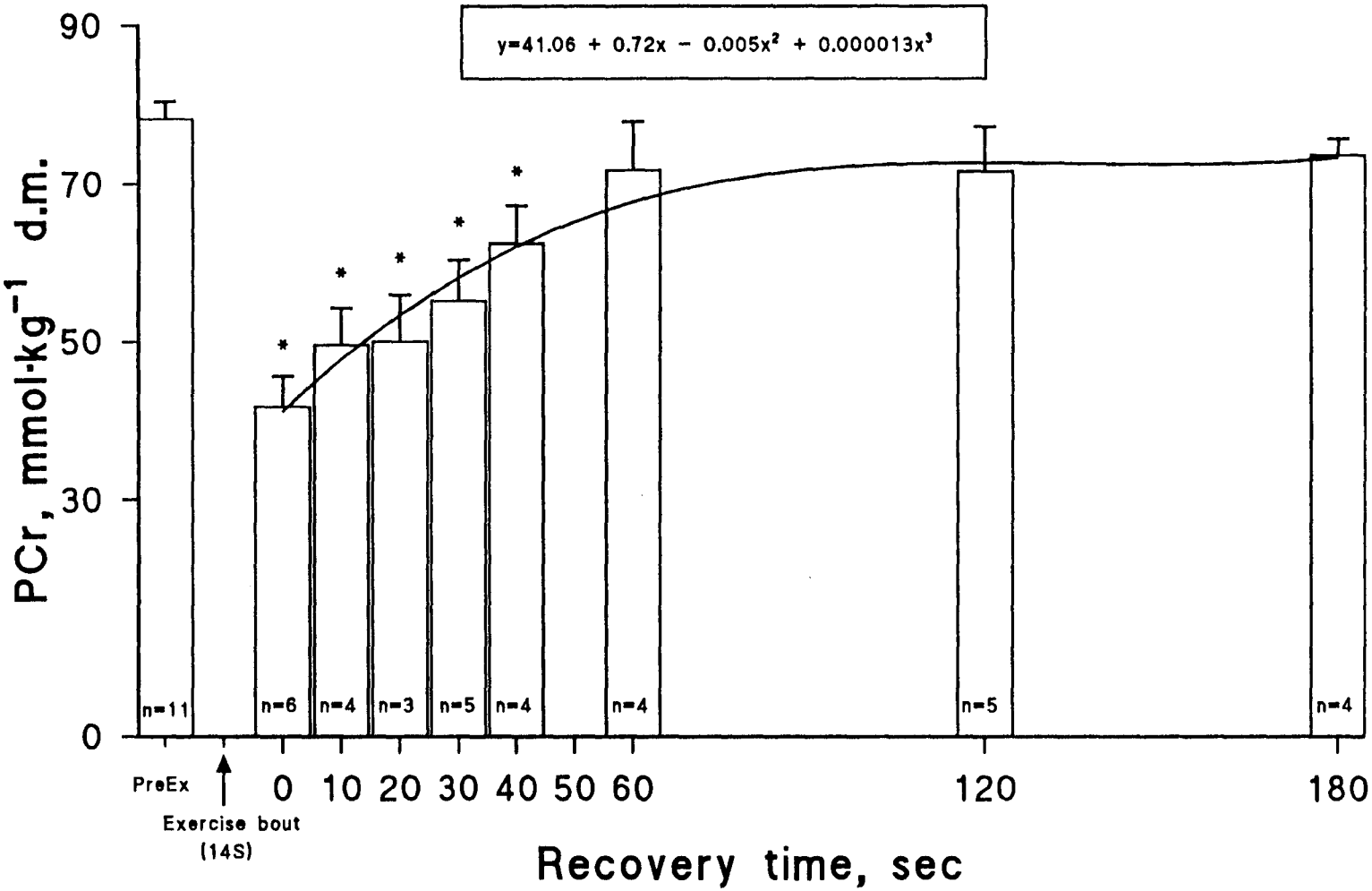
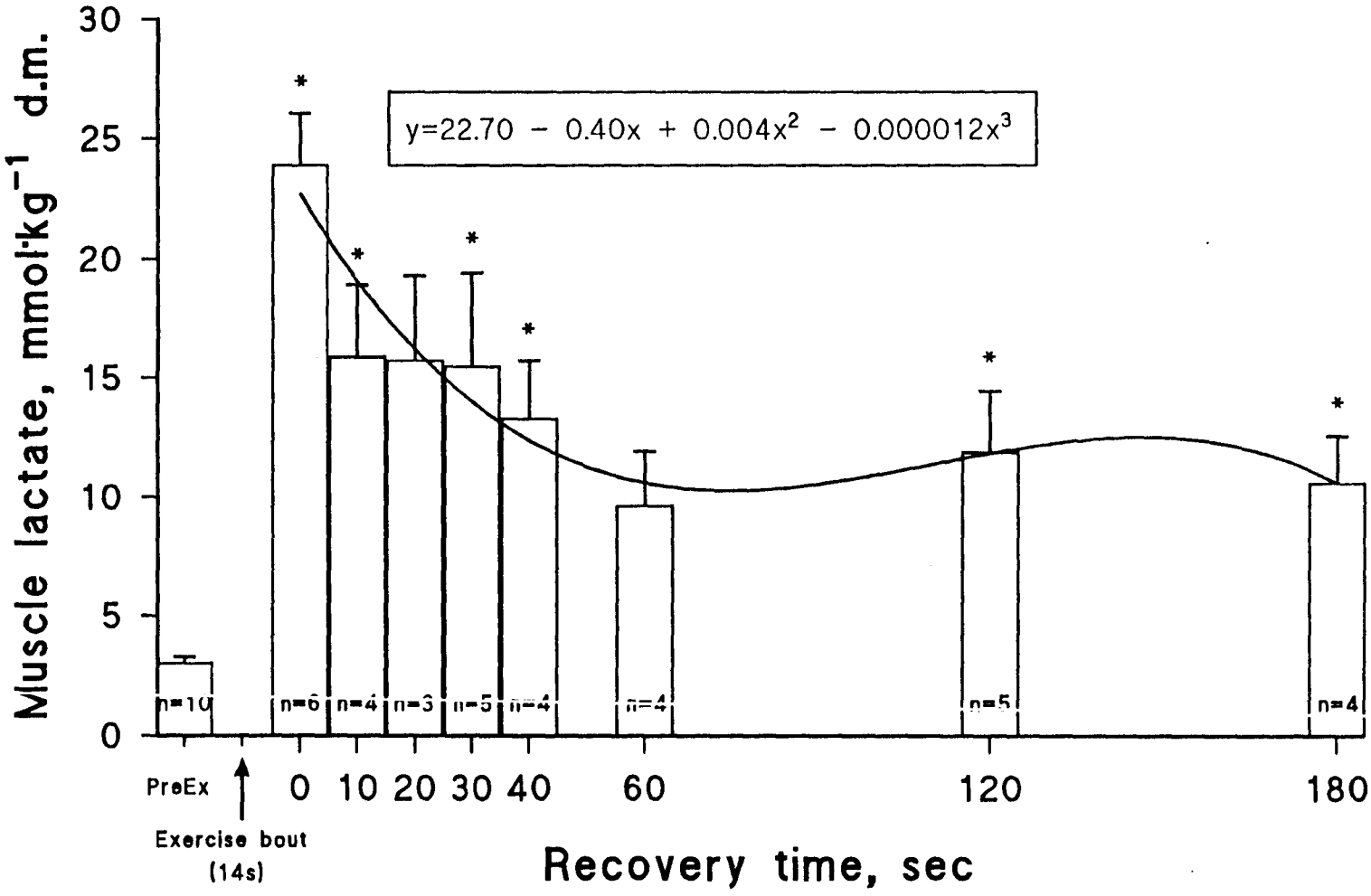

y=41.06 + 0.72x − 0.005x² + 0.000013x³
PCr, mmol·kg⁻¹ d.m.
90
70
50
30
0
n=11
n=6
n=4
n=3
n=5
n=4
n=4
n=5
n=4
*
PreEx
Exercise bout
(14S)
0 10 20 30 40 50 60
120
180
Recovery time, sec

Figure 2.10 The time course of lactate clearance during recovery from 14s of maximal sustained voluntary contraction of the quadriceps muscle group with circulation occluded for 30s prior to contraction. Values are means ± SE.


Muscle lactate, mmol·kg⁻¹ d.m.
30
25
20
15
10
5
0
y=22.70 − 0.40x + 0.004x² − 0.000012x³
n=10
n=6
n=4
n=3
n=5
n=4
n=4
n=5
n=4
PreEx
0
10
20
30
40
60
120
180
Exercise bout
(14s)
Recovery time, sec

2.4 DISCUSSION

This study investigated energy provision during voluntary isometric contractions of the quadriceps muscle group. Maximal contractions were sustained for durations between 2 and 14s under occluded circulation, and anaerobic ATP production was estimated from direct measures of muscle metabolites. In addition, ATP turnover rates were estimated and the relative contributions of the PCr and glycogenolytic systems to ATP provision were determined for the 14s time span.

2.4.1 METABOLITE CHANGES

Figs. 2.2, 2.3, and 2.4 all demonstrate the changes in PCr and lactate concentration which occurred during contraction. The relationship between lactate concentration and contraction duration was linear throughout the contraction ($r^2=0.843$, $p\leq0.05$). As mentioned previously, the 'semi-repeated measures' design made analysis of the results difficult, however, the lactate concentration appeared to increase at a steady rate of 1.52 ± 0.10 $mmol \cdot kg^{-1}dm \cdot s^{-1}$. This value was taken from the regression equation:

$$y = 1.52\pm0.10(x) + 3.64\pm0.21 \ mmol \cdot kg^{-1}dm \cdot s^{-1}$$

which was determined by taking the mean value of each subject's individual regression equation (Fig 2.6b). This

equation can be used to predict muscle lactate concentration following maximal isometric contractions of the quadriceps muscle group for durations up to 14s. This mean equation is very similar to the equation determined from the scatterplot of lactate concentration values over time (Fig 2.5b).

The rate of PCr depletion was highest during the initial 2s (4.87 $mmol \cdot kg^{-1}dm \cdot s^{-1}$), after which, PCr concentration appeared to decline at a steady rate. Despite this appearance, multiple regression analysis did not cause a significant increase in the r^2 value (0.624, $p \leq 0.05$) determined for the linear relationship illustrated in Fig 2.6a. Similar to the analysis for lactate concentration changes, a mean regression equation was calculated from individual data to express the relationship between PCr concentration and contraction time (Fig 2.5a). From this equation, the mean rate of PCr depletion was 2.53 ± 0.18 $mmol \cdot kg^{-1}dm \cdot s^{-1}$. Similar to the equation calculated for lactate concentration above, this equation can be used to predict PCr concentration during short duration MVC's of the quadriceps.

2.4.2 ATP TURNOVER

The highest rate of anaerobic ATP resynthesis occurred during the initial 2s of exercise (7.26 ± 1.94 $mmol \cdot kg^{-1}dm \cdot s^{-1}$). During this period there was no measurable decrease in muscle

ATP, and PCr degradation was estimated as providing 67% and glycogenolysis 33% of the required ATP. Continuation of contraction led to a decrease in ATP turnover rate concomitant with a decrease in muscle force. After 14s of contraction, muscle force decreased to 91±1.74% of MVC, while ATP turnover rate decreased to 68% of the initial rate. It appears that the decrease in ATP turnover rate was mainly due to a decrease in the proportion of ATP production from PCr degradation. PCr provided ATP at a rate of 4.87±1.5 $mmol \cdot kg^{-1}dm \cdot s^{-1}$ during the initial 2s, but the mean rate for the entire 14s time period was 2.62±0.28 $mmol \cdot kg^{-1}dm \cdot s^{-1}$. In contrast, the rate of glycogenolysis (estimated from lactate accumulation), reached a rate of 2.39±0.24 $mmol \cdot kg^{-1}dm \cdot s^{-1}$ during the initial 2s, fluctuated between mean rates of 2.21±0.24 and 2.95±0.20 $mmol \cdot kg^{-1}dm \cdot s^{-1}$ as contraction continued, and resulted in a mean rate of 2.31±0.23 $mmol \cdot kg^{-1}dm \cdot s^{-1}$ for the entire 14s. The rate of glycogenolysis thus reached a steady state within the initial 2s, and maintained that rate for the duration of the contraction. Despite the slight decrease in ATP resynthesis that occurred from PCr degradation as contraction proceeded, the data reveal that there was an almost equal contribution to ATP provision from the 2 anaerobic energy-delivery pathways during the contraction protocol, and that (with the exception of the first 2 seconds) the proportion changed very little over time.

The slight decrease in torque which occurred during the course of the contraction is probably not due to inhibition of glycogenolysis, since the estimated rate of ATP provision from this pathway remains steady throughout the duration of exercise. It is also unlikely that the decrease in torque production resulted from the decrease in ATP production from PCr, because there was no concomitant decrease in ATP concentration. Therefore, lack of energy cannot be postulated to be the main reason for fatigue in this contraction protocol.

Although motor unit contractile properties were not examined in the present investigation, previous literature has indicated that during sustained voluntary contractions, mean firing rates decline progressively and are accompanied by concomitant reductions in muscle relaxation rates and lowered tetanic fusion frequencies (muscle wisdom) (Bigland-Ritchie et al., 1983). Muscles can therefore remain fully activated by the CNS despite reduced excitation rates (Bigland-Ritchie et al., 1983). Potentially the decrease in ATP turnover rate which occurred can be explained by a decrease in energy demand.

No allowance was made for possible oxidative metabolism in the calculations used to estimate ATP turnover. Harris et al. (1975) determined that the upper limit for oxygen stored in an occluded muscle is 2 mmol $O_2kg^{-1}dm$, which

is capable of producing 12 mmol·kg^{-1}dm ATP through oxidative metabolism. Thirty seconds of occlusion prior to contraction might be expected to deplete one-twelfth of the stored O_2 (Harris et al., 1975; Blei et al., 1993), and the remaining O_2 has been estimated to produce ATP at an average rate of 1.1 mmol·kg^{-1}dm·s^{-1} for the initial 10s of exercise (Hultman and Sjöholm, 1983a), after which metabolism is expected to be purely anaerobic.

The present results are consistent with early investigations of voluntary isometric exercise. In an investigation in 2 subjects, Bergström et al. (1971) showed an ATP turnover rate of ~ 6-7 mmol·kg^{-1}dm·s^{-1} during a maximal isometric quadriceps extension contraction held for 6.6s. Karlsson and Ollander (1972) reported that 30s isometric quadriceps contractions held at 75% of MVC led to an increase in muscle lactate to an average of 10.3 mmol·kg^{-1} wet muscle in three subjects. When this value is converted to an equivalent concentration of lactate relative to dry muscle weight (assuming muscle wet weight is ~ 80% water), the associated rate of ATP production from glycogenolysis can be calculated to equal ~ 2.25 mmol·kg^{-1}dm·s^{-1}, which is analogous to the rate determined in the present experiment. Similar results were reported by Ahlborg et al. (1972) following brief maximal isometric contractions.

2.4.2 (i) Comparison with dynamic exercise

As discussed above, very few studies exist which have examined energy provision during maximal isometric contractions of brief duration. Comparably, few studies have investigated dynamic exercise of less than 15s in duration, and the estimated ATP turnover rates associated with dynamic exercise appear to be higher than those associated with isometric exercise (see Table 1.1). Jacobs et al. (1983) were the first to systematically demonstrate that lactate accumulation occurred before the PCr store was depleted. Following 10s of supramaximal cycle exercise, muscle lactate values of 46.1±15.2 (SD) $mmol \cdot kg^{-1}dm \cdot s^{-1}$ in males and 25.2 $mmol \cdot kg^{-1}dm \cdot s^{-1}$ in females were reported. Exact ATP turnover rates could not be determined, however, since resting lactate values were not measured. Boobis et al. (1982) found that 19.11 $mmol \cdot kg^{-1}dm$ of muscle lactate accumulated and 29.48 $mmol \cdot kg^{-1}dm$ of PCr was utilized during 6s of maximal cycle exercise. The ATP turnover rate from PCr could then be calculated to be 4.91 $mmol \cdot kg^{-1}dm \cdot s^{-1}$, and from glycogenolysis to be 4.77 $mmol \cdot kg^{-1}dm \cdot s^{-1}$.

More recently, Hirvonen et al. (1987) measured muscle metabolites following maximal sprint running exercise 5-11s in duration. In 5.5s muscle PCr decreased by 5.5 $mmol \cdot kg^{-1}$ wet weight, which is approximately equivalent to 27.5 $mmol \cdot kg^{-1}dm$. The ATP turnover from PCr can then be calculated to be 5

mmol·kg^{-1}dm·s^{-1}. Muscle lactate increased ~4.2 mmol·kg^{-1} wet weight (corresponding to ~21 mmol·kg^{-1}dm) during the same time period. ATP turnover from glycogenolysis was ~5.7 mmol·kg^{-1}dm·s^{-1}. These rates and those indicated in Table 1.1 are higher than those determined from isometric exercise in the present investigation.

It should be understood that the nature of maximal dynamic exercise makes it extremely difficult to quantify precise metabolic changes which occur during brief efforts. Firstly, a closed system cannot be implemented, thus oxygen is available to the muscles, and metabolites can be released to the blood. Secondly, the time period between the completion of exercise and muscle sampling will inevitably be larger following dynamic exercise than that which would occur following isometric contraction. Thirdly, warm-up periods are often implemented prior to dynamic exercise in order to prevent injury, and these warm-up periods may cause metabolic disturbances (i.e. increased muscle lactate, decreased PCr concentration) that are not taken into account when determining ATP turnover rates. Therefore, comparison of the present results to those obtained following dynamic exercise is difficult. Certainly, differences in energy metabolism would be expected to occur between dynamic and static exercise because relaxation of muscle and the reconstitution of high

force both require more energy than does maintenace of tetanic contraction (Loiselle and Walmsley, 1982).

2.4.3 COMPARISON WITH ELECTRICALLY STIMULATED EXERCISE

Several experiments have investigated energy metabolism during isometric contractions elicited through electrical stimulation protocols. The present results correspond well with estimates of ATP turnover during electrical stimulation at 20 Hz (Hultman and Sjöholm, 1983a; Spriet, 1992). Similar to the present study, continuous stimulation of the quadriceps (20 Hz), which produces a fused tetanus that represents 70-75% of MVC force (Sjöholm et al., 1983), resulted in an ATP turnover rate of 5.6 $mmol \cdot kg^{-1}dm \cdot s^{-1}$ during the initial 10s, 60% of which was provided by PCr splitting and 40% by glycogenolysis. Absolute values of PCr depletion and lactate accumulation were also very similar to the present results for the 1st 15s of stimulation (Hultman and Sjöholm, 1983a).

In contrast, studies which have utilized electrical stimulation protocols at 50 Hz (Hultman and Sjöholm, 1983b; Hultman et al., 1990) have shown much higher rates of ATP turnover. 1.26s of stimulation (50 Hz - which produces 95% MVC force (Sjöholm et al., 1983)) resulted in an ATP turnover rate of 11 $mmol \cdot kg^{-1}dm \cdot s^{-1}$, 80% of the ATP provided by PCr, and 20% from glycogenolysis (Hultman and Sjöholm, 1983b). A

subsequent study from the same laboratory (Hultman et al., 1990) estimated ATP resynthesis rates of 5.3 and 4.2 $mmol \cdot kg^{-1}dm \cdot s^{-1}$ from PCr degradation, and 4.4 and 4.5 $mmol \cdot kg^{-1}dm \cdot s^{-1}$ from glycogenolysis after 5s and 10s, respectively, of intermittent stimulation at 50 Hz (1.6s on / 1.6s off) (Hultman and Greenhaff, 1991). The rates reported above are much higher than those measured in the present investigation, however, the results from these stimulation experiments are difficult to interpret since neither study gave the number of subjects involved in experimentation, nor the equations used to calculate the rates of ATP turnover.

A more recent study investigated muscle metabolism in type I and type II fibers of the human quadriceps, and also utilized an intermittent stimulation protocol (20 Hz and 50 Hz) (Söderlund et al., 1992). Anaerobic ATP turnover was found to be much greater in type II fibers than type I fibers at both stimulation frequencies, and the rates given are specific to each fiber type. In addition, rather than estimating the proportion of ATP production by glycogenolysis from the accumulation of lactate (as in the present study), Söderlund et al. (1992) used an open circulation and estimated the ATP production per glucosyl unit metabolized from the decrease in muscle glycogen which occurred. The ATP turnover rates given by Söderlund et al. (1992) for stimulation durations of 10 and 20s are again much higher than those

determined in the present study, however, due to the variations in the protocol mentioned above, comparison between the two studies is difficult.

It appears that energy provision during voluntary sustained maximal contractions is similar to the energy provision which occurs during continuous stimulation at 20 Hz but less than that which occurs intermittently at 50 Hz. In the past, force has been shown to fall more rapidly with continuous stimulation at high-frequencies (50-80 Hz) than during voluntary contraction (Bigland-Ritchie et al., 1978). This phenomenon which has been explained by failure of electrical propagation at the muscle fiber membrane (Bigland-Ritchie et al., 1979), is often referred to as "high-frequency" fatigue, and is accompanied by changes in the wave form of the muscle action potential (Jones and Bigland-Ritchie, 1986). This seems to indicate that during voluntary activity, fewer changes in electrical transmission will occur than during high-frequency stimulation. Such changes may have an adverse effect on energy metabolism, thus leading to an increased requirement for ATP. This factor makes the comparison between voluntary maximal activity and high-frequency stimulation difficult. In the present study, voluntary force decreased 10% in 14s, which appears to be similar to the force loss resulting from intermittent stimulation (50 Hz) (Hultman et al., 1990). Neuromuscular

block is not probable in this situation (Hultman et al., 1990), since the stimulation was intermittent.

There is evidence to suggest that the energy cost of intermittent contraction exceeds that of sustained contraction. Spriet et al. (1988) found that intermittent stimulation (20 Hz) resulted in a faster decline in force production, and higher utilization of energy than did continuous stimulation (20 Hz). These stimulation studies were similar to the present study in that circulation to the quadriceps was occluded 30s prior to contraction, and ATP turnover was estimated from changes in metabolites measured by muscle biopsy sampling. However, it is difficult to identify whether the increased rate of ATP turnover associated with the intermittent protocol was due solely to the experimental treatment, because, during the intermittent stimulation, the blood flow was occluded for twice the duration than during the sustained protocol. Therefore, even though the contraction duration was the same between the 2 protocols (continuous vs. intermittent) it is possible that, during the intermittent protocol, changes could have occurred within the muscle to cause an increased energy cost associated only with increased duration of circulatory occlusion, but had little to do with the energy requirements for contraction. In this situation, ATP turnover rates calculated for the intermittent protocol may be artificially elevated. Evidence to support Spriet et

al. (1988), comes from Bergström and Hultman (1988) who found that fatigue developed more rapidly during intermittent contractions of short duration compared to intermittent contractions of long duration. However, similar to the Spriet et al. (1988) paper, duration of circulatory occlusion differed between the contraction protocols.

Despite the complications involved in comparing sustained and intermittent exercise, an increased energy requirement during intermittent exercise certainly exists (Spriet et al., 1988; Bergström and Hultman, 1988; Chasiotis et al., 1987). Established mechanisms for increased energy cost during intermittent exercise are enhanced Ca^{++}-transport ATPase activity during the relaxation phase (Spriet et al., 1988), as well as enhanced actomyosin ATPase activity during initiation of each contraction (Bridges et al., 1991). It was our aim in the present investigation to use a voluntary exercise protocol that taxed the energy systems to the greatest extent, but also allowed accurate measures of muscle metabolites. Second by second intermittent maximal voluntary contraction would exhibit low reliability, and voluntary dynamic exercise would not allow for multiple biopsy sampling. Therefore, for our purposes, sustained isometric maximal contraction was the protocol of choice.

2.4.4 THE RECOVERY PROCESS

2.4.4 (i) Phosphocreatine resynthesis

Following contraction, PCr resynthesis occurred rapidly such that after 60s of recovery PCr concentration was not significantly different from the resting value ($n=4$, $p \leq 0.05$). Multiple regression analysis was required to determine a curve-fit for the relationship between PCr concentration and recovery time (Fig 2.9) because the rate of PCr resynthesis was rapid during the first 40s of recovery, but then appeared to level off. The nature of the experimental design did not allow comparison between recovery times (i.e. between 60s and 180s), however, following 60s of recovery there did not appear to be any further increase in PCr concentration.

These results correspond well with previous investigations of PCr resynthesis (Harris et al., 1976; Bangsbo et al., 1993).

2.4.4 (ii) Lactate clearance

Muscle lactate concentration was measured up to 180s of recovery following a MVC (14s). Paired *t*-tests were performed to compare lactate concentration values at each recovery time point with the PreEx value. After 180s of recovery, the muscle lactate concentration was still

significantly higher (n=4, $p \leq 0.05$) than the resting value. The small sample size at some recovery time points (20s, n=3; 60s, n=4), resulted in non-significant differences. Previous literature has indicated that approximately 1 hr is required to remove most of the muscle lactate accumulated during intense exercise (Karlsson and Saltin, 1971).

Fig 2.10 reveals that a decrease in muscle lactate concentration appeared to occur in the initial 10s of recovery. This was to be expected. Prior to contraction the circulation was occluded for 30s, and although the cuff was released at the initiation of contraction, occlusion should have persisted throughout contraction due to the high muscular pressures produced during isometric exercise, thus resulting in a high muscle-blood gradient which would favour the efflux of lactate. Previous literature has reported that following isometric contraction to fatigue (66% MVC), where muscle lactate concentration reached 91 $mmol \cdot kg^{-1}dm$, the initial rate of lactate disappearance was 26.6 $mmol \cdot kg^{-1}dm \cdot min^{-1}$ (Harris et al., 1981). In the present investigation, 14s MVC's produced a mean muscle lactate concentration of 23.89 ± 2.17 $mmol \cdot kg^{-1}dm$, which would not be expected to produce as much driving force as 91 $mmol \cdot kg^{-1}dm$ reported previously (Harris et al., 1981), however, lactate clearance to the blood stream would still be expected to lead to a decrease in muscle lactate concentration.

2.4.5 SUMMARY AND CONCLUSIONS

Results from the present investigation indicate that during 14s of maximal isometric contraction, PCr concentration decreased to 53.3% of the PreEx value at a rate of 2.53 $mmol \cdot kg^{-1}dm \cdot s^{-1}$. There was a concomitant increase in muscle lactate to approximately 8 times the initial value, at a steady rate of 1.52 $mmol \cdot kg^{-1}dm \cdot s^{-1}$. Following contraction, PCr was resynthesized rapidly, and returned to the PreEx value after 60s of recovery, while muscle lactate concentration appeared to decrease, but was still significantly elevated above resting concentration after 180s of recovery.

Perhaps the most important conclusion which can be drawn from the present results is that, in a voluntary isometric contraction protocol, PCr and glycogenolysis appear to contribute equally to energy provision throughout the duration of the task (with the exception of the first 2s). This is in contrast with early research which predicted PCr would supply the majority of the energy required during intense exercise up to 10 or 15s (Margaria et al., 1964, 1969). It is this relationship which is depicted in Fig. 1. The present results are also in contrast with previous data which suggested that PCr is depleted early, and most of the ATP should be provided through anaerobic glycogenolysis (Hirvonen et al., 1987; Boobis, 1987). These previous data, however, come from dynamic exercise, which, as described

above, is difficult to compare to isometric exercise. Nonetheless, the present results are the first to give a systematic, second by second, estimation of energy provision during maximal voluntary exercise.

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McMASTER UNIVERSITY
HAMILTON, ONTARIO, CANADA

COMMITTEE ON
THE ETHICS OF RESEARCH ON HUMAN SUBJECTS

TO: Dr. Duncan Mac Dougall / M. Odland

RE: _____

TITLE: The time course and relative contribution of phosphocreatine splitting and anaerobic glycolysis to energy provision during a sustained maximal voluntary contraction.

The above named applicant has submitted an application to the Committee on Ethics of Research on Human Subjects.

The Committee has reviewed this request and finds that it meets our criteria of acceptability on ethical grounds. The review has been conducted with a view toward insuring that the rights and privacy of the subject have been adequately protected; that the risks of the investigation do not outweigh the anticipated gain; and that informed consent will be appropriately obtained.

We concur in all necessary endorsements of the application.

Jane Synge
Jane Synge

Date: 6th Dec 93

For the Committee on the Ethics of Research on Human Subjects

C.K. Bart, Associate Professor, Business
T. Beckett, Judge, Unified Family Court
I.M. Begg, Professor, Psychology
R.A. Brown, Associate Professor, School of Social Work
B. Donst, Ecumenical Chaplain, Chaplains' Office
R. Howard, Professor, Sociology
T. Kroeker, Lecturer, Religious Studies
R.J. Preston, Professor, Anthropology
C. Riach, Associate Professor, Department of Kinesiology
J. Synge, Associate Professor, Sociology (Chair)

THE TIME COURSE AND RELATIVE CONTRIBUTION OF PHOSPHOCREATINE SPLITTING AND ANAEROBIC GLYCOLYSIS TO ENERGY PROVISION DURING A MAXIMAL ISOMETRIC CONTRACTION.

INFORMATION AND CONSENT FORM

The principal investigator for the project is Maureen Odland, under the supervision of Dr. Duncan MacDougall. A detailed verbal description of the procedures in the study will be given in addition to this written information. After carefully reading the following information, please sign it if you wish to be a subject for the study.

A. PURPOSE

The purpose of this study is to determine how the depletion of phosphocreatine (PCr) and the production of lactate combine to provide energy during a maximal isometric contraction of the quadriceps muscle.

B. PROCEDURES

You will be seated with your lower leg positioned 90 to the horizontal. Your upper and lower leg will both be firmly secured to the dynamometer with velcro straps. As you maximally contract your quadriceps extensors, torque will be measured via a force plate located just above and in front of your ankle. Visual feedback of muscle force will be provided on a computer screen and you will be trained to ensure maintenance of maximal force output throughout each contraction (max 14s).

You will be required to attend the lab on two separate occasions. During each session, three small samples of muscle will be taken from your quadriceps muscle by what is known as the needle biopsy procedure. One sample will be a resting sample, while the remaining two samples (one from each leg) will be taken following exercise of a pre-determined duration (between 2 and 14s). On your second trip to the lab, the resting sample will be taken from the opposite leg as the first resting sample.

Prior to each biopsy sample (and exercise), a small incision will be made in your skin (see section C), and a blood pressure cuff will be inflated around your thigh for 30s to occlude the

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blood flow to your quadriceps muscle. This procedure prevents oxygen from reaching the muscle, and also prevents metabolites from escaping the muscle following contraction. Once the cuff has been inflated for 30s, you will be asked to contract maximally for the pre-determined time period. As soon as you relax, the muscle sample will be taken. Following the biopsy, the cuff will be removed and immediately placed around your opposite thigh in order to obtain the second test sample. We wish to minimize the time period between samples.

Muscle samples will be analyzed for concentrations of ATP, ADP, PCr, total creatine, glycogen, G-6-P, and lactate.

C. POSSIBLE RISKS of the NEEDLE BIOPSY PROCEDURE

This procedure involves the local injection of an anaesthetic into the skin of the quadriceps area, after which a small (4mm) incision will be made and a small (50-100mg) piece of muscle will be removed with a special needle. After the procedure a suture will close the skin and pressure will be applied to minimize bruising. Most people report little discomfort with the procedure. It will be performed by a physician who is familiar with the technique.

Complications with the procedure are rare. However, in our experience, fewer than 1 in 400-500 subjects experience a local skin infection, 1 in 30-40 have a temporary (up to 4 months) localized loss of sensation in the skin at the site and a few subjects have mild bruising around the incision for 4-5 days. There is also the very rare (one in a million) chance that you may be allergic to the local anaesthetic.

D. OCCLUSION OF BLOOD FLOW

Since the pressure cuff will only be in place for a total duration of less than two minutes, there are no harmful effects associated with this part of the study, although you may experience a sensation of numbness towards the end of the procedure. This will disappear as soon as the pressure is released.

E. CONFIDENTIALITY

The data collected will be used in preparation of reports to be published in scientific journals. Subjects will not be identified by name in these reports. You will have access to your own data when it is available, for your own interest.

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F. REMUNERATION

You will receive a minimum honorarium of $100 to help compensate you for your time commitment.

G. FREEDOM TO WITHDRAW

You are free to withdraw from the study at any time. If, after reading the above information, you are interested in participating as a subject you should read the statement below and sign in the space provided.

I HAVE READ AND UNDERSTAND THE ABOVE EXPLANATION OF THE PURPOSE AND PROCEDURES OF THE PROJECT AND AGREE TO PARTICIPATE AS A SUBJECT.

Signature:_____

Witness: _____

Date: _____

Appendix II Paired t-test analysis

EX - PCr and lactate concentration changes with contraction time

Time	n	df	PCr t	PCr p	PCr significance	Lactate t	Lactate p	Lactate significance
0 to 2s	7	6	3.256	0.017	yes	-4.367	0.0047	yes
0 to 4s	7	6	6.042	9.32e-4	yes	-8.055	1.96e-4	yes
0 to 6s	6	5	4.603	0.006	yes	-8.474	3.76e-4	yes
0 to 8s	6	5	7.192	8.08e-4	yes	-9.762	1.92e-4	yes
0 to 10s	5	4	10.404	4.82e-4	yes	-19.188	4.35e-5	yes
0 to 12	6	5	11.054	1.06e-4	yes	-9.132	2.64e-4	yes
0 to 14s	6	5	9.368	2.34e-4	yes	-10.655	1.26e-4	yes

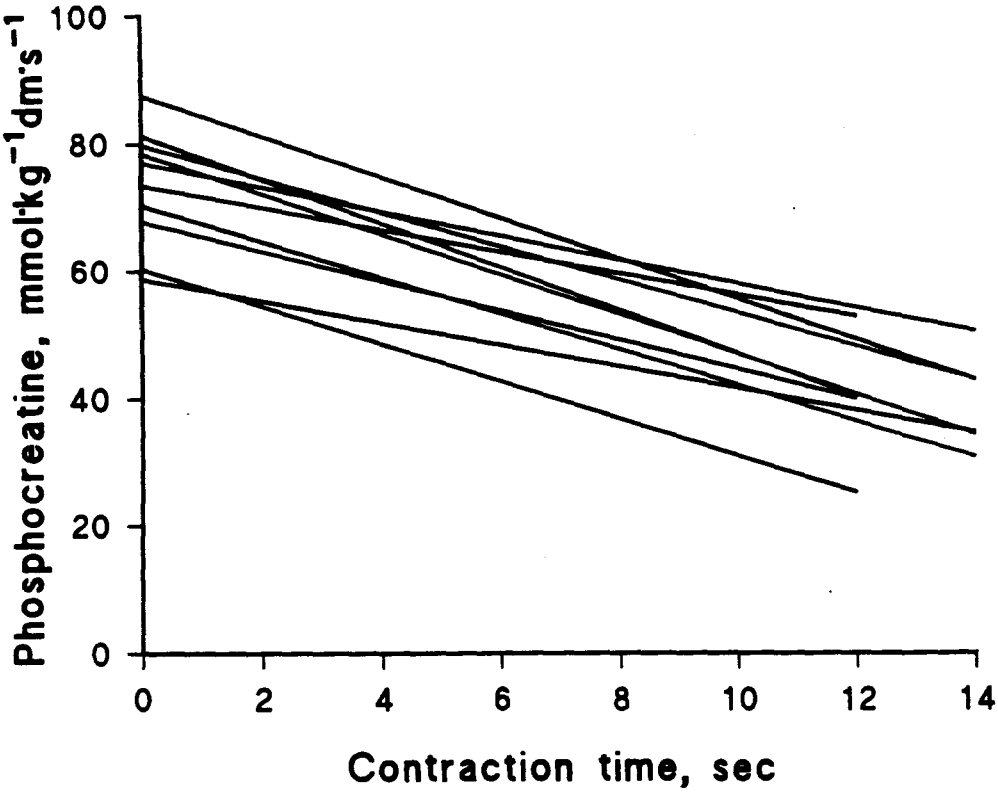
REC - PCr and lactate concentration changes with recovery time

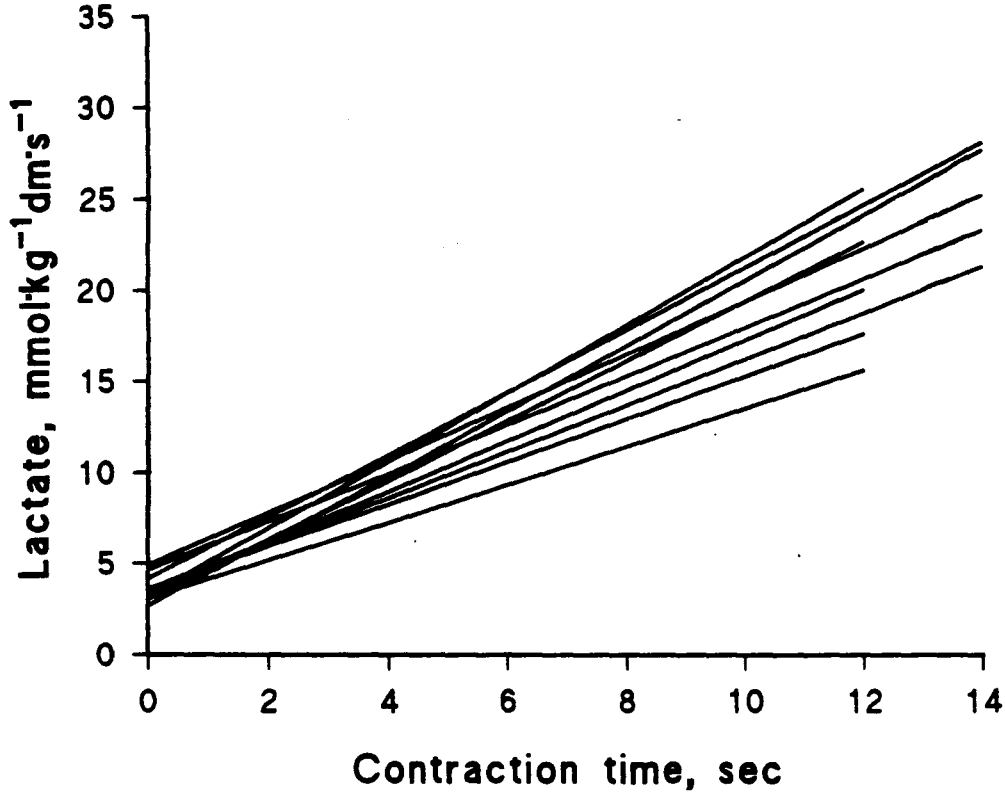
Time	n	df	PCr t	PCr p	PCr significance	Lactate t	Lactate p	Lactate significance
0 to 10s	4	3	6.345	0.008	yes	-4.237	0.024	yes
0 to 20s	3	2	5.972	0.027	yes	-3.501	0.073	no
0 to 30s	5	4	6.021	0.004	yes	-3.225	0.031	yes
0 to 40s	4	3	3.470	0.040	yes	-4.359	0.022	yes
0 to 60s	4	3	0.974	0.402	no	-2.448	0.092	no
0 to 12	5	4	1.159	0.311	no	-3.558	0.024	yes
0 to 18	4	3	1.431	0.248	no	-3.219	0.047	yes

Appendix III Individual linear regression equations

Lactate increase	y = mx + b	
SUBJECT	m	b
SD	1.7147	4.1682
MB	1.3842	3.4822
TN	1.0491	3.0908
JK	1.7905	2.6842
IM	1.6426	3.0412
JM	1.8647	3.2321
SC	1.3351	4.6677
MS	1.4531	4.9185
CW	1.2684	3.5981
SI	1.1722	3.6129
MG	2.0584	3.5726
mean	1.521182	3.642591
SE	0.095246	0.207621

PCr decrease	y = mx + b	
SUBJECT	m	b
SD	-1.6968	58.6808
MB	-2.8041	70.2482
TN	-1.6954	73.3644
JK	-3.1758	87.5245
IM	-2.9153	60.2762
JM	-3.4019	81.2012
SC	-2.619	79.7743
MS	-1.8788	76.9448
CW	-3.1326	78.4108
SI	-2.2985	67.7145
MG	-2.18	74.32
mean	-2.52711	73.49634
SE	0.184951	2.646711


Phosphocreatine, mmol·kg⁻¹dm·s⁻¹
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Contraction time, sec


Lactate, mmol·kg⁻¹dm·s⁻¹
35
30
25
20
15
10
5
0
0
2
4
6
8
10
12
14
Contraction time, sec

Appendix IV EX Metabolite concentration raw data

SUBJECT	SAMPLE TIME	AVG G3P	CONC ADP	NORM Cr	NOR G6P	CONC TCr	NORM ATP	NORM PCr	AVG LACTATE
SD	0.000	1.765	1.429	43.629	1.101	111.525	26.503	73.236	2.437
	2.000	2.846	1.498	48.150	2.677	98.071	28.468	68.715	6.815
	4.000	3.559	2.486	49.102	2.938	104.493	28.242	67.763	12.203
	8.000	2.034	2.479	57.886	4.152	116.865	27.286	58.979	9.915
	12.000	4.745	2.491	70.547	6.067	102.841	26.000	46.318	22.032
MB	0.000	0.257	1.786	50.013	0.300	122.553	25.681	72.540	2.229
	4.000	0.491	1.577	58.512	0.817	86.046	25.780	64.041	6.470
	6.000	1.331	2.725	48.465	2.313	132.287	25.981	69.318	9.653
	8.000	3.581	1.058	64.385	3.811	109.918	23.231	58.168	15.301
	12.000	1.098	2.150	60.845	3.921	122.242	24.945	51.893	13.273
TN	0.000	0.480	2.213	47.712	0.919	126.774	25.790	86.013	2.571
	2.000	3.383	2.199	47.721	1.522	133.724	27.484	86.004	3.044
	6.000	0.858	1.759	75.592	2.749	112.698	26.470	58.132	17.804
	10.000	1.920	3.536	66.484	1.763	102.892	27.450	67.240	21.896
	14.000	6.433	1.595	95.117	5.994	125.126	25.033	38.607	25.403
JK	0.000	0.668	2.183	43.400	1.757	108.040	26.573	65.847	3.252
	2.000	2.468	1.629	57.789	2.515	103.513	26.043	51.458	7.701
	6.000	2.440	2.178	73.500	0.550	69.230	26.397	35.746	10.281
	10.000	9.001	2.223	75.494	6.376	109.247	26.440	33.753	22.783
	12.000	1.750	2.070	82.128	2.746	77.511	23.686	27.119	21.067
IM	0.000	1.713	2.119	49.591	0.799	116.805	25.212	79.781	4.936
	2.000	0.550	2.228	52.850	1.840	128.842	26.093	76.522	5.247
	6.000	2.403	1.894	66.474	3.230	119.478	26.290	62.898	15.827
	8.000	2.737	1.513	79.599	4.403	108.804	25.269	49.773	15.215
	12.000	2.849	2.050	87.593	5.352	129.372	23.565	41.779	27.148
JM	0.000	1.184	1.657	45.479	0.402	110.002	27.347	82.013	2.862
	4.000	3.706	1.179	56.704	2.183	113.154	27.056	70.821	11.877
	6.000	0.589	2.039	66.503	3.955	113.889	26.462	61.022	13.582
	10.000	3.542	1.462	79.505	5.107	127.525	26.275	48.020	18.121
	14.000	1.666	2.166	79.575	6.190	114.974	26.423	47.950	22.365

Appendix IV EX Metabolite concentration raw data

SUBJECT	SAMPLE TIME	AVG G3P	CONC ADP	NORM Cr	NOR G6P	CONC TCr	NORM ATP	NORM PCr	AVG LACTATE
SC	0.000	0.067	1.147	49.210	1.362	123.365	26.102	83.344	2.538
	4.000	0.986	1.417	71.521	4.582	118.855	27.584	61.033	9.532
	8.000	2.009	1.612	69.114	4.592	118.432	26.894	63.440	16.536
	12.000	1.556	1.720	85.542	6.470	132.554	24.816	47.012	24.980
	14.000	0.683	4.334	59.195	5.722	105.706	26.048	58.500	20.411
MS	0.000	0.210	1.460	35.110	0.294	113.148	27.334	81.288	2.153
	2.000	0.430	2.332	46.166	1.541	116.398	24.573	70.232	6.042
	4.000	1.419	2.003	52.314	3.869	115.495	27.416	64.084	8.261
	10.000	1.800	2.067	69.154	6.832	125.524	23.330	47.244	22.644
	14.000	0.809	0.943	81.171	8.281	76.153	25.092	35.227	16.942
CW	0.000	0.310	1.667	38.223	0.904	96.908	29.719	76.728	3.445
	2.000	0.460	1.650	61.579	0.394	98.773	26.292	53.372	8.208
	4.000	1.130	2.231	57.920	3.400	114.951	28.983	57.031	7.352
	8.000	2.689	1.997	50.676	3.178	88.757	25.507	49.629	14.709
	12.000	3.320	3.250	72.899	7.133	111.398	27.360	42.052	16.475
SI	0.000	0.670	2.510	44.016	1.822	106.274	25.421	62.258	3.672
	2.000	1.696	1.795	48.167	2.988	92.603	25.518	50.632	7.672
	6.000	2.501	2.017	55.029	0.000	102.080	30.744	49.222	15.775
	14.000	5.000	2.309	70.993	8.691	104.654	30.032	35.281	32.455
MG	0.000	0.460	4.240	41.067	1.281	109.952	27.634	75.216	3.010
	4.000	0.253	2.217	61.622	4.244	115.713	29.028	54.662	10.938
	8.000	2.488	1.658	72.892	3.143	110.441	27.676	43.391	19.072
	10.000	1.911	2.264	73.903	8.642	116.283	26.259	42.380	23.808
	14.000	3.493	1.871	81.639	9.570	101.062	24.404	34.644	25.743

Appendix V REC metabolite concentration raw data

SUBJECT	SAMPLE TIME	AVG LACTATE	NORM Cr	AVG TCr	NORM ATP	NORM PCr
MB	0.000	2.229	50.013	122.553	25.681	72.540
	10.000	8.823	70.574	101.118	25.742	51.980
	40.000	6.471	53.320	108.343	25.778	69.233
	30.000	6.137	61.449	102.495	26.259	61.104
	120.000	5.160	47.616	111.391	27.439	74.937
TN	0.000	2.571	43.726	126.774	25.790	86.013
	180.000	10.812	58.581	93.804	26.811	69.803
JK	0.000	3.252	48.686	108.039	26.573	65.847
	30.000	29.883	72.499	90.587	25.279	36.998
	120.000	19.157	61.561	108.851	23.038	54.370
	40.000	13.685	65.910	65.672	25.101	50.494
SC	0.000	2.538	45.497	123.365	28.715	84.695
	10.000	17.022	70.916	99.670	27.414	56.272
	40.000	14.694	57.261	120.938	27.441	71.153
	20.000	17.251	78.612	106.371	27.287	47.886
	120.000	15.780	57.657	101.843	27.627	70.721
MS	0 000	2.153	36.966	113.148	27.334	81.288
	40 000	18.213	60.476	91.346	25.925	58.960
	180 000	10.761	48.796	85.151	25.534	70.053
CW	0.000	3.445	40.862	94.190	29.867	76.624
	20.000	21.004	78.875	111.409	24.353	40.969
	60.000	14.904	54.407	88.235	26.919	63.919
	30.000	15.798	66.311	97.369	26.574	52.754
	120.000	11.629	49.744	102.593	26.892	68.292
MG	0.000	3.010	43.281	109.952	27.634	75.216
	10.000	23.482	85.189	125.686	21.918	35.453
	60.000	11.963	54.708	119.061	22.549	64.374
	30.000	12.147	61.704	96.519	23.355	57.736
	180.000	15.148	41.844	99.394	25.260	76.580
MH	0.000	3.311	44.302	125.105	24.687	81.974
	20.000	8.912	64.034	112.833	25.054	61.304
	60.000	6.244	36.768	128.385	25.956	89.867
	30.000	13.356	57.890	128.048	24.311	67.740
	120.000	7.593	34.945	125.040	25.861	89.386
JC	0.000	4.479	49.603	74.406	28.848	76.686
	10.000	14.167	70.595	128.829	24.579	54.619
	180.000	5.505	48.775	124.395	26.635	77.556
	60.000	5.400	52.120	108.797	26.924	74.040

Appendix VI Reproducibility Measurements

Coefficients of Variation (CV) - Peak Torque
CV = SD of repeated trials/mean of trials X 100

SUBJECT	Ex - 4 trials CV	SUBJECT	Rec - 2 trials CV
TN	13.27	TN	17.47
SD	4.80	JM	2.70
JM	12.26	MG	6.93
IM	6.17	JK	0.95
MG	4.54	SC	14.05
JK	8.52	SI	0.29
SC	7.70	MB	0.29
SI	4.72	CW	14.05
MB	5.74	MS	8.56
CW	6.35	MH	5.90
MS	5.23	JC	5.17
MEAN	7.21	MEAN	6.94
SE	0.91	SE	1.81

Coefficients of Variation - metabolite assays

CV = SD of repeated trials/mean of trials X 100

Extract CV - 6 repeated trials with same extract
Aliquot CV - 4 repeated trials with different aliquots from the same biopsy sample

Metabolite	Extract	Aliquot
[PCr]	4.23	5.89
[ATP]	2.64	4.65
[LACTATE]	4.86	6.84
[GLYCOGEN]	4.13	9.42

APPENDIX VII - Human muscle tissue metabolite assays

TISSUE POWDERING TECHNIQUE

1. Break biopsy apart on kleenex or kimwipe to allow the absorption of blood onto the tissue paper.

2. Transfer the tissue to a ceramic bowl or Petri dish.

3. Remove large pieces of connective tissue.

4. Pulverize the muscle biopsy by crushing between the ends of 2 large pairs of tweezers.

5. Carefully take fractions of pulverized tissue and search for connective tissue.

6. When a clump of tissue is found that may contain connective tissue, remove it by scraping it on the inner rough edge of a pair of large tweezers until all of the muscle flakes away. The tissue that does NOT flake away will be connective tissue.

7. Weigh out the powdered tissue into eppendorf tubes or small test tubes for extraction of glycogen (>2 mg) or metabolites (>5 mg).

MUSCLE METABOLITE EXTRACTION PROCEDURE

1. Bring freeze-dried samples to room temperature in a desiccated container (the samples should be in capped 12 x 75 disposable glass tubes). Precool the Sorval centrifuge to +4°C.

2. Use the appropriate formula to determine the volume of 0.5 M PCA (1 mM EDTA) to add

   Rest samples: muscle dry wt (mg) x 40 = vol (ul)
   Exercise samples: muscle dry wt (mg) x 80 = vol (ul)

   Minimum muscle weight for rest samples should be 10 mg and for exercise, 5 mg. If less muscle is available, use 400 ul PCA. Round up to the nearest multiple of 25, eg. if required 465 ul, add 475 ul.

3. Add PCA as quickly as possible to 10 samples or less. Vortex the samples gently to ensure all muscle is reached by the PCA, while making sure muscle adherence to the tube wall above the fluid line is minimal. Store samples on ice during the extraction. Vortex 3 times during 5 minutes of extraction. Do not extract greater than 10 samples at one time.

4. Centrifuge the samples (4°C at 7,000 rpm for 5 minutes. Remove the supernatant with Pasteur pipettes carefully and transfer to a tared 12 x 75 glass test tube and weight the PCA extract obtained on the Sartorius digital balance (round to the nearest mg - eg. 643 mg).

5. Divide PCA extract mass by 1.025 to give the extract volume. Divide the volume by 4 to calculate the required $KHCO_3$ (2.2 M) needed for neutralization. Add the $KHCO_3$ and vortex 2-3 times or until bubbling stops. Remember that during steps 4 and 5 samples should be maintained on ice.

6. Centrifuge for 15 min (4°C) at 7,000 rpm. Remove supernatant and transfer to labelled test tubes (glass or plastic). Samples are now ready for assays or immediate freezing at -80°C if not proceeding with assays.

ATP AND PCr

$$\text{Pcr} + \text{ADP} \xrightarrow{\text{CPK}} \text{Cr} + \text{ATP}$$

$$\text{ATP} + \text{Glu} \xrightarrow{\text{HK}} \text{ADP} + \text{Glu-6-P}$$

$$\text{Glu-6-P} + \text{NADP} \xrightarrow{\text{G6PDH}} \text{P-Gluconolactone} + \text{NADPH}$$

REAGENTS Wt/Vol	Conc of Stock mmol/1	Cuvette Conc mmol/1	x20 ul	x30 ul	x40 ul	x60 ul	x80 ul	x120 ul
Triethan- 18.6g/ olamine 100 ml pH 7.5-7.6	1000	100	500	750	1000	1500	2000	3000
Mg(Ac)2. 2.2g/100 ml 4H20	100	10						
EDTA.Na2. 0.4g/100 ml 2H20	10	1						
DTT 7.8mg/ml	50	1	100	150	200	300	400	600
NADP.Na2. 20.9mg/ml 4H20	25	1	200	300	400	600	800	1200
ADP.Na. 5.1mg/ml	10	0.04	20	30	40	60	80	120
Glucose 22.5mg/ml	125	5	200	300	400	600	800	1200
G6PDH (undil)		.7	15	23	30	45	60	90
Water (deion)			4 ml	6 ml	8 ml	12 ml	16 ml	24 ml

Enzymes

1. G6PDH - Glucose-6-Phosphate Dehydrogenase (Boehr. 127 035), use undiluted in reagent.

2. HK - Hexokinase (Boehr. 127 175), dilute 1:1 with water and use 2 ul.

3. CPK - Creatine Phosphokinase (Boehr. 127 566), dissolve 15 mg/ml and use 2 ul.

Procedure

1. Prepare reagent. Add 25 ul muscle extract to cuvettes and then add 225 ul reagent. Mix. Place in photometer. Monitor background absorbance.

2. Add 2 ul HK. Monitor absorbance.

3. Add 2 ul CPK. Monitor absorbance.

Creatine

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

$$\text{ADP} + \text{PEP} \xrightarrow{\text{PK}} \text{ATP} + \text{Pyruvate}$$

$$\text{CR} + \text{ATP} \xrightarrow{\text{CPK}} \text{PCr} + \text{ADP}$$

REAGENTS Wt/Vol	Conc of Stock mmol/1	Cuvette Conc mmol/1	x20 ul	x40 ul	x60 ul	x80 ul	x120 ul
Glycine 2.4 g/100 ml	320	100	2000	4000	6000	8000	12000
Mg(Ac)2. 0.4g/ 4H20 100 ml pH 9.0-9.1	16	5					
KC1 15.0g/100 ml	2000	30	100	200	300	400	600
ATP.Na2 15.4mg/ml 3.5 H20	25	1.5	400	800	1200	1600	2400
PEP 12.3mg/ml	25	1.0	300	600	900	1200	1800
NADH 9 mg/ml	12	0.15	90	180	270	360	540
LDH (undil)			10	20	30	40	60
PK (undil)			10	20	30	40	60
Water (deion)			3.1 ml	6.2 ml	9.3 ml	12.4 ml	18.6 ml

CREATINE 2

Enzymes

1. LDH - Lactate Dehydrogenase (Boehr. 107 034), use undiluted in reagent.

2. PK - Pyruvate Kinase (Boehr. 128 155), use undiluted in reagent.

3. CPK - Creatine Phophokinase (Boehr. 127 566), dissolve 15 mg/ml. Use 10 ul in assay.

Procedure

1. Prepare reagent. Add 20 ul muscle extract to cuvettes and then add 300 ul reagent. Mix. Place in photometer. Measure background absorbance.

2. Add 10 ul CPK. Monitor absorbance.

Technical Notes

In calculations use 330 ul sample size (10 ul from enzyme).

The reaction can begin to drift at approximately 7 min. It is best, therefore, to monitor absorbance at 2 min. intervals (eg. 5', 7', 9', and 11') and determine when the change/unit time becomes constant. The final delta is then at the last time point before this drift begins.

Lactate (Glycerol-3-P)

DHAP + Hydrazine -----> DHAP - Hydrazone

Glycerol-3-P + NAD $\xrightarrow{\text{G3PDH}}$ DHAP + NADH

Pyruvate + Hydrazine -----> Pyruvate - Hydrazone

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

v = up to 150 ul for rest samples
up to 50 ul after moderate exercise
up to 25 ul after intense exercise
+ water to make up to 150 ul

REAGENTS Wt/Vol	Conc of Stock mmol/1	Cuvette Conc mmol/1	x20 ul	x40 ul	x60 ul	x80 ul	x120 ul
Glycine 7.5 g/100 ml	1000	300	1500	3000	4500	6000	9000
Hydrazine 6.5 g/ Sulphate 100 ml	500	150					
EDTA.Na2. 0.2 g/ 2H20 100 ml pH 9.5	5	1.5					
NAD 16.6mg/ml	25	2.5	500	1000	1500	2000	3000

LACTATE 2

Enzymes

1. G3PDH - Glycerol-3-Phosphate Dehydrogenase (Boehr. 127 752), dilute 1:1 with water and use 2 ul.

2. LDH - Lactate Dehydrogenase (Boehr. 107 034), use 2 ul undiluted.

Procedure

1. Prepare reagent. Add muscle extract and water to 150 ul to cuvettes and then add 100 ul reagent. Mix. Place in photometer and read background absorbance.

2. Add 2 ul G3PDH. Monitor absorbance.

3. Add 2 ul LDH. Monitor absorbance.

Technical Notes

1. Due to the high concentration of lactate in perspiration, extreme care must be taken when handling pipette tips, oxford pipettes (for stirring), civettes, and test tubes.

2. For absorbance changes of greater than 200, 12 minutes may be required for the reaction to complete.

ADP and PYR 1

Pyruvate, ADP, AMP

$$1/2\ AMP + 1/2\ ATP \xrightarrow{MK} ADP$$

$$ADP + PEP \xrightarrow{PK} ATP + Pyruvate$$

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

REAGENTS Wt/Vol	Conc of Stock mmol/1	Cuvette Conc mmol/1	x20 ul	x40 ul	x60 ul	x80 ul	x120 ul
Triethan- 18.6g/ olamine 100 ml pH 7.5-7.6	1000	300	1500	3000	4500	6000	9000
	500	150					
Mg(Ac)2. 2.2 g/ 4H20 100 ml	5	1.5					
EDTA.Na2 0.4 g/ 2H20 100 ml	25	2.5	500	1000	1500	2000	3000
KC1 15.0 g/100 ml							
PEP 12.3 mg/ml							
NADH 9 mg/ml							

ADP and PYR 2

Enzymes

1. LDH - Lactate Dehydrogenase (Boehr. 107 034), dilute 1:11 with water and use 2 ul.

2. PK - Pyruvate Kinase (Boehr. 128 155), dilute 1:10 with water and use 2 ul.

3. MK - Myokinase (Boehr. 107 506), dilute 1:4 with water and use 2 ul.

Procedure

1. Prepare reagent. Add up to 200 ul muscle extract to cuvettes and then add 50 ul reagent. Mix. Place in photometer and monitor background absorbance.

2. Add 2 ul LDH. Monitor absorbance.

3. Add 2 ul PK. Monitor absorbance.

4. Add 2 ul MK. Monitor absorbance.

Technical Notes

1. Note that 1/2 AMP ultimately results in the reduction of 1 NADH so AMP concentrations must be divided by 2.

2. When running standards AMP and ADP, they must be run separately due to AMP contamination of ADP. Also, 2 ul of ATP must be added to each cuvette after PK and a background taken before MK addition. This is because the standards have no excess ATP to run the MK reaction, and ATP does have ADP contamination.

Glycogen Extraction Procedure (Animal and Human Skeletal Muscle)

1. Turn on the water bath to 80°C. Remove freeze-dried samples from the freezer and allow equilibration to room temperature in a desiccated container (samples should be in Eppendorf tubes).

2. Prepare solutions (G5, G6, and G7 are usually already made up). Stock:

   a. G5 - 0.1 M NaOH
   b. G6 - 0.1 M HCl
   c. G7 - 0.2 M citric acid; 0.2 M $Na_2.HPO_4$ (pH=5.0)
   d. Amyloglucosidase (Boehr. 208 469)

   a,b. Once G5 and G6 are made, check that equal volumes neutralize each other (e.g., 5 ml of NaOH and 5 ml HCL). This is needed only when first making up these solutions. If unequal volumes are required to obtain pH = 7, refer to steps b,c).

   b.c. Add G6 and G7 in the ratio of 1 part:3 parts. If G5 and G6 do not neutralize with equal volumes, amend at this step. Also check the pH of G7 periodically as it tends to become more acidic with time.

   c. G7

	conc.	gm/100ml
Citric acid.H_2O ($C_6H_8O_7.H_2O$)	0.2 M	4.20
$Na_2HPO_4.7H_2$)	0.2 M	5.36

   Initial pH is 3.9, uses 5 or 10 M NaOH to pH to 5. Dissolve chemicals in 75-80 ml of H_2O and then pH if making up 100 ml.

   d. AGS - Amyloglucosidase (Boehr. 208 469), dissolve 200 mg AGS per ml of G7 (takes some time to fully dissolve, vortex periodically).

3. **Extraction Procedure**

a. Add appropriate amount of G5 (0.1 M NaOH) to muscle samples according to the table below. Make sure that all muscle is reached by the solution by gently vortexing.

Dry muscle (mg)	G5 (NaOH) (ul)	G6,G7 (HCl, citrate) (ul)	AGS (ul)
0 -2.0	100	400	15
2.01-3.0	120	480	15
3.01-4.0	160	640	20
4.01-5.0	200	800	25

b. Incubate the samples for 10 min at 80°C. This step destroys background glucose and hexose monophosphates.

c. Neutralize the samples by adding the appropriate amount of G6,G7 (see table) after allowing the samples to cool. Close Ependorf tubes and gently shake or invert.

d. Add AGS as outlined in table, gently vortex and incubate samples at room temperature for 60 min. glycogen degradation occurs at this step.

e. Freeze samples at -20°C or measure glycogen (glucose) content directly.

Glycogen (Glucose Assay)

$$\text{Glu 6-P} + \text{NAD} \xrightarrow{\text{G6PDH}} \text{6-P-Gluconolactone} + \text{NADH}$$

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{Glu-6-P} + \text{ADP}$$

REAGENTS Wt/Vol	Conc of Stock mmol/1	Cuvette Conc mmol/1	x20 ul	x40 ul	x60 ul	x80 ul	x120 ul
Triethan- 7 g/ olamine 100 ml	375	100	1600	3200	4800	6400	9600
KOH 0.8 g/100 ml	150	40					
Mg(Ac)2. 2.4 g/ 2H20 100 ml	112.5	30					
EDTA.Na2 0.14 g/ 2H20 100 ml (adjust to pH 8.2 with further KOH)	3.75	1					
ATP 27.7mg/ml	45	0.75	100	200	300	400	600
DTT 9.36mg/ml	60	1	100	200	300	400	600
NAD 19.9mg/ml	30	1	200	400	600	800	1200
Water (deion)			3000	6000	9000	12ml	18ml

Enzymes

a. G6PDH - Glucose-6-Phosphate Dehydrogenase (Sigma G-5885, 100 NADP units or 228 NAD units), dilute in 200 ul water.

b. HK - Hexokinase (Sigma H-4502, 200 units), dilute in 200 ul water.

** Mix equal volumes of HK and G6PDH and use 2 ul in assay.

*** It takes approximately two weeks to order these enzymes from the U.S. and often HK can be back ordered for 4-6 weeks!!

Procedure

1. Add 50 ul sample and 250 ul reagent to cuvettes. Use 25 ul sample, 25 ul water and 250 reagent for resting human samples.

2. Monitor background absorbance.

3. Add 2 ul HK/G6PHD enzyme mix and monitor absorbance for 5-10 min. If samples are frozen, allow them to thaw and vortex in Eppendorf centrifuge to pull sediment to tube bottoms and ensure particle free extracts for the assays.

Note: Due to the variability in the measure of muscle glycogen content two aliquots of a given muscle are extracted and assayed separately. Acceptable variation between muscle aliquots is 10%.

Metabolite Concentrations

$$\text{conc. in muscle (umoles/g dry wt)} = \frac{A \times TV\ (ul)}{e \times SV\ (ul)} \times DF\ (ul/mg)$$

where: A = absorbance
e = extinction coefficient for NADH or NADPH;

VITATRON: is fixed at 366 nm, e = 3.40 cm2/mole
DU70 : may be set to 366 nm, e = 3.40 cm2/mole but greater sensitivity is achieved at 340 nm, e = 6.22 cm2/mole.

TV = total cuvette volume in ul
SV = sample volume in ul
DF = dilution factor in ul/mg

(Remember 1 = path length through cuvette = 1.0 cm and so it is not seen above)

Calculation for Glycogen

Dilution:

$$DF\ (ul/mg) = \frac{mg(dry\ muscle) + ul(NaOH) + ul(G6+G7) + ul\ (AGS)}{mg(dry\ muscle)}$$

Concentration: (similar to metabolites)

$$\text{[glycogen] in muscle (umoles glucosyl units/g dry wt)} = \frac{A \times TV\ (ul)}{e \times SV\ (ul)} \times DF\ (ul/mg)$$

Standard Check Recipes

Metabolite	Stock No.	M.W.	Corrected M.W.	Conc. Required	Recipe	Together
Lactate	Sigma 826-10	4.44 uM/ml		.75 uM/ml	168.9 ul std into 1 ml	168.9 ul LA 10 ul (A)
Glyc-3-P	Sigma G4631	418.3	422.5	.74 uM/ml	A. 31.4mg/ 10ml	821 ul H_2O 1.0 ml
ATP	Sigma A-5394	611.3	659.4	250 nm/ml	B. 30.6mg/ 2 ml 15.3 mg/ ml	10 ul (B) 10 ul (C)
PCr	boehr 127 574	338.9	380.4	350 nm/ml	C. 23.8 mg/ 2 ml 11.9 mg/ ml	980 ul H_2O 1.0 ml
F16DP	boehr 104 795	555.9	565.5	25 nm/ml	D.34.75mg/ 25 ml volumetric flask	10 ul (D) 990 ul H_2O 1.0 ml
G6P	Sigma G-7879	287.9	294.1	300 nm/ml	E.21.58mg/ 5 ml F. 19.2 mg/ 5 ml	20 ul (E) 10 ul (G) 10 ul (H) 100 ul (I) 860 ul H_2)
G1P	boehr 105 279	383.9	426.6	10 nm/ml	100 ul (F) into 10 ml(G)	10 ml
F6P	boehr 104 850	304.1	378.2	60 nm/ml	H. 18.3 mg/ 10 ml	
Glucose	Sigma 635-100	5.56 nM/l	4.51 nM/l	100 nm/ml	I. 25 ul stock soln + 114 H_2O	

Metabolite	Stock No.	M.W.	Corrected M.W.	Conc. Required	Recipe	Together
Pyruvate	Sigma 726-70	.45 uM/ml	.48 uM/ml	32 nm/ml	66.7 ul/ 1.0 ml	stds must be run separated for PYR, ADP, AMP ***
ADP	boehr 102 199	304.1	378.2	60 nm/ml	19.8 mg/ 10 ml 10 ul—> 1.0 ml	
AMP	boehr 102 199	475.5	504.2	5 nm/ml	25.2 mg/ 10 ml 100 ul—> 1.0 ml (J) 10 ul(F) —> 1.0 ml	
Creatine	Sigma 03630	131.1 87%	150.6	1.2 uM/ml	37.4 mg/ 3 ml (12.5	15 ul (K) 985 ul H_2O 1.0 ml

Appendix VIII Step by step procedure to measure ATP and PCr.

1) Lyophilize muscle tissue previously frozen in liquid N_2.

2) Powder all tissue (see instructions), and divide muscle powder into aliquots ≈5-10mg and place in 12 X 75 testtubes or eppendorf tubes. If you are not going to extract the metabolites immediately, tissue aliquots can be stored desiccated at -70°C.

3) Prior to extracting metabolites, have all the required solutions for the PCr/ATP assay prepared, and make up your reagent. Specifically, prepare your TEA buffer (with $Mg(Ac)_2$ and EDTA), and ensure the pH is 7.5-7.6. Store TEA buffer (clearly labelled) in the fridge - this solution should be good for 2-3 months. The DTT, ADP, and glucose solutions can be made in large volumes, and then divided into small aliquots (≈1.5 ml or less) and frozen at -20°C in small freezer tubes. When preparing the reagent on the day of the assay, simply defrost 1 aliquot of each, and add the correct volume to the TEA buffer. NADP, HK and CPK must be prepared fresh daily. The standard solution should also be prepared fresh daily (see recipe).

4) See instructions to extract metabolites. Before beginning the extraction procedure, be sure PCA and $KHCO_3$ solutions neutralize each other. Ensure to prepare $KHCO_3$ fresh daily.

5) Because PCr and ATP are acid labile, be sure not to leave the tissue in the PCA solution for too long. Practise the assay with resting tissue until you can consistently get PCr values close to 80 $mmol \cdot kg^{-1}$ dry weight.

6) Your samples are now ready for analysis. Ensure cuvettes are clean and dry. Six cuvettes fit in the spectrophotometer at a time. On your first run, run 2 blanks, 2 standards, and 2 samples (in duplicate). Add the correct volumes of samples and reagents, and stir. Place in the spec, and monitor the absorbance (abs). When the abs is stable, add HK and stir. Monitor abs until stable (≈2-4min), then add CPK. The CPK rxn can take 5-10 min.

8) Calculate the metabolite concentrations from the recorded abs changes using the calculations given. It's a good idea to use a spreadsheet for ease and quickness.