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Lactate Metabolism and Transport in Skeletal Muscle

by

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the degree of Doctor of Philosophy

at

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This thesis is dedicated to my parents and grandparents:

John and Janet McDermott, Arthur and Edith Archer,
Michael and Beatrice McDermott.

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Abstract

Lactate metabolism and transport were studied in skeletal muscle. Experiments examining the metabolism of inactive muscle during exercise revealed that lactate accumulates in inactive muscle due to increased glycogenolytic flux. This glycogenolysis is dissociated from the muscle energetic demand. Further studies in the perfused rat hindlimb showed that lactate is taken up by muscle and can be used to synthesize glycogen (glyconeogenesis). The rate of glyconeogenesis is related to the fiber type distribution of the muscle (FG>FOG>SO). Glyconeogenesis is sensitive to pH but is not altered by hormones (Corticosterone and Insulin). To determine the nature of the membrane permeability to L-lactate, the transmembrane flux of lactate was studied in two preparations: a) purified plasma membrane vesicles, and b) intact muscle strips. Transport (zero-trans) and uptake experiments indicated saturability with increasing L-lactate concentrations, stereospecificity, sensitivity to inhibitor compounds such as pyruvate and N-ethylmaleimide, and stimulation by an inwardly directed pH gradient. These data implicate a carrier mediated transport sytem as the predominant mode for lactate transport in skeletal muscle. Further studies on the lactate transport system suggest that, in contrast to glucose uptake in muscle, lactate uptake is not increased after an acute bout (30min.) of contractile activity. Lactate transport studies using skeletal muscle sarcolemmal vesicles (SLV) isolated from rats that were subjected to chronic exercise (CE) for five weeks, revealed that lactate transport was enhanced in the CE membranes compared to membranes from a sedentary control group (S), when the experiments were performed at a low concentration of L-lactate (1mM), however, at an external concentration of 50mM L-lactate there was no difference in membrane transport between the CE and S groups. In summary, lactate may be produced in a muscle without increased contractile activity, and it can be used as a precursor for glyconeogenesis. Lactate transport in muscle is mediated by a carrier system which displays similar characteristics to the lactate carrier described for several other tissue types. However, lactate transport is not altered by muscle contractility, although some adaptation in transport capacity is evident after a period of chronic exercise.

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Finally, my deepest thanks are conveyed to my wife, Nicola, who has unceasingly supported my endeavour with much love and understanding.

SECTION 1. INTRODUCTION

Since the 1920's much attention has been focused on the production of lactate in muscle, usually from the perspective of lactate as a fatigue-inducing metabolite during anaerobiosis (Gollnick, Bayly et al. 1986; Gladden 1989; Graham 1988). The understanding of skeletal muscle lactate physiology is important because, under non-pathogenic conditions, skeletal muscle is by far the most important determinant of whole body lactate dynamics (Roth and Brooks 1990). More recent'y there has been a reappraisal of the role of lactate as an 'intermediary' metabolite, rather than as a metabolic 'end product' in skeletal muscle (Connett, Honig et al. 1990; Brooks 1986; Connett, Gayeski et al. 1984). The challenge, therefore, is to understand the role of lactate as a metabolic intermediate, and the role it may play in skeletal muscle physiology when it is not involved as a fatigue-inducing end product. Viewed as a metabolic intermediate, lactate can have several fates. In skeletal muscle the primary means of disposal include; a) re-conversion back to glycogen (glyconeogenesis), b) oxidation to CO_2, or c) efflux into the circulation to join the oxidizable/gluconeogenic carbon pool.

The use of lactate as a precursor for glycogen synthesis in skeletal muscle (glyconeogenesis) has been the subject of considerable debate (Bar and Blanchaer 1965; Connett 1979; Hermansen and Vaage 1977; Hill 1924; Meyerhof 1920; Meyerhof, Lohman et al. 1925). However, it has now been determined that glyconeogenesis constitutes a significant process in mammalian

skeletal muscle (McLane and Holloszy 1979; Bonen, McDermott et al. 1990c; Talmadge, Scheide et al. 1989), and the relative contribution of lactate as a precursor for glycogen synthesis in muscle, compared to the major glycogenic precursor glucose, has been elucidated (Bonen, McDermott et al. 1990c).

It has been recognized for some time that lactate is an important substrate for the normally functioning mammalian heart (Katz 1977), and it has been suggested that lactate is a more preferable substrate for the heart than glucose (Drake, Haines et al. 1980). Lactate consumption in skeletal muscle has perhaps not received as much attention as the role of lactate in muscle fatigue, even though there is good evidence that lactate can be used as a substrate for muscle metabolism (Mazzeo, Brooks et al. 1986; Jorfeldt 1970), and requires a minimal number of enzymatic reactions to enter the energy generating pathways.

Key studies by Jorfeldt (Jorfeldt 1970) showed that circulatory lactate oxidation was augmented by mild exercise, and further studies linked this to lactate oxidation in skeletal muscle (Issekutz, Shaw et al. 1976; McGrail, Bonen et al. 1978; Depocas, Minaire et al. 1969). More recently, Richter et al. (Richter, Kiens et al. 1988) reported that net lactate efflux from muscle was reversed to net uptake when the circulatory lactate concentrations exceeded 6mM. Adding to the complex nature of net lactate movements between different tissue compartments are reports that lactate efflux has been observed from non-exercising muscle during exercise, due to an apparent glycogen breakdown (Ahlborg 1985; Ahlborg, Hagendfeldt et al. 1975). Such glycogenolysis in non-exercising muscle has been observed during exercise in humans (Ahlborg 1985; Bonen, Ness et al. 1985) and animals (McDermott, Elder et al. 1987).

Despite the importance of lactate movement between tissues for whole body

lactate dynamics (Buchalter, Crain et al. 1989), minimal consideration has been given to the transport of lactate in skeletal muscle. Yet, the plasma membrane of skeletal muscle fulfills a crucial role in mediating the metabolic, contractile and electrophysiological functions of muscle cells (Clausen 1986). A primary function of this membrane is the transport of ions into and out of the cell to maintain ionic gradients necessary for cell function (Clausen 1986; Miller 1983). Since lactate is ubiquitous, the concentration of this metabolite in any body compartment is related to the tissue membrane permeability to either the acid and/or the ionized form. Therefore, a potential regulatory site for lactate disposal resides at the membrane level. The existence of specific channels or carriers has been demonstrated for the transport of many different ions in skeletal muscle cells (Clausen 1986; Miller 1983). At physiological pH, lactate is almost completely dissociated and in this ionized state would be expected to traverse the hydrophobic membrane matrix quite slowly. Functionally, a membrane- bound transport system for the lactate ion would be advantageous to the muscle cell. In fact, the transmembrane movement of lactate in skeletal muscle has been implicated as a rate limiting step for lactate dissipation from muscle, leading to intracellular acidosis and altered muscle function when glycolytic flux (lactate production) is high (Mainwood and Renaud 1985; De Hemptinne, Marrannes et al. 1983).

Clearly, the conventional view of lactate as a fatigue provoking metabolite is being re-assessed. In particular, the idea that lactate is a convenient substrate for skeletal muscle has only begun to be addressed. Therefore, in this thesis, the following aspects of lactate physiology will be considered: a) the role of non-exercising muscle in producing lactate as a substrate for other tissues; b) the

glyconeogenic capacity of skeletal muscle with specific reference to the muscle fiber type and pH regulation; c) the existence of a specific transporter for L-lactate in muscle and whether the transport of lactate could be rate limiting for metabolism; and e) the regulation of lactate transport by pH, and by acute and chronic contractile activity.

SECTION 2. LACTATE PRODUCTION BY INACTIVE MUSCLE

2.1 Introduction

The possibility of enhanced metabolism in non-exercising muscles has been addressed in several studies (Ahlborg, Felig et al. 1974; Bonen, Ness et al. 1985; McDermott, Elder et al. 1987). In humans a lactate efflux has been observed from non-exercising muscle during exercise, and its appearance was attributed to glycogen breakdown in the non-exercising muscle (Ahlborg 1985). It has also been observed that glycogenolysis occurs in non-exercising muscles during exercise in humans (Ahlborg 1985; Bonen, Ness et al. 1985), and animals (McDermott, Elder et al. 1987). Since glycogen losses were not observed in exercising and nonexercising muscles in adrenalectomized animals, epinephrine has been implicated as provoking glycogenolysis (McDermott, Elder et al. 1987). This implies that non-specific substrate mobilization occurs during exercise.

Whether epinephrine - induced glycogenolysis in non-exercising muscle (McDermott, Elder et al. 1987) results in complete glycogen metabolism to lactate or CO_2 is not known. Epinephrine perfusion of rat hindlimb muscles increases the accumulation of glycolytic intermediates (Chiasson, Shikama et al. 1981) and enhances resting metabolism (Swann 1984), whereas in humans a lactate efflux from presumptive non-exercising muscle has been observed

(Ahlborg 1985). It seems likely, however, that both effects may occur in non-exercising muscle, namely that glycolytic intermediates accumulate, and that glycogen is metabolized to lactate or CO_2, due to the mass action effect of increased glycolytic flux in non-exercising muscles (Newsholme and Leech 1983).

Speculation about the enhanced metabolism of glycogen in the non-exercising muscle necessitates careful quantitation of the muscle's contractile activity, a requirement that has not been adequately met in some studies (Ahlborg 1985; Ahlborg, Hagendfeldt et al. 1975). For example, In the aforementioned human studies the nonexercising muscles have been forearm muscles which were gripping the handlebars during leg exercise (Ahlborg 1985; Ahlborg, Hagendfeldt et al. 1975). Therefore, some contractile activity was presumably present in these forearm muscles, and may challenge the findings of these studies. In previous work from this laboratory, in which animals exercised on their forelimbs while the hindlimbs were suspended above the treadmill, hindlimb muscle activity appeared to be reduced (McDermott, Elder et al. 1987), although this evidence was descriptive, since EMG data were only presented on one animal. However, procedures have since been developed to quantitate muscle activities *in vivo*, via a computer-based, continuous, on-line monitoring system (Bonen, Blewett et al. 1990). This permits accurate quantification of muscle activities at rest, during exercise, and also in muscles that are not exercising during exercise.

In order to determine the fate of glycogen in non-exercising muscle the activities of exercising and non-exercising muscles were quantified using a recently developed on-line muscle monitoring system (Bonen, Blewett et al. 1990), these muscle activities were compared to the depletion of glycogen, and the accumulation of glycolytic intermediates and lactate in exercising and non-exercising, soleus and plantaris muscles during exercise.

2.2 Methods

Male Sprague Dawley rats, weighing 200-225g, were used in this study. All animals were kept in an air conditioned room, maintained on a 12h light:dark cycle and fed a diet of Purina chow and water ad libitum.

To prevent selected skeletal muscles from exercising during the treadmill exercise a noninvasive hindlimb suspension system was used to elevate the hindlimbs so that treadmill running could be performed on the forelimbs (McDermott, Elder et al. 1987). Briefly, the harness used for suspension was made of a canvas belt that was fastened around the animals' lower abdominal region. Two leg straps were then passed around the upper part of the hindlimb and attached to the main harness. An additional strap was then placed around the caudal end of the main harness to keep it stable. The rats were suspended from the posterior part of the harnes so that the hindlimbs were suspended approximately 2cm above the treadmill. Before the experiments all animals were familiarized with treadmill exercise while wearing the harness on four occasions

for 10min/occasion. This procedure allowed a comparison between muscle metabolism and muscle activity patterns in rat hindlimb muscles, when the same muscles were either exercising or were not exercising, while the animal was running on a treadmill. Similar endocrine responses are observed during both modes of exercise (McDermott, Elder et al. 1987).

Experimental Procedure. Exercise in the suspended exercise (SE) group consisted of running on the treadmill at 15m/min up an 8% grade for 90 min. using only the forelimbs while the hindlimbs were suspended. In the normal exercise (NE) group, exercise was performed on all four limbs at 15 m/min up an 8% grade for 90 min. In the running groups animals were killed at the point at which exercise would have commenced (t=0) or after the selected running period (t=90min.). In the control groups animals were killed at the point when exercise would have commenced (t=0) or after 90 min. of suspension above the treadmill at rest, or after 90 min. of normal rest on the treadmill. All experiments were performed between 8:30 and 10:00 am to control for the known diurnal effects on glycogen metabolism (McDermott, Elder et al. 1987).

Blood collection and tissue sampling. At the appropriate time rats were killed by an overdose injection (intraperitoneal) of pentobarbitol sodium (85mg/kg). On induction of surgical anesthesia before the animal died (~2min) the soleus and plantaris muscles of one hindlimb were rapidly dissected, frozen in liquid nitrogen, and stored at -80°C for the determination of glycogen, lactate and glycolytic intermediate concentrations. After the muscle dissection the thoracic cavity was opened and blood was collected in a syringe via cardiac

puncture (18 gauge).

Analytical Procedures. Muscle samples were lyophilized at -50°C until completely dehydrated (~ 5hrs). Extraction was carried out for the muscle metabolites as follows; a 5-10 mg piece of lyophilized muscle was extracted with 150ul of 3M $HClO_4$ for 10 min, neutralized with 250ul of 2M $KHCO_3$. The neutralized extract was then analysed enzymatically for glucose-1-phosphate (G-1-P), glucose -6-phosphate (G-6-P), fructose -6-phosphate (F-6-P), fructose -1,6-Phosphate (F-1,6-P), glyceraldehyde-3-phospate (Gly-3-P) and lactate (La)(Bergmeyer 1965). Muscle glycogen was assayed on a separate piece of muscle according to the method of Passoneau and Lauderdale (Passonneau and Lauderdale 1974). This method avoids the problem of losing 'acid labile' glycogen during the metabolite extraction (Jansson 1981).

Two mls of blood were added to iced tubes containing an anticoagulant. Plasma was separated and stored at -80° C for the determination of glucose and lactate. Plasma glucose was measured as previously described using a Beckmann glucose analyzer (McDermott, Elder et al. 1987). Lactate was measured enzymatically according to Bergmeyer (Bergmeyer 1965)(see appendix 1 for assay details).

For the determination of muscle fiber composition of the muscles under investigation four male Sprague Dawley rats were used. Muscles were dissected out, cut in the midbelly region, covered in embedding compound (Tissue Tek OCT) to prevent dehydration, mounted on cork chucks, and frozen rapidly in isopentane cooled in liquid N_2. Several sections (10μm) were cut in a cryostat

at -20°C and reactions for myosin ATP ase at pH 9.4 and 4.3 were performed to determine muscle fiber types according to Peter et al. (Peter, Barnard et al. 1972). Additional sections were stained for succinate dehydrogenase (SDH) by the method of Nachlas et al.(Nachlas, Tsou et al. 1957) to enable classification of the subtypes of fast-twitch fibers as fast twitch oxidative-glycolytic (FOG), and fast twitch glycolytic (FG).

EMG recordings. In a separate group of rats (n=5) muscle activity patterns during the four experimental treatments were determined as recently described in detail elsewhere (Bonen, Blewett et al. 1990)(see appendix 11 for copy of this manuscript). Briefly, bipolar fine wire electrodes (Cooner stainless steel wire, AS632) were used to record the EMG activity. The electrodes were implanted while the rats were under anesthesia (Somnotol 65mg/kg) under aseptic conditions approximately 3 weeks before the experiments. A lateral incision <4mm long was made to expose the mid-region of the soleus, and a medial incision exposed the lower one-third of the plantaris. Electrodes were placed across the bellies of the muscles at approximately right angles to the fibers (electrodes were 2mm apart with the recording surface 3.5mm long). The electrodes were connected via a shielded cable to a Grass wideband a.c. preamplifier (7P5) and driver amplifier (7DA).

For determinations of motor unit action potentials (MUAP's) the procedures of Nandedkar et al. were used (Nandedkar, Sanders et al. 1986a; Nandedkar, Sanders et al. 1986b). Briefly, the EMG signals were low and high pass filtered

between 10 and 40 KHz and digitized on line using an A-D board (AI13 Interactive Structures) mounted on an Apple IIe. The program sampled each channel for 500 ms in sequence, with a sampling rate of 2KHz. A turn was defined as any positive potential having an amplitude greater than a preset threshold set above the noise level of the signal (normally ~ 100uV). The fall in amplitude with a subsequent increase was interpreted as a new turn (Nandedkar, Sanders et al. 1986a).

The EMG was recorded at rest, for 30 min. when the muscles were weight bearing. The activity patterns in the soleus and plantaris muscles were studied throughout the following experimental procedures: a) running on the treadmill (15 m/min, 8% grade) on all four limbs (normal exercise- NE), and b) exercising with the forelimbs (15 m/min, 8% grade) while the hindlimbs were suspended above the treadmill (suspended exercise-SE). The aforementioned exercise bouts were performed over several days. Muscle activity patterns (turns and their amplitudes) were monitored continuously (20 sec/min /channel) for the entire 90 min experimental periods. A total muscle activity index during the exercise experiments was calculated as the sum of the MUAP frequencies for the 90 min. period (Σturns /90 min.).

Statistical analysis. An analysis of variance (ANOVA) was used to determine whether the treatment (SE,NE, NR, SR) exerted a significant effect on the parameters measured. When a significant F ratio was found, significantly different means were located by the Fisher PLSD post hoc test (Fisher 1949). All data are reported as means $\pm$SE. Significance was established at $p < 0.05$.

2.3 Results.

The hindlimb muscles studied differed with respect to fiber type composition (Table 1) and are similar to fiber distributions reported elsewhere (Armstrong and Phelps 1984.).

Table 1. Muscle fiber composition of selected skeletal muscles of Sprague-Dawley rats.

	Muscle Fibers%		
Muscle	SO	FG	FOG
Soleus	72.2±4.3	0.6±0.5	27.2±4.2
Plantaris	5.8±0.4	65.8±5.0	28.4±4.8
Red Gastrocnemius	20.9±3.6	10.9±1.1	68.2±1.0
White Gastrocnemius	–	86.2±1.0	13.3±1.0

Values are means ± SE; n=4. SO, slow-twitch oxidative; FG, fast-twitch glycolytic; FOG, fast-twitch oxidative-glycolytic.

In one group of animals (n=5) the muscles' motor unit activity patterns (MUAP) were monitored continuously over the 90 min experimental periods for four different experimental treatments (Fig. 1+2).

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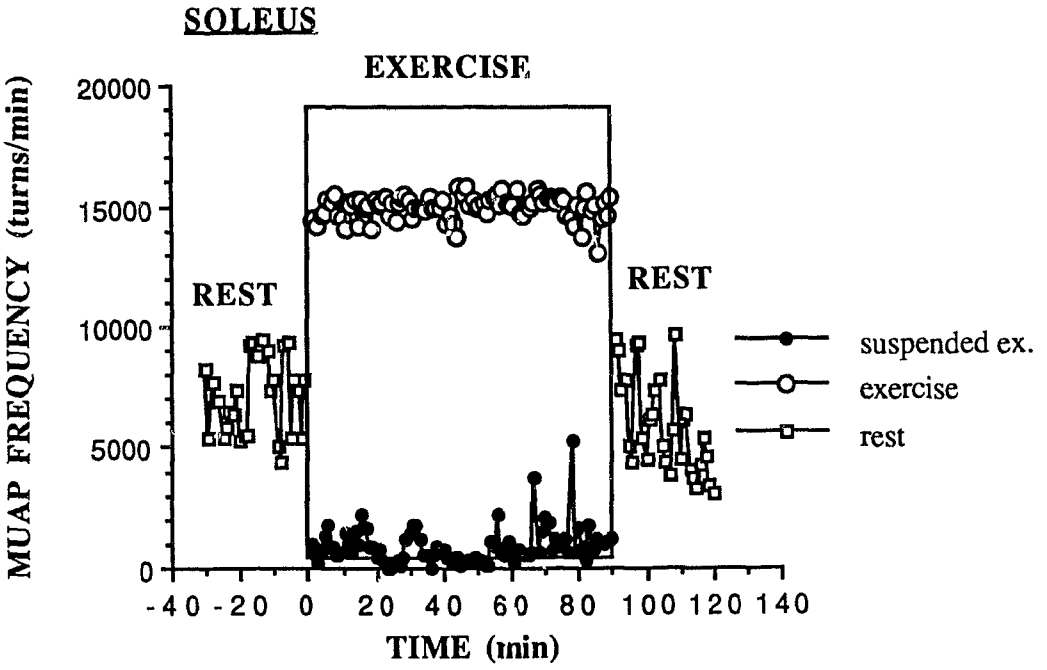


***Fig. 1** Frequency of motor unit action potentials (MUAP) in the soleus muscle during normal rest, normal exercise (15m/min, 8% grade), and suspended exercise (15m/min, 8% grade). Data are the means from 5 animals, the data plotted for each minute.*

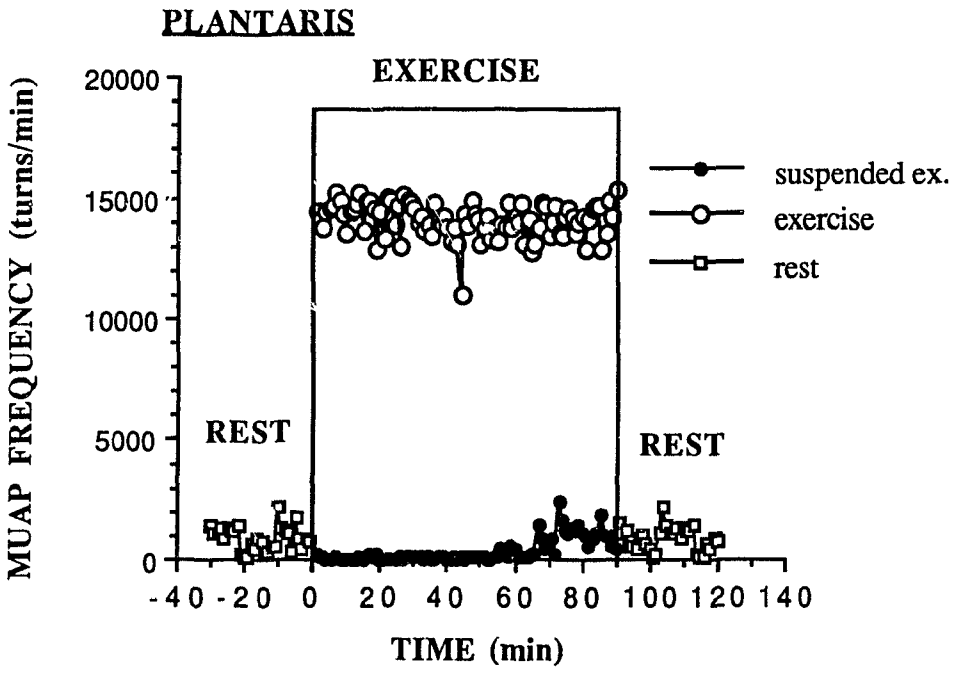


***Fig. 2** Frequency of motor unit action potentials (MUAP) in the plantaris muscle during normal rest, normal exercise (15m/min, 8% grade), and suspended exercise (15m/min, 8% grade). Data are means from 5 animals, data are plotted for each minute.*

MUAP pattern (soleus) These data demonstrate that the MUAP frequencies at rest while weight bearing were 600-700% greater than when weight bearing is relieved during suspended exercise (see Fig 1). MUAP frequencies were 15 fold greater in the exercising than in the nonexercising soleus muscles (Fig 1).

MUAP pattern (plantaris) The plantaris muscle showed a similar trend to the activity patterns observed in the soleus muscle (Fig 2). However, the MUAP frequency in the plantaris was considerably less than the soleus at rest, probably due to the greater postural role played by the soleus under normal weight bearing conditions (Fig 2). During normal exercise a marked increment in the plantaris MUAP frequencies occurred approximating the levels seen in the soleus muscle during normal exercise (Fig 2).

Blood glucose and lactate. Blood glucose was significantly elevated in the NE and SE groups after the 90 min exercise period compared to the respective control groups at both 0 and 90 min. ($p<.05$ see Table 2) . Blood lactate concentrations were not different in the exercise conditions compared to the respective control groups ($p<.05$ see Table 2)

Table 2. Blood metabolite concentrations.

Treatment	Lactate ($mmol \cdot l^{-1}$)	Glucose (mg/dl)
Control (0)	2.5 ± 0.05	164 ±5.7
Control (90)	2.3 ± 0.16	153 ±8.6
Exercise (90)	2.3 ± 0.40	178 ±4.6 *
Suspended Exercise (90)	2.4 ± 0.31	179 ±4.9 *
Suspended Control (90)	2.3 ± 0.20	162 ±17.2

Values are means ± SE. n=5 for each group.
Number in parenthesis is the time of sampling (min.).
See methods for explanation of treatment conditions.
** different from control t= 0 (p<.05)*

Glycogen. Muscle glycogen changes in the rats exposed to the different treatments are shown in Figs. 3 and 4. In the normal or suspended conditions at rest, no loss of glycogen occurred in any of the muscles (p>.05), between 0 and 90 min. During forelimb exercise in which the hindlimbs were suspended and during normal exercise on all four limbs, glycogen concentrations were significantly reduced in the hindlimb muscles after 90 min of exercise (p<.05 Fig 3 and 4). The extent of the depletion was generally larger in the exercising as opposed to the nonexercising muscle (see Fig 3 and 4). However, the main

observation is that a marked decrease ($p<.05$) occurred in the glycogen stores in exercised and non-exercised muscles, an effect which would not be expected based on the drastically different activity profiles (Fig 1and 2) observed for the two exercise modes.

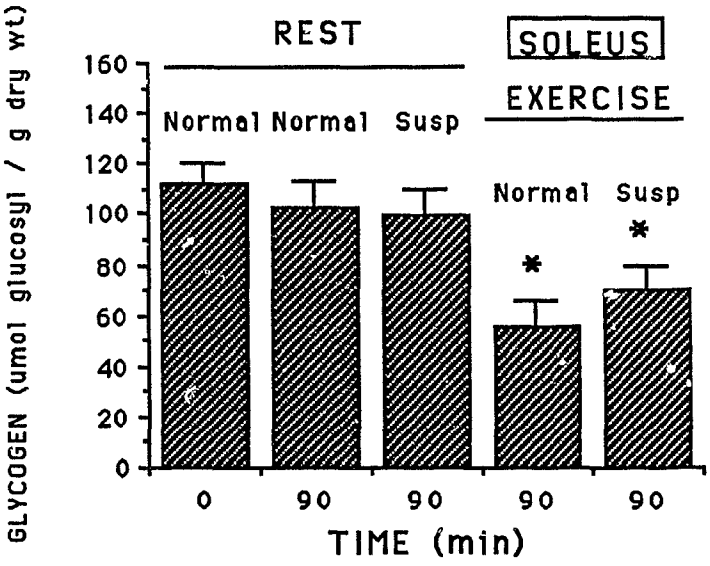


Fig. 3 Glycogen concentrations in the soleus muscle of rats before and after 90 min of exercise (15 m/min, 8% grade) performed on all four limbs (normal exercise) or with hindlimbs suspended above the treadmill (suspended exercise) while running was performed on the forelimbs. Values are means ± SE; n=8 muscles per point. Significantly different to control 0 ($p<.05$).*

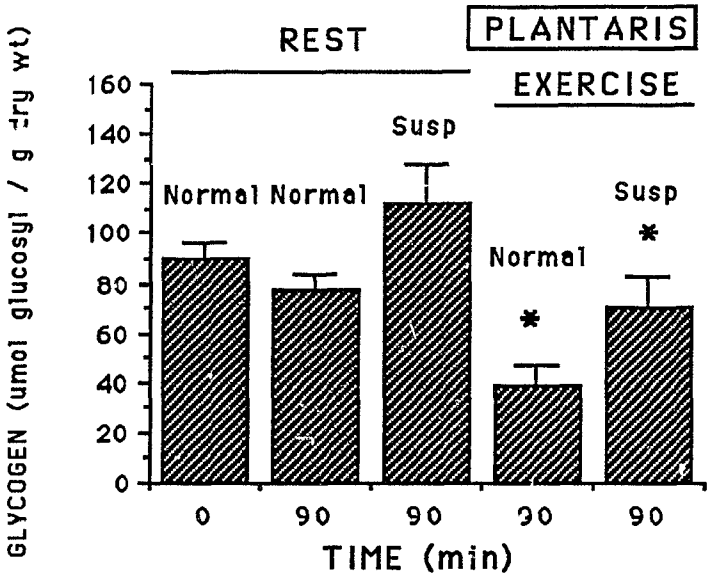


Fig. 4 Glycogen concentrations in the plantaris muscle of rats before and after 90 min of exercise (15 m/min, 8% grade) performed on all four limbs (normal exercise) or with hindlimbs suspended above the treadmill (suspended exercise) while running was performed on the forelimbs. Values are means ± SE; n=8 muscles per point. Significantly different to control 0 ($p<.05$).*

Glycolytic intermediates. The concentrations of glycolytic intermediates (G-1-P, G-6-P, F-6-P, F-1,6-P, G-3-P) and La in the experimental groups are shown in Tables 3 and 4 for the soleus and plantaris muscles, respectively. A significant increase in the intramuscular concentration of La was observed in the soleus in the NE mode ($p<.05$), and in the soleus in the SE group ($p<.05$). No consistent trend was observed in the data for the glycolytic intermediates in the sampled muscles. In some instances the data indicated that there might be an increase in the exercise conditions in the hexose monophosphates. However, the trends were mostly non-significant ($p>0.05$), or the changes were not consistent across the different muscles and were very small compared to the glycogen changes over the 90 min. period.

Table 3. Metabolite concentrations in the SOLEUS muscle.

	Metabolite concentration (umol/g dry wt)						
Treatment	Glycogen	G-1-P	G-6-P	F-6-P	F-1,6-P	G-3-P	La
Control (t=0)	112.3 ±7.6	0.102 ±0.005	0.069 ±0.005	0.008 ±0.002	0.068 ±0.037	0.077 ±0.008	7.0 ±1.5
Control (t=90)	102.2 ±10.5	0.071 ±0.018	0.183 ±0.034	0.024 ±0.004	0.134 ±0.024	0.097 ±0.030	12.2 ±1.2
Exercise (t=90)	55.3 [a,b] ±10.5	0.096 ±0.021	0.157 ±0.047	0.406 [a,b] ±.111	0.206[a] ±0.033	0.091 ±0.031	16.8[a] ±3.8
Suspended Exercise (t=90)	70.3[a,b] ±8.7	0.004 ±0.002	0.300 ±0.219	0.025 ±0.016	0.167 ±0.058	0.107 ±0.031	20.0[a,b] ±2.4
Suspended Control (t=90)	98.9 ±10.4	0.013 ±0.001	0.013 ±0.035	0.016 ±0.006	0.047 ±0.030	0.072 ±0.044	9.3 ±2.4

Values are means ±SE. G-1-P, glucose-1-phosphate; G-6-P, glucose-6-phosphate; F-6-P, fructose-6-phosphate; F-1,6-P, fructose-1,6- bisphosphate; G-3-P, glyceraldehyde-3-phosphate; La, lactate. [a] *- significantly different to control 0 group (p<.05),* [b] *- Exercise group is significantly different to the corresponding temporal control condition (p<.05).*

Table 4. Metabolite concentrations in the PLANTARIS muscle

	Metabolite concentration (umol/g dry wt)						
Treatment	Glycogen	G-1-P	G-6-P	F-6-P	F-1,6-P	G-3-P	La
Control (t=0)	90.3 ±6.0	0.136 ±0.044	0.304 ±0.087	0.032 ±0.016	0.142 ±0.028	0.065 ±0.021	26.5 ±2.9
Control (t=90)	77.1 ±6.6	0.102 ±0.033	0.209 ±0.043	0.031 ±0.008	0.091 ±0.011	0.042 ±0.015	26.8 ±4.3
Exercise (t=90)	38.4[a,b] ±8.5	0.177 ±0.044	0.425 0.041	0.151[a] ±0.037	0.105 ±0.020	0.11 ±0.040	39.1 ±5.2
Suspended Exercise (t=90)	70.1[a] ±12.2	0.150 ±0.033	0.366 ±0.140	0.052 ±0.008	0.063[a] ±0.020	0.033 ±0.009	46.1[a] ±4.1
Suspended Control (t=90)	111.4 ±16.4	0.087 ±0.021	0.450 ±0.027	0.055 ±0.006	0.042 ±0.019	0.077 ±0.039	49.1 ±4.6

Values are means ±SE. G-1-P, glucose-1-phosphate; G-6-P, glucose-6-phosphate; F-6-P, fructose-6-phosphate; F-1,6-P, fructose-1,6- bisphosphate; G-3-P, glyceraldehyde-3-phosphate; La, lactate. [a] *- significantly different to control 0 group (p<.05),* [b] *- Exercise group is significantly different to the corresponding temporal control condition (p<.05).*

Comparison of glycogen decrements with muscle activity. In the exercising soleus and plantaris muscles a large muscle activity index, compared to the resting conditions, is correlated with a substantial glycogen decrement, as would be expected for prolonged exercise of this intensity and duration (Gollnick, Karlsson et al. 1974). In contrast, in the nonexercising soleus and plantaris muscles a low muscle activity index is observed (Fig 5), yet a large decrement in glycogen occurs, which approaches the loss seen in the normally exercising muscle during 90 min. of exercise (Fig 5). In addition, in the non-exercising muscle the amount of glycogen loss that could be attributed to the accumulation of glycolytic intermediates and lactate was 14 and 27 % of the total glycogen decrement in the soleus and plantaris muscles, respectively (Fig 3).

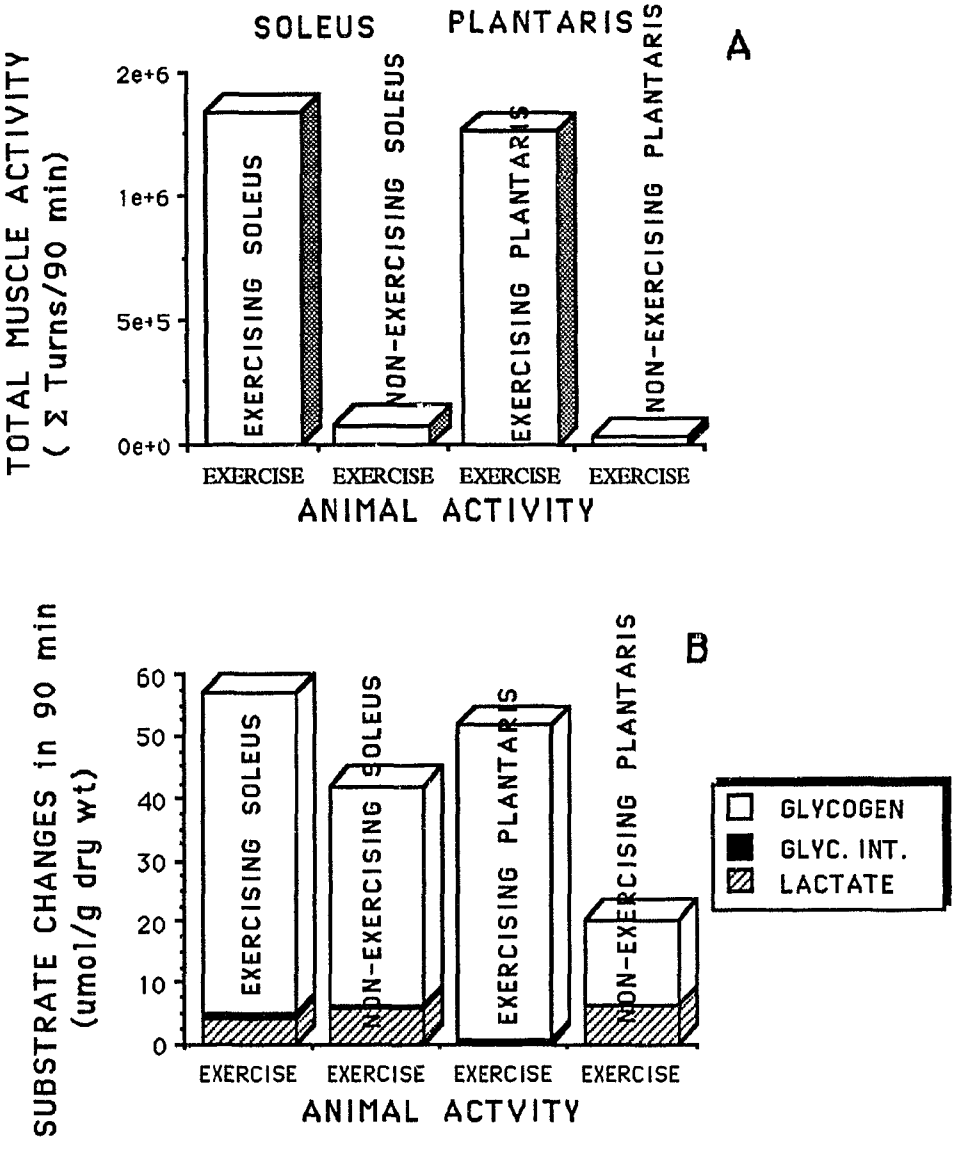


Fig 5 Comparison of total muscle activity (Σ Turns in 90 min) during the exercising and suspended exercise (A)with concomitant changes over the 90 min experimental period in muscle glycogen, lactate, and glycolytic intermediates (sum of G-1-P, G-6-P, F-6-P, F-1,6-P (B)in the soleus and plantaris muscles . The summed totals of the accumulated glycolytic intermediates (GI) converted to glucosyl units are shown within the Δ glycogen bars.

Calculations:

Δ Glycogen = [glycogen (t = o)] - [glycogen (t=90)]

Δ Lactate = [lactate (t=90)] - [lactate (t=0)]

Total GI = [ΣGI (t=90)] - [ΣGI (t=0)]

2.4 Discussion

A procedure was used which permitted a comparison of muscle metabolism with muscle activity patterns in rat hindlimb muscles. This comparison was made when the same hindlimb muscles were either; a) exercising normally on all four limbs or b) not exercising whilst the animal was running on a treadmill with it's hindlimbs suspended above the treadmill. Similar endocrine responses are observed during both modes of exercise (McDermott, Elder et al. 1987). Using this suspended exercise mode, to establish non-exercising muscle during exercise, the muscle contractile activity in the suspended hindlimb musculature was negligible compared with exercising muscle (Fig 1,2) in both the soleus and plantaris muscles throughout the 90 min. of SE. The similarity in the net glycogen depletion after 90 min in both exercise modes despite vastly different activity profiles (Fig 3,4) indicates a marked dissociation between contractile activity and glycogenolysis. The notion that glycogenolysis is always associated with contractile activity is prevalent in the literature (Armstrong, Saubert et al. 1974; Delp and Armstrong 1988; Delp and Armstrong 1988; Armstrong and Ianuzzo 1977; Gollnick 1985). However, these data provide persuasive evidence that in some situations the glycogen loss in a muscle is not necessarily related to the activity pattern of the muscle.

The present study reconfirms a previous study (McDermott, Elder et al. 1987) illustrating that glycogenolysis occurs in non-exercising muscle in rats during exercise (Fig 3,4). This is in accord with similar observations in

humans (Ahlborg 1985; Bonen, Ness et al. 1985). The intriguing question to consider, given that glycogenolysis does occur in nonexercising muscle, concerns the subsequent fate of this glycogen in the organism. Several hypotheses have been formulated with respect to its fate : a) it could be partially catabolized to expand the pool of glycolytic intermediates which recycle b k to glycogen on cessation of the glycogenolytic stimulus, b) it could be completely oxidized to CO_2 within the non-exercising muscle, or c) it could be metabolized to lactate which could then be effluxed from the muscle to join the oxidizable/ gluconeogenic carbon pool in the circulation.

The lack of changes in the glycolytic intermediate concentrations in the nonexercising muscle refutes hypothesis (a) since only a small expansion in G-6-P and a modest increment in intra-muscular lactate occurred in the nonexercising muscles (Tables 3 and 4) . The increments in the glycolytic intermediates including lactate within the muscle summed as glucosyl units only account for 15-27% of the glycogen lost in nonexercising muscle (Fig 5). Therefore the amount of glycogen catabolized in both exercising and non-exercising muscles was far more than could be attributed to the accumulated intramuscular glycolytic intermediates and lactate (Fig 5). Thus, the possibility of glycogen remaining within the muscle in an altered metabolic form seems unlikely.

From a bioenergetic viewpoint the second hypothesis (b) is unlikely on the basis that the glycogen decrement in non-exercising muscle, if completely oxidized to CO_2, would produce a large amount of extra ATP despite a

reduction in the demand for ATP in these unloaded muscles. It can be calculated that the aerobic utilization of this fuel would generate ~1369 umoles ATP/ g dry wt/ 90 min. (assuming 37 umol ATP/ umol glucosyl unit, correcting for the accumulation of glycolytic intermediates). However it is also possible that this additional ATP production is dissipated as heat. It was recently postulated that epinephrine mediates facultative carbohydrate - induced thermogenesis in skeletal muscle and that beta blockade via propranolol negated this effect (Astrup 1986).

The final hypothesis (c) that glycogen is partially catabolized to lactate effluxed from the muscle, and used elsewhere in the organism also has some experimental support. L-lactate can be efficiently transported via a carrier mediated transport system (see section 4) and oxidized in skeletal muscle (McDermott and Bonen 1989; Mazzeo, Brooks et al. 1986; Jorfeldt 1970). In addition, lactate can also serve as a gluconeogenic precursor in the liver, in fact recent reports suggest that it is the primary substrate for hepatic gluconeogenesis (Katz, Kuwajima et al. 1986). Although gluconeogenesis is recognised to be an important pathway for glucose production during exercise (Ahlborg, Felig et al. 1974), the contribution of non-exercising muscle to this process has not been previously considered. Therefore, either by direct muscle to muscle redistribution (via lactate oxidation) or indirectly via the liver (hepatic gluconeogenesis), the non-exercising muscle glycogen store could be shunted towards tissues with higher energetic demands. This hypothesis is supported by some circumstantial evidence in that exercising muscle glycogenolysis and

lactate production decrease as long term exercise proceeds (Karlsson and Saltin 1970). However, despite the reduction in lactate production from exercising muscle, arterial lactate values increase (Ahlborg, Felig et al. 1974). Ahlborg (Ahlborg 1985) postulated that the increment in arterial lactate in the face of a decrease in exercising muscle lactate production is due to glycogenolysis and subsequent lactate efflux from nonexercising muscle. It has been substantiated that lactate production can occur in muscle when oxygen is not rate limiting to mitochondrial function, as indicated by the mitochondrial redox state (Graham and Saltin 1989; Connett, Gayeski et al. 1984). In the present study blood lactate concentrations were not different after the exercise conditions when compared to controls (Table 2), however, this is not suprising as this only reflects the net concentration of circulating lactate which does not provide a sensitive indication of the rates of appearance and disappearance of lactate in the blood (Eldridge, T'So et al. 1974).

This mobilization of glycogen in nonexercising muscle may have important implications for whole body metabolism since such a large amount of glycogen is stored in the skeletal muscle mass (~ 70% of total body glycogen (Bonen, McDermott et al. 1989). Previously it was thought that the large mass of glycogen stored in nonexercising muscle could not be mobilized during exercise due to the lack of contractile stimulation (Gollnick, Karlsson et al. 1974). However, recent data in animals (present study, Bonen, Blewett et al. 1990; McDermott, Elder et al. 1987) and studies in humans (Ahlborg 1985; Bonen, Ness et al. 1985), indicate that there is a substantial mobilization of glycogen from nonexercising muscle during long term moderate intensity exercise.

Several studies have recently drawn attention to an apparent mismatch between sustrate mobilization and contractile activity during exercise (Kjaer, Farrell et al. 1986; Richter and Galbo 1986). Richter and Galbo (Richter and Galbo 1986) showed that the mobilization of glycogen stores during electrical stimulation of perfused rat hindquarter muscles varied depending on the initial pre-stimulation glycogen concentrations. Spriet at al. (Spriet, Ren et al. 1988) found that the infusion of epinephrine during electrical stimulation of human vastus lateralis caused an enhanced glycogenolysis without any alteration in force production. Similarly, Jansson et al.(Jansson, Hjemdahl et al. 1986) also showed that epinephrine infused into one leg during two legged cycling enhanced muscle glycogenolysis and increased lactate accumulation in the muscles of the infused leg. It is also possible that the non-specific aspect of substrate mobilization is neurally regulated in a feed-forward manner as proposed by Vissing et al. (Vissing, Wallace et al. 1989a) for hepatic glucose mobilization. This could be mediated via the ventromedial hypothalamus (VMH) as in the liver (Vissing, Wallace et al. 1989a), since anatomic mapping has identified connections between the peripheral sympathetic nervous system and the VMH (Vissing, Wallace et al. 1989b).

In conclusion, this investigation has reconfirmed that glycogenolysis occurs in nonexercising muscle during prolonged moderate intensity exercise. This glycogenolysis is clearly shown to be uncoupled from the contractile activity of the muscle. Of the glycogen decrement which occurs in non-exercising muscle only a small portion remains trapped within the muscle in the glycolytic pathway, an accumulation of lactate in these muscles indicates that the glycogen

may be metabolized via this substrate pool. At present one cannot discount two possible hypotheses concerning the fate of the rest of the glycogen lost from nonexercising muscle ; the glycogen could be either oxidized within the muscle, or the glycogen might be partially catabolized to lactate, effluxed from the muscle and used elsewhere in the organism as a gluconeogenic / oxidizable substrate.

Section 3. GLYCOGEN RESTORATION FROM LACTATE AND GLUCOSE IN SKELETAL MUSCLE

3.1 Introduction

Glucose as a substrate for glycogen synthesis (glycogenesis) has been known for some time (for review see Bonen, McDermott et al. 1989b). However, glycogen can also be synthesized from lactate (glyconeogenesis). This reconversion of lactate into glycogen was first postulated by Meyerhof ((Meyerhof 1920; Meyerhof, Lohman et al. 1925) and Hill (Hill 1924) for frog skeletal muscle, and this has been reconfirmed by Connet (Connett 1979). Glyconeogenesis has now also beeen observed in mammalian skeletal muscle (Bonen, McDermott et al. 1990c, McLane and Holloszy 1979). McLane and Holloszy (McLane and Holloszy 1979) showed that non-contracting, perfused rat hindlimb muscles synthesized glycogen from lactate, and Johnson and Bagby (Johnson and Bagby 1988) demonstrated that glyconeogenesis occurred in the post-exercise period in rats. More recently, Talmadge et al.(Talmadge, Scheide et al. 1989) postulated that lactate can be used as a glycogen precursor in active as well as resting muscle.

Estimations of the relative contribution of glucose and lactate to glycogen synthesis vary widely, ranging from 50 to 95% in human muscle (Hermansen and Vaage 1977) and from 10 to 66% in rat muscles (Bonen, McDermott et al. 1990c; McLane and Holloszy 1979). These approximations are gross estimates only and require a considerable number of arguable assumptions in humans

(Hermansen and Vaage 1977), and in animals (McLane and Holloszy 1979). Several studies have implied differences in the rates of glycogenesis and glyconeogenesis in muscle depending on the fiber type distribution of the muscle.

The perfused rat hindlimb provided the opportunity to examine the relationship between muscle fiber composition and glyconeogenesis in muscles with known heterogeneity in muscle fiber composition and metabolic capacities. Alternatively, since the incubated mouse muscle preparation provides greater experimental efficiency and allows a larger number of experimental perturbations to be undertaken, it seemed preferable to examine the possible physiological regulation of glyconeogenesis by selected hormones in this preparation. In both preparations it was possible to study glyconeogenesis in muscles with different fiber composition. Moreover, it was also possible to compare rates of glyconeogenesis and glycogenesis to obtain estimates of the relative contribution of glucose and lactate to the synthesis of muscle glycogen.

3.2 Methods

Studies on the rat were undertaken using a cell-free perfusate rather than with the commonly used (Hood, Gorski et al. 1986; Spriet, Matsos et al. 1985; Ruderman, Houghton et al. 1971; Ruderman, Goodman et al. 1977.) rejuvenated aged human erythrocytes or bovine erythrocytes, which are difficult to obtain locally. Therefore, preliminary studies with the perfused rat hindlimb preparation were undertaken to assess the metabolic viability of muscles perfused with a cell-free perfusate. Specifically, insulin stimulation of glycogenesis involves a number of complex, sequentially linked physiologic

and biochemical steps (including; hormone-receptor interaction, recruitment of glucose transporters, transport of glucose, phosphorylation of glucose to glucose-6-phosphate, and the UTP dependent synthesis of glycogen in the presence of glycogen synthase). Therefore, the rate of glycogen synthesis from glucose, with and without insulin, provided an ideal means to assess the metabolic integrity of muscles perfused by a cell-free medium. Once the concerns of using this type of perfusate were satisfied the glyconeogenic capacity of the muscles in the perfused rat hindlimb was determined.

In the preliminary experiments it was found that the fiber type dependence of glyconeogenesis observed in the perfused rat hindlimb (FG>FOG>SO) was similar to that observed in the incubated mouse muscle preparation (FT>ST). Because of the similarity in the data between these types of studies, and the logistic simplicity of the *in vitro* incubated mouse muscle (soleus and EDL muscles) compared to the rat hindlimb perfusion, further studies were undertaken using the incubated mouse muscle preparation.

Studies in rat skeletal muscles

Hindlimb perfusion procedures: Male Sprague Dawley rats weighing 330± 6g fed ad libitum were used in all experiments. The rat hindlimb preparation was essentially the same as described by Ruderman et al.(Ruderman, Houghton et al. 1971) with minor modifications as adapted by Gorski et al. (Gorski, Hood et al. 1986.) and Hood et al. (Hood, Gorski et al. 1986)(see appendix 10 for schema).

Rats were anaesthetised with sodium pentobarbitol (65mg/kg, ip). After induction of anesthesia a midline abdominal incision was made from the pubic

symphysis to the xiphoid process. The testes, bladder, seminal vesicles, prostate gland and intestines were ligated and excised. To minimize perfusate flow to other tissues, apart from the hindlimb, the following arteries were subsequently cauterized: inferior mesenteric, pudic epigastric, abdominal and superior vesicle arteries. After separation of the abdominal aorta and vena cava loose ligatures were placed around these vessels to ligate the artery and vein proximal to the catheters after cannulation, and to secure the catheters during the perfusion. A ligature was also placed around the iliac artery of one hindquarter which was tied shortly before perfusion was initiated to restrict perfusate flow to the other hindlimb.

The arterial catheter (18 gauge) was placed in the abdominal aorta approximately 2-3mm proximal to the aortic bifurcation. The venous catheter (14 gauge) was inserted in the vena cava parallel to the aortic catheter. Flow rate was quantitated by timed collection of the venous effluent which was then returned to the arterial reservoir. Rapid facilitation of homogeneous perfusate flow can help to prevent the onset of edema, this can be achieved by : a) the inclusion of a colloid in the perfusate such as albumin (4mg/100ml) or dextran (0.6 mM)(Bomzon and Naidu 1985), b) the use of heparin, either injected systemically before cannulation (2000 U) or injected via the arterial catheter shortly after cannulation (100 U), to prevent coagulation in the hindlimb microvasculature before the onset of perfusate flow (Gorski, Hood et al. 1986.), and c) rapid activation of perfusate flow (3-4 $ml.min^{-1}$) directly after arterial cannulation to minimize the period of ischemia and absence of flow through the hindlimb for even the brief period before the venous cannula is positioned (present observations). Rippe et al (Rippe, Haraldsson et al. 1985)

showed that even a brief increase in venous pressure can cause an increase in capillary hydrostatic pressure leading to a filtration of fluid from the perfusate into the tissue, thus indicating the importance of establishing homogeneous perfusate flow rapidly after cannulation.

The perfusate was maintained at 37°C and was pumped through an artificial 'lung', which consisted of a plexiglass reservoir maintained at 37°C containing 10ft of silastic tubing (Dow Corning 602-235) permeant to oxygen and carbon dioxide. From the 'lung' the oxygenated perfusate passed through the arterial pressure monitor prior to entering the arterial catheter of the preparation. The preparation was enclosed in a plexiglass cabinet maintained at 37°C. The perfusate consisted of (concentrations in mM): 115 NaCl, 5.9 KCl, 1.2 $MgCl_2$, 1.2 NaH_2PO4, 1.2 Na_2SO_4 , 2.5 $CaCl_2$ and 25 $NaHCO_3$, 4% bovine serum albumin, 10 mM glucose and maximal concentrations of porcine insulin (4.2 mU/ml) where applicable.The perfusate (pH 7.4) was gassed with 95% O_2, 5% CO_2.The oxygen tension of the perfusate was 1.09 umol/ml.

Metabolic viability of a cell-free perfusate.

To assess glucose incorporation into glycogen in muscle 10 uCi's of [5-^{3} H] -D- glucose were included in the perfusion volume of 300 ml. The first 25-30 ml of venous perfusate medium was discarded. An initial equilibration period of 15 mins. preceded each experiment, thereafter the flow rate was progressively increased every 15 mins. for a 60 min. period, to establish the relationship between flow rate (5-30ml/min) and oxygen uptake.

Arterial and venous (A-V) perfusate was sampled for oxygen in the last five minutes of each 15 min. period, and A-V glucose was sampled at the 18-

21ml/min flow rate. The Fick principle (A-V difference * flow rate) was used to determine oxygen and glucose uptake. On cessation of the experiment the soleus (SO), red (RG) and white gastrocnemius (WG) were rapidly excised, frozen in liquid nitrogen and stored at -80°C. Glycogen was extracted from the muscle samples as described elsewhere (Chan and Exton 1976.) and counted for [5-^{3}H] - D- glucose incorporation into glycogen (see appendix 7). Protein content of the muscle samples was determined by the method of Bradford (see appendix 8 (Bradford 1976).

Glyconeogenesis in perfused rat hindlimb muscles: In these studies, the perfused rat hindlimb was used as the experimental model. For determination of glyconeogenesis an identical perfusate composition was maintained as in the glycogenesis studies, except that 5mM glucose was used in the pre-perfusion period but not in the experimental perfusate, which contained 12 mM lactate and 10 uCi of [U-^{14}C] L- lactate. The perfusion duration was 20 min. and a single pass mode was used, therefore no re-circulation of the perfusate occurred. After the perfusion period the hindlimb muscles were rapidly dissected and frozen in liquid nitrogen. Glycogenesis and glyconeogenesis were determined from D-[U-^{14}C] glucose and L-[U-^{14}C] lactate, respectively, that were incorporated into glycogen. The incorporation of the radiolabelled substrate into glycogen was determined by precipitating the glycogen from the muscle and quantitating the amount of ^{14}C label in glycogen by liquid scintillation counting, as previously described (see appendix 7). Muscle protein determinations were performed with the method described by Bradford (see appendix 8 Bradford 1976).

Glyconeogenesis and glycogenesis in mouse muscles incubated in vitro: In all studies intact muscles from mice (28-30g body weight) were incubated *in vitro*. These incubation procedures have been used extensively in this laboratory (Bonen, McDermott et al. 1990; Bonen and Tan 1989; Bonen, Tan et al. 1984; Bonen, Tan et al. 1981) and elsewhere (Nesher, Karl et al. 1985; Newsholme, Leighton et al. 1986; Wallberg-Henriksson 1987; Young, Wallberg-Henricksson et al. 1985) for studies of glucose metabolism. Mice were anaesthetised with sodium pentobarbitol (100mg/kg ip) and the soleus and EDL muscles were removed from both hindlimbs, mounted on clips and placed in buffer. After a 30 min preincubation in Krebs-Henseleit bicarbonate buffer (KHBB) (pH 7.4)(see Appendix 9) containing fat-free bovine serum albumin and palmitate (0.5mM), muscles were incubated in fresh buffer containing [U-^{14}C] -L-lactate (1uCi/vial) or [U-^{14}C]-D-glucose (0.25uCi/vial) and appropriate quantities of unlabelled lactate or glucose as the sole substrate. Muscles were gassed with 95% O_2/5% CO_2 for 10 min. at the beginnning of the incubation period in all experiments. Glycogenesis and glyconeogenesis were determined from [U-^{14}C]-D-glucose and [U-^{14}C]-L-lactate, respectively, that were incorporated into glycogen. This was accomplished by precipitating the glycogen from the muscle and determining the ^{14}C content in glycogen, as previously described (appendix 7). Preliminary studies established that after the 30 min preincubation period both glyconeogenesis and glycogenesis increased linearly from 5-30 min. in soleus and EDL muscles at 37^{O}C . Therefore, all experiments were conducted with a 30 min preincubation followed by a 15 min incubation period at 37°C. Muscle protein determinations were performed with the method

described by Bradford (see Appendix 8, Bradford 1976).

In the hormonal studies muscles were incubated in pairs either without hormone or with various concentrations of corticosterone (10^{-12} to 10^{-6}M). To determine the effects of insulin on glyconeogenesis the studies were performed on muscle pairs, with one muscle receiving no insulin and the other 10nM of insulin during the 30min preincubation and the 15 min incubation period.

Statistical comparisons in all studies were based on analyses of variance or t-tests, depending on the experiment under consideration. All data are reported as means $\pm$ SE.

3.3 Results

Metabolic viability of a cell-free perfusate in the perfused rat hindlimb

The oxygen uptake characteristics of our preparation indicate that adequate oxygenation of the hindlimb occurred at flow rates > 20 ml.min-1 (Fig. 1), since a plateau in oxygen uptake occurred despite an increase in perfusate flow rates.

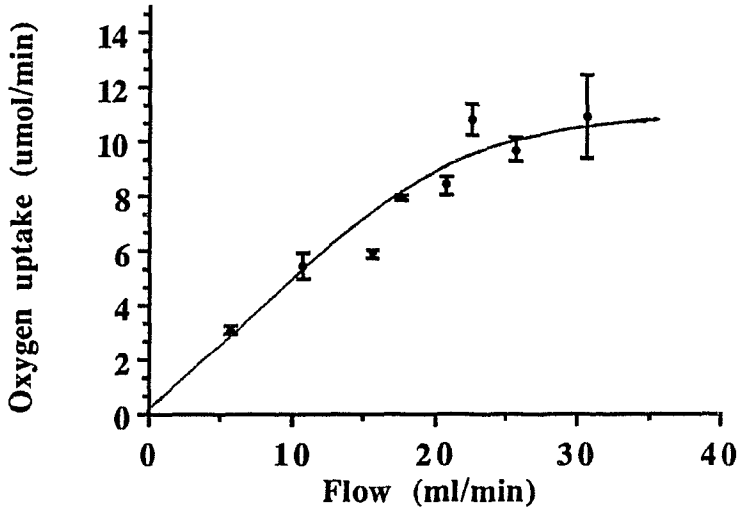


Fig. 1 Oxygen uptake as a function of flow rate in the perfused rat hindlimb preparation . Data are means±SE from 12 perfusion experiments.

At a flow rate of 20 ml·min^{-1} the oxygen consumption of this preparation (≈ 8 umol. min^{-1}. hindlimb^{-1}) is in agreement with other studies utilizing more complex perfusion media (Hood, Gorski et al. 1986; Rennie and Holloszy. 1977; Spriet, Matsos et al. 1985). However, in some studies (Rennie and Holloszy 1977; Ruderman, Goodman et al. 1977; Spriet, Matsos et al. 1985) resting oxygen consumption was attained at a lower flow rate than in the present study. The higher perfusion flow rates used in our study are not unreasonable when compared to blood flow in vivo. An approximation of the tissue flow rate in our experiment is 0.38 ml . min $^{-1}$. g $^{-1}$ [perfusate flow(ml.min^{-1})/ muscle mass perfused(g)] at a flow rate of 20 ml. min^{-1}.

From the comprehensive blood flow data in rats reported by Laughlin and Armstrong (Laughlin and Armstrong 1983.) it can be calculated that the average flow rate at rest in the rat hindlimb in vivo (based on the sum of the individual muscle flow rates and muscle weights of 23 hindlimb muscles) is 0.53 ml.min^{-1}.g^{-1}. This indicates that the flow rate at which oxygen uptake is independent of flow rate (> 20 ml. min $^{-1}$) used in this experiment is comparable to or higher than the flow rates used in other perfusion experiments, and approximates normal flow rates in vivo (see Table 1 for summary).

Table 1. A summary of different perfusion flow rates in several experiments compared to the present study

Study	Expt Conditions	Blood Flow (ml/min/g)
Laughlin and Armstrong (1983)	*In Vivo*	0.53
Spriet et al. (1985)	*P,RBC*	0.13
Rennie and Holloszy (1977)	*P,RBC*	0.16
Ruderman et al. (1977)	*P,RBC*	0.31
Gorski et al. (1986)	*P,RBC*	0.35
Shiota and Sugano (1986)	*P,CF*	0.39
Present Study	*P,CF*	0.38

(P= perfusion, CF =cell-free perfusate, RBC=red blood cell perfusion medium). Mass of perfused hindquarter muscle was approximated as 16.6% of mean body weights reported in each experiment (as reported by Ruderman et al. 1977)

Muscle blood flow ($ml.min^{-1}.g^{-1}$) was calculated as follows:

[perfusate flow ($ml.min^{-1}$) / muscle mass perfused (g)]

In vivo blood flow was calculated from the data of Laughlin and Armstrong (1983) as follows:

*Flow in each muscle ($ml.min^{-1}.mg\ wt^{-1}$) * each muscle wt(mg) = Total flow for each muscle ($ml.min^{-1}$)*

Σ of Total flow rates for each muscle ($ml.min^{-1}$) / total mass of hindlimb muscles (g) = average flow in the hindlimb based on the individual flow rates of 23 hindlimb muscles. ($ml.min^{-1}.g^{-1}$).

In the present study the rates of basal glycogenesis and insulin-stimulated glycogenesis were greater in the SOL and RG than in WG (Fig.2). This confirms other reports concerning the heterogeneous glycogenic capacity among different muscle fiber types both in vivo (James, Jenkins et al. 1985; James, Burleigh et al. 1986) and in the perfused rat hindquarter (Richter, Ploug et al. 1985).

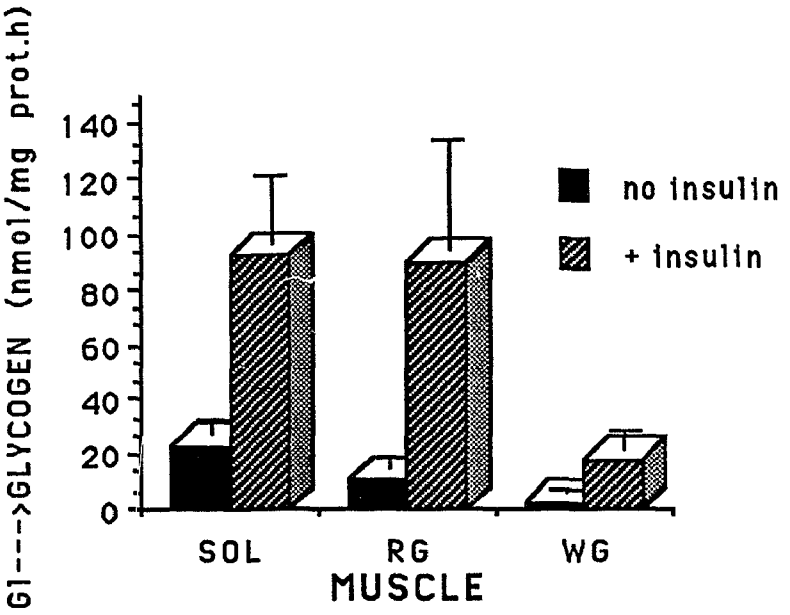


Fig.2. Glycogenesis in perfused hindlimb muscles (mean ±SE; n= 5 animals/ data point). Perfusions were performed with cell free media for 60 min (glycogenesis). RG, red gastrocnemius; WG, white gastrocnemius; SOL, soleus.

Glycogenesis; no insulin SOL>RG>WG ($p<0.05$, t test);

+ insulin (10nM), SOL>WG, RG>WG ($p<0.05$, t test).

These results suggest that an erythrocyte-free medium can be used in the rat hindlimb perfusion preparation to investigate resting muscle glucose metabolism. This medium can provide adequate tissue oxygenation at flow rates comparable to those in vivo. However, caution is necessary to avoid the development of tissue edema which may negate the normal response to insulin, and possibly other hormones (McDermott, Hutber et al. 1989).

Comparison of glyconeogenesis and glycogenesis in the perfused rat hindlimb

The rates of glycogenesis and glyconeogenesis in the perfused rat hindlimb are presented in Fig.2 and Fig.3 , respectively. The rates of glyconeogenesis were WG>RG>SOL (Fig3). Conversely, the rates of glycogenesis without insulin present in the perfusate were SOL>RG>WG, and the relative rates were SOL~RG>WG when insulin (4.2mU/ml) was present (Fig2).

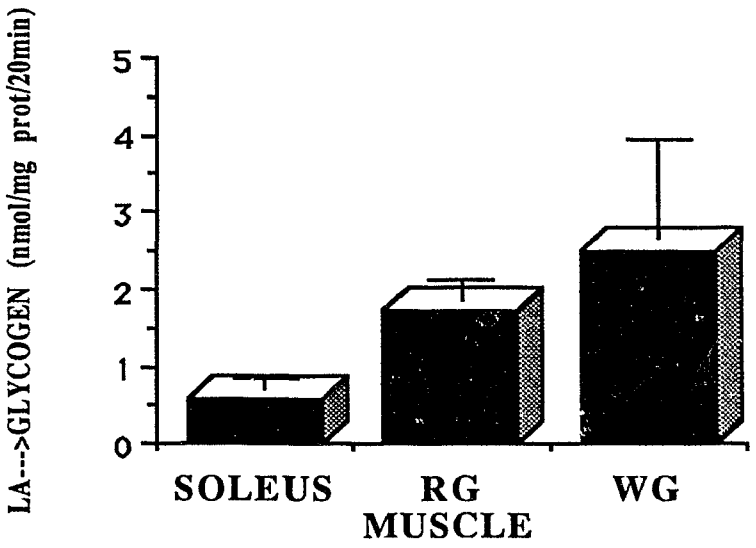


Fig.3. Glyconeogenesis in perfused hindlimb muscles (mean ±SE; n=3-5 animals/ data point). Perfusions were performed with cell free media for 20 min (glyconeogenesis). RG, red gastrocnemius; WG, white gastrocnemius; SOL, soleus.

Glyconeogenesis RG>SOL, WG>SOL ($p<0.05$, t test)

Direct comparison of glyconeogenesis and glycogenesis indicates that the amount of lactate used to synthesize glycogen is negligible compared to the amount of glycogen derived from glucose in the soleus and red gastrocnemius muscles (Table 2, Figs. 2 and 3), whereas in the white gastrocnemius the amount of lactate used for glycogen synthesis may be substantial, being as much as 25% of that derived from glucose in the presence of insulin (Table 2), when compared to the amount of glycogen derived from glucose.

Table 2. Comparison of the rates of glycogenesis and glyconeogenesis in the perfused rat hindlimb.

	(nmol/ glucose equiv/h)*					
Muscle	Glyconeogenesis				Glycogenesis	
					no INS	+INS
		%	Corr	%		10nM
SOL	0.75	<1	1.12	1	24.0	93.4
RG	2.55	3	3.95	4	11.4	89.1
WG	3.75	24	5.88	37	2.76	15.6

** Comparison is facilitated by the conversion of lactate moles into glucose equivalents (ie. 1 mole of glucose = 2 moles lactate). Corr - are corrected values for glyconeogenesis based on the dilution of label (see text). % refers to the % of glycogen formed from lactate compared to glycogen formed from glucose with 10 nM insulin (100%).*

The relationship between muscle fiber type composition and the rates of glycogenesis and glyconeogenesis are shown in Figs. 4 and 5, respectively. These data demonstrate that basal glycogenesis is related to the percentage of slow twitch fibers in a muscle (Fig 4). Conversely, glyconeogenesis appears to be related to the fast twitch fiber composition of a muscle (Fig.5).

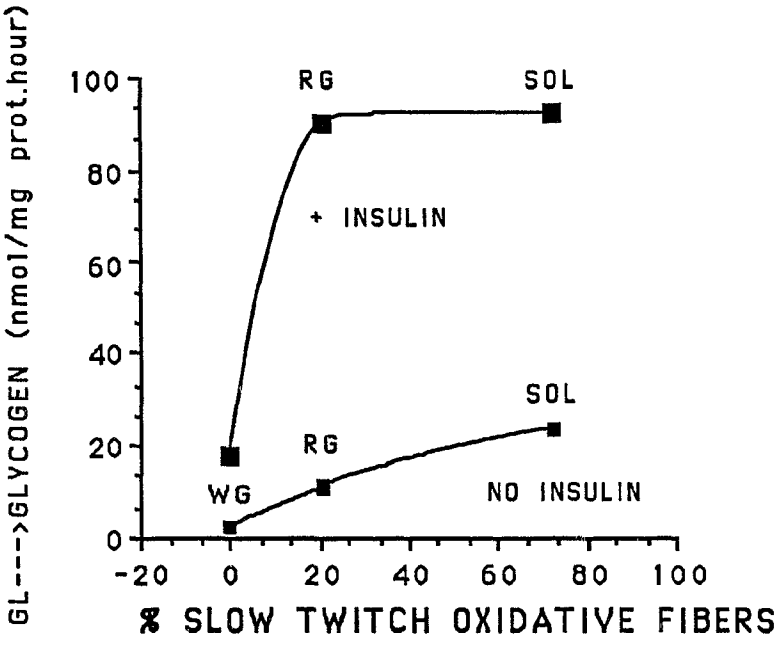


Fig.4. Rate of glycogenesis in relation to muscle fiber composition of the hindlimb muscles. Glycogenesis data are from Fig 2 and muscle fiber type data are from McDermott et al. 1987 . RG, red gastrocnemius; WG, white gastrocnemius; SOL, soleus.

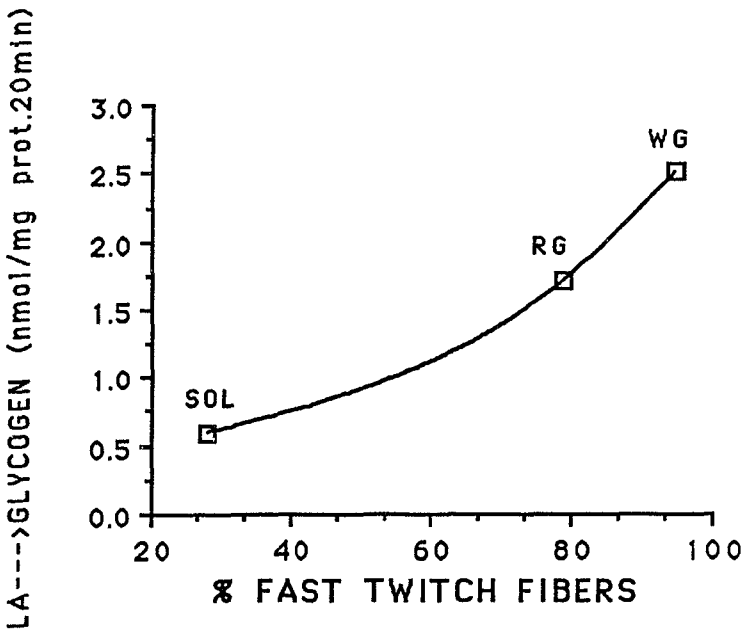


Fig.5. Rate of glyconeogenesis in relation to muscle fiber composition of the hindlimb muscles. Glyconeogenesis data are from Fig 3 and muscle fiber type data are from McDermott et al. 1987. RG, red gastrocnemius; WG, white gastrocnemius; SOL, soleus.

*Glycogenesis and glyconeogenesis in mouse muscles in vitro.*The rates of glycogenesis and glyconeogenesis were determined during 15 min incubation periods (the rates of glycogenesis and glyconeogenesis are linear up to 30 min.). Over the physiological range of lactate concentrations (5-20mM) glyconeogenesis increased linearly in the soleus and EDL muscles (Fig.6).

Similarly, glycogenesis also increased in a linear fashion when glucose concentrations were increased from 2.5-10 mM (Fig.7). Therefore, in subsequent experiments glyconeogenesis and glycogenesis were studied at concntrations of 10 mM lactate and 5 mM glucose, respectively.

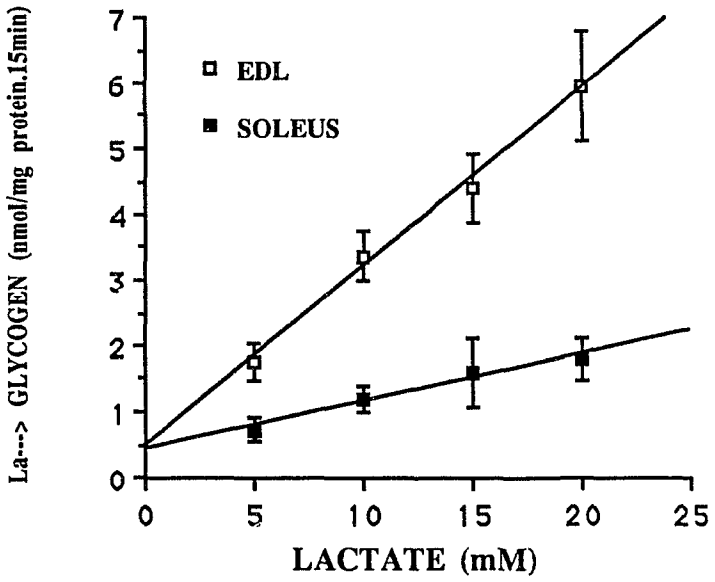


Fig.6 Effects of lactate concentration on glycogen formation in EDL and soleus muscles (means ±SE). (Incubation conditions pH 7.4, 15 min incubations; data based on 5-8 muscles at each point).

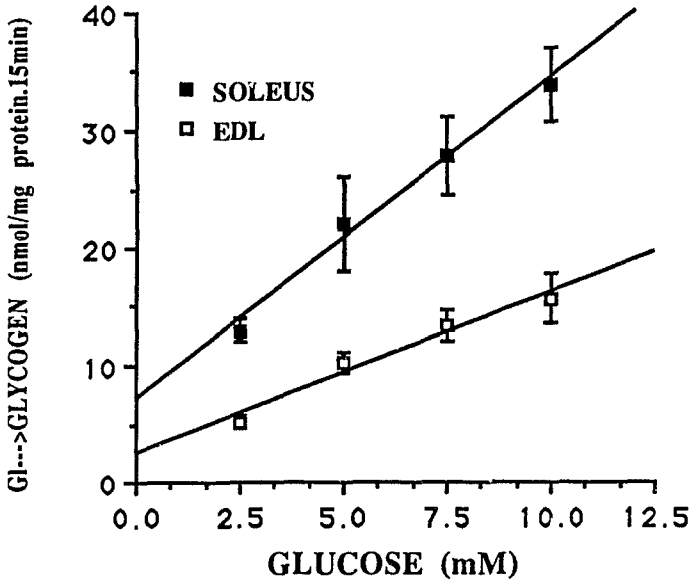


Fig. 7 Effects of glucose concentration on glycogen formation in EDL and soleus muscles (means ±SE). (Incubation conditions pH 7.4, 15 min incubations; data based on 5-8 muscles at each point).

The rate of glyconeogenesis was approximately 3-4 fold greater in the EDL than in the soleus and this rate was not altered by insulin (Fig 8). In contrast, the rate of glycogenesis in EDL was about half that observed in the soleus in the absence of insulin (Fig. 9). A marked increase in glycogenesis occurred in both muscles when incubated with insulin ($p<0.05$; Fig.9).

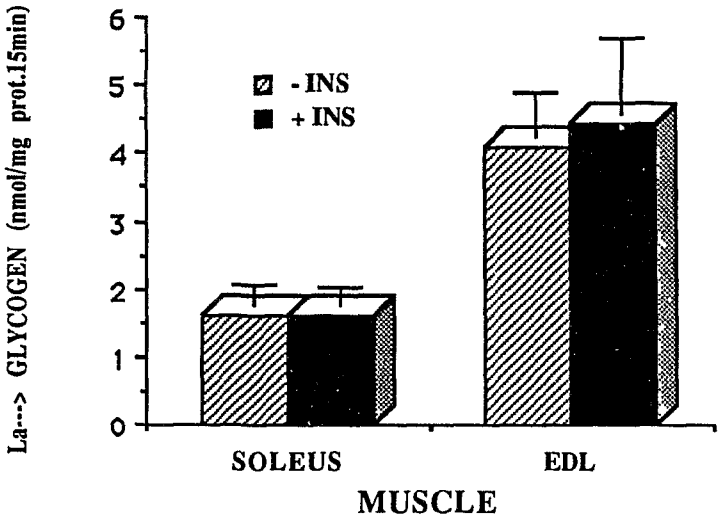


Fig. 8. Effects of insulin (10nM) on glyconeogenesis in EDL and soleus muscles (means ± SE) (incubation conditions pH 7.4, 15 min incubations, 10mM lactate ; data are based on 10 muscle pairs.

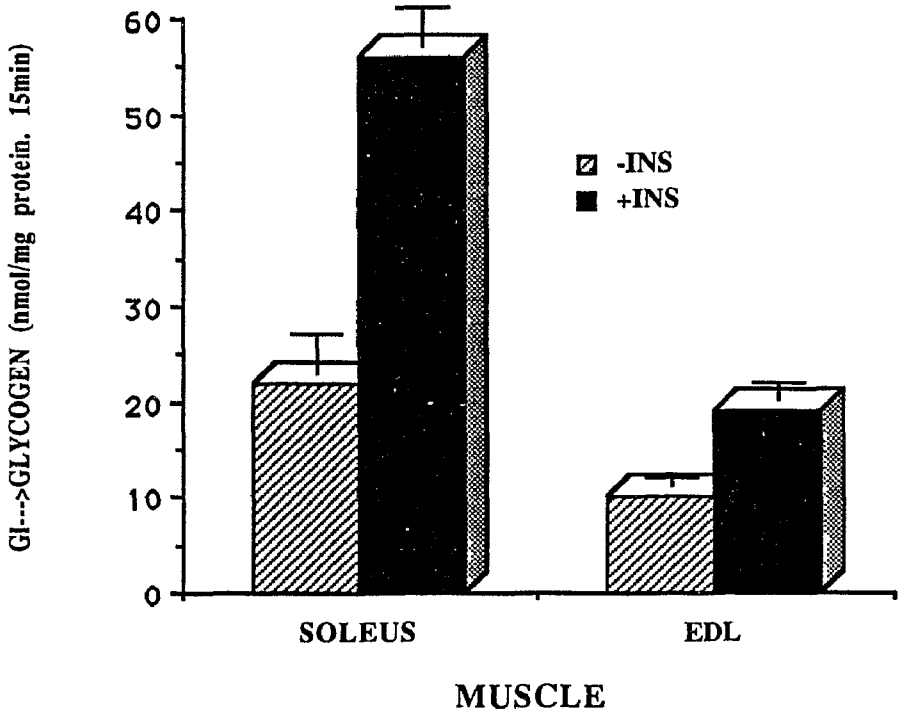


Fig.9. Effects of insulin (10nM) on glycogenesis in EDL and soleus muscles (means ± SE) (incubation conditions pH 7.4, 15 min incubations, 10mM lactate ; data are based on 8 muscle pairs .

Over a wide range of substrate concentrations, direct comparisons of glyconeogenesis and glycogenesis indicate that very little of the lactate is synthesized to glycogen in the soleus muscle (Fig. 10), whereas in the EDL a substantial proportion of glycogen is derived from lactate (Fig.11).

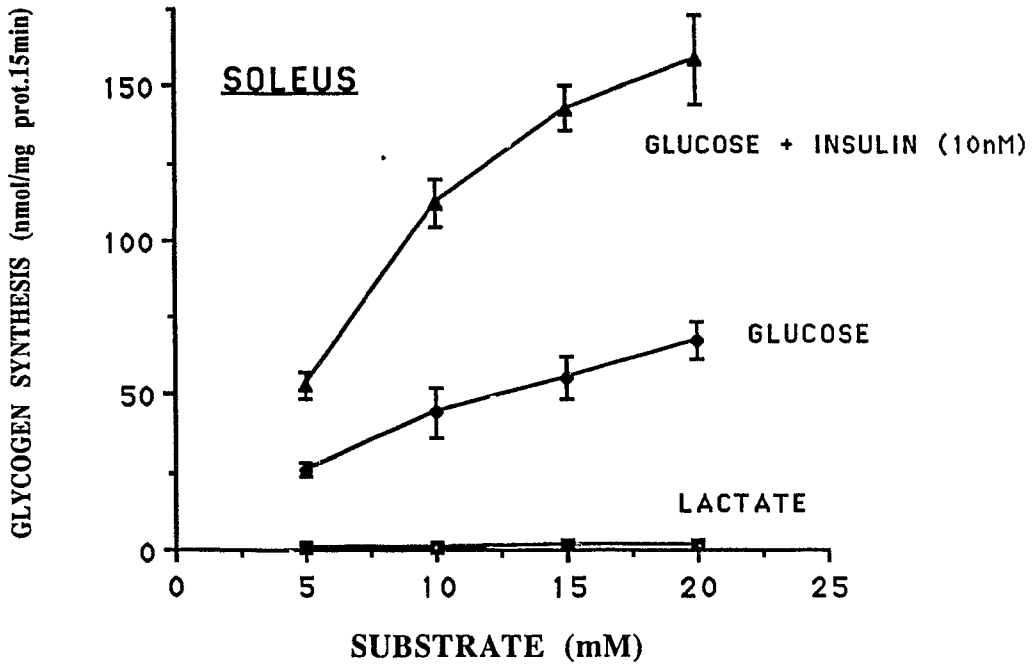


Fig.10. Comparison of glycogenesis and glyconeogenesis in soleus muscles (means ± SE). For direct comparison purposes the glucose data have been converted to lactate equivalents (1mM glucose = 2mM lactate)

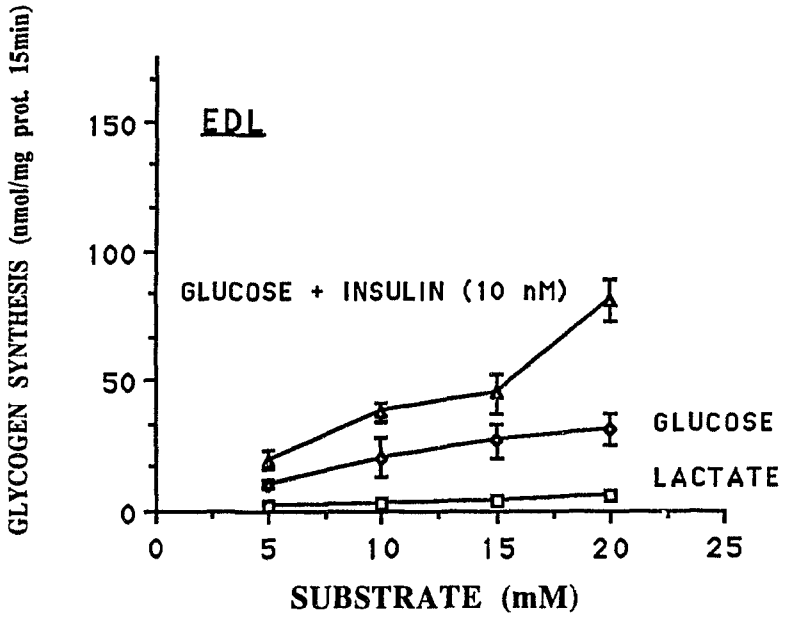


Fig.11 Comparison of glycogenesis and glyconeogenesis in EDL muscles (means ± SE). For direct comparison purposes the glucose data have been converted to lactate equivalents (1mM glucose = 2mM lactate)

In the soleus only 1-2% of the glycogen is formed from lactate compared to what can be formed from glucose in the presence of maximal levels of insulin (100%) (Fig.12), and in the EDL lactate formation of glycogen ranges from 9-16% compared to maximal rates of glycogen synthesis (100%) from 5 mM glucose in the presence of maximal concentrations of insulin (10nM) (Fig. 12).

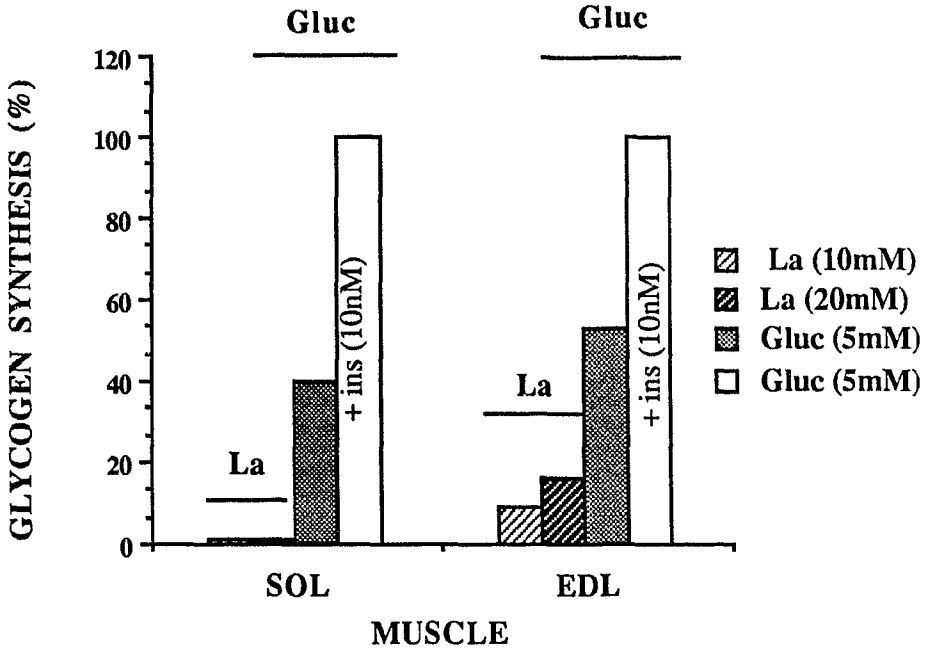


Fig. 12 Relative contributions (%) of lactate and glucose to the formation of glycogen in soleus and EDL muscles. Basal glycogenesis (5mM glucose, no insulin), and glyconeogenesis at two lactate concentrations (10 and 20 mM) are shown in relation to the maximal rate of glycogenesis (100%: 5mM glucose, 10nM insulin)

Incubations with varying concentrations of corticosterone did not alter glyconeogenesis in either muscle (Fig. 13 and 14).

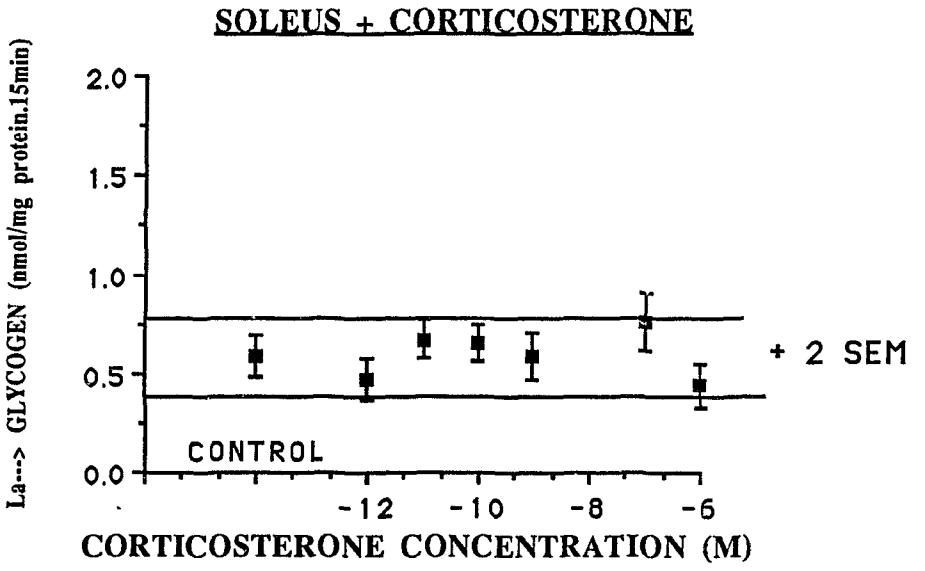


Fig. 13 Effects of corticosterone on glyconeogenesis in the soleus muscle (means ± SE) (incubation conditions pH 7.4, 15 min incubations; data are based on 28 control muscles and 4 muscles at the various hormone concentrations (logM).

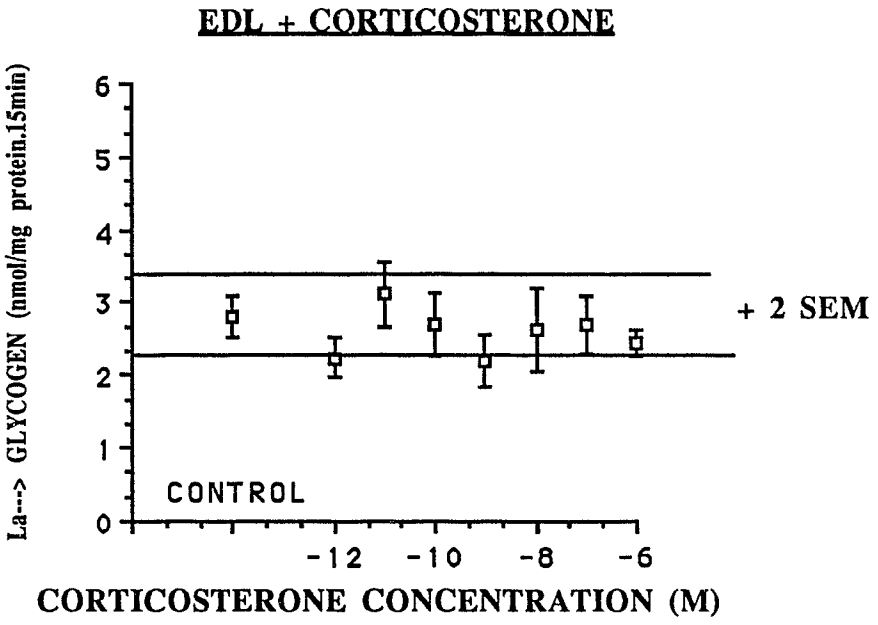


Fig. 14 Effects of corticosterone on glyconeogenesis in EDL muscle (means ± SE) (incubation conditions pH 7.4, 15 min incubations; data are based on 28 control muscles and 4 muscles at the various hormone concentrations (log M)).

A large change in the rate of glyconeogenesis occurred when the pH was altered. Optimum glyconeogenesis occurred at a pH of 6.5-7.0 (Fig. 15). At pH 6.5 glyconeogenesis was considerably greater than at pH 7.4 in both the soleus (+49%) and EDL (+39%) muscles.

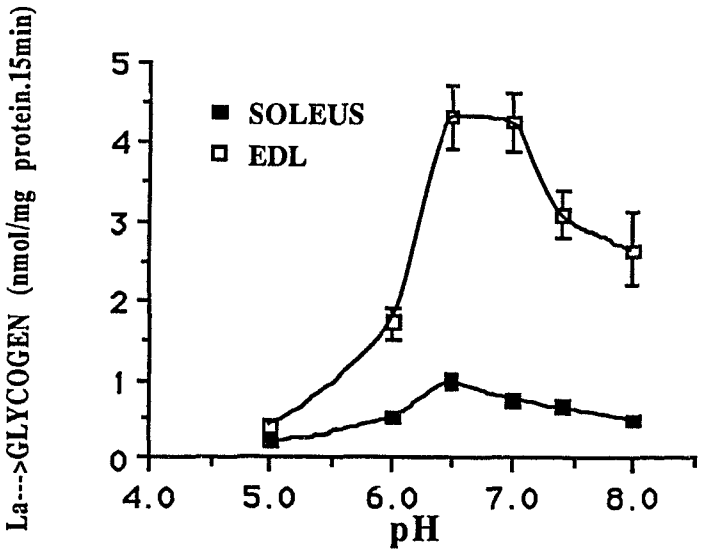


Fig.15. Effects of external pH on glyconeogenesis in the EDL and soleus muscles (means ±SE). (data are based on 10-16 muscles at each point).

3.4 Discussion

Glycogenesis and glyconeogenesis in perfused rat muscles: The studies were performed in the perfused rat hindlimb because the hindlimb muscles have markedly different muscle fiber compositions (see section 2, Table 1) and these differences in fiber composition are associated with dissimilar metabolic and functional capacities (Baldwin, Hooker et al. 1978; Baldwin, Klinkerfuss et al. 1972; McDermott and Bonen 1990; Barnard, Edgerton et al. 1971). The present study confirms previous reports of differences in the glycogenic and glyconeogenic rates in skeletal muscle composed of different fiber types (McLane an ˜ Holloszy 1979; James, Jenkins et al. 1985.). In contrast to the rates of glycogenesis in muscle, glyconeogenesis was much greater in muscles rich in FOG and FG fibers compared to a muscle rich in SO fibers (Figs.4 and 5). It is interesting to note that a significant relationship can be observed between the rate of glyconeogenesis and the muscles' percentage of fast twitch fibers (FG+FOG fibers) (Fig.5). Conversely, glycogenesis appears to be related to the proportion of slow twitch fibers in a muscle but this relationship is dissociated when insulin is present (Fig. 4). These data are consistent with 1) data on the relative rates of glycogenesis and glyconeogenesis in mouse skeletal muscles incubated in vitro (see incubated mouse muscle results in this section) and 2) previously reported differences concerning the glycogenic (James, Jenkins et al. 1985; Bonen, Tan et al. 1981)) and glyconeogenic (McLane and Holloszy 1979) capacities of muscle with different fiber type distributions.

Glycogenesis and glyconeogenesis in mouse muscles in vitro: These studies were performed with intact mouse soleus and EDL muscles because they have markedly different muscle fiber compositions (soleus: 70% SO, 28% FOG, 2% FG; EDL: 59% FOG, 41% FG), and also this preparation is logistically more efficient than the perfused rat hindlimb. In contrast to the rates of glycogenesis, the rates of glyconeogenesis were greater in the EDL than in the soleus muscle. This difference in glycogenesis and glyconeogenesis between these two types of the mouse skeletal muscle parallels a similar difference between rat fast twitch muscles (red and white gastrocnemius) and a rat slow twitch muscle (soleus) in the perfused hindlimb (see above).

No hormonal effects on glyconeogenesis were observed, whereas glycogenesis is increased by insulin. The lack of effect of corticosterone on muscle glyconeogenesis contrasts with this hormone's potent effect on hepatic gluconeogenesis(for review see Cherrington and Vranic 1986). The lack of effect of insulin on muscle glyconeogenesis has also been reported by McLane and Holloszy (McLane and Holloszy 1979). We conclude therefore that in contrast to glycogenesis, glyconeogenesis is likely not under endocrine regulation by the hormones that are known to affect glucose metabolism in muscle or gluconeogenesis in the liver (Cherrington and Vranic 1986).

An effect of pH on glyconeogenesis was found in the present studies (Fig.15). The observation that the optimal rates of glyconeogenesis occurred at pH 6.5-7.0 is intuitively attractive. First, pH decrements occur in skeletal muscles during

intense exercise, and secondly increased lactate availability after intense exercise would enhance the rate of glyconeogenesis. The mechanism for this pH sensitivity of glyconeogenesis is not clear. However, it is known that lactate transport is sensitive to pH gradients (see lactate transport, section 4). Thus, an increase in lactate transport may account for the increased glyconeogenesis in the present studies when the external pH is reduced.

The reason for these differences in glyconeogenesis in muscles composed of different fiber types, may be related to the greater activity of fructose 1,6 bis - phosphatase (McLane and Holloszy 1979). Whether the activity of this enzyme is critical to glyconeogenesis is not known, since the pathway for muscle glyconeogenesis (in contrast to glycogenesis) is not known. The currently favoured hypothesis is that glyconeogenesis in mammalian muscle occurs by the energetically 'unfavourable' reversal of the pyruvate kinase reaction to permit the formation of phospoenolpyruvate directly from pyruvate (McLane and Holloszy 1979; Shiota, Golden et al. 1984), rather than via the 'malic enzyme' route proposed by Connet (Connett 1979) for frog sartorius muscle. One of the factors that may determine the rate of glycogenesis in muscle is the membrane transport of glucose (for review see Bonen, McDermott et al. 1990b), whether the transport of lactate is a factor in determining the rate of glyconeogenesis has yet to be determined. However, it is interesting that the data from the present study indicate that an acidic external pH (6.5) stimulated glyconeogenesis and also under similar conditions (ie. an inwardly directed proton gradient) lactate transport is enhanced (see section 4). The physiological significance of this may be that glyconeogenesis is increased in the post-exercise period when systemic

lactate levels are high and pH gradients are directed into the muscle.

The present studies demonstrate that the rates of glycogenesis and glyconeogenesis are dependent on the substrate supply (Figs. 6 and 7). Johnson and Bagby (Johnson and Bagby 1988) have suggested that the glyconeogenesis observed by McClane and Holloszy (McLane and Holloszy 1979) and Shiota, Golden and Katz (Shiota, Golden et al. 1984) occurred under rather unphysiological circumstances, namely with muscles that were partly depleted of glycogen by contraction and then perfused by media containing no glucose or low concentrations of glucose and high concentrations of lactate. They (Johnson and Bagby 1988) and others (Stevenson, Mitchell et al. 1987) have suggested that high levels of lactate are necessary to observe glyconeogenesis. However, in the present studies the following has been observed; glyconeogenesis occurs in non-glycogen depleted muscles, and the presumed requirement for high lactate concentrations does not concur with the observations in the present studies, since under very 'unfavourable' conditions measurable rates of glyconeogenesis (7.5 ± 0.98 fmol/mg prot/5min, n=8) can occur, ie. in glycogen rich muscles that are not especially well predisposed towards glyconeogenesis (soleus) at low concentrations of lactate (0.15mM) in a relatively short time period (5min). It appears therefore that even small quantities of lactate can be synthesized to glycogen in skeletal muscles.

The data in this study indicate that the rate of glycogenesis far exceeds the rate of glyconeogenesis (Figs. 10 and 11). However, the use of a ^{14}C label to assess glyconeogenesis may lead to an underestimation of the process (Shiota, Golden et al. 1984). Data from this laboratory indicate that the rate of glyconeogenesis in the EDL muscle when determined radiochemically is ~65% of that observed

when this process is measured analytically using an enzymatic, fluorometric method to determine glycogen synthesis (in Table 2 the glyconeogenesis data are corrected for this underestimation). This may reflect some dilution of the radiolabelled substrate with endogenous pools of glycolytic intermediates. This has been reported by McLane and Holloszy (McLane and Holloszy 1979) and Shiota et al.(Shiota, Golden et al. 1984). Caution is therefore warranted when using the radiochemical approach, however, it is still considered to be the most suitable approach for estimating glyconeogenesis in muscle (Shiota, Golden et al. 1984; Connett 1979).

Despite the potential for glyconeogenesis, the perfused rat hindlimb studies have shown that at best only about 37% (corrected for the dilution of the label from our previous experiments) of muscle glycogen can be obtained from lactate (12mM) compared to glucose (10mM glucose), and then only in FT muscles in the rat (Table 2). The in vitro incubation studies largely concur with these data indicating that approximately 25% of glycogen can be derived from lactate in FT muscle from the mouse (EDL). In the soleus muscle negligible quantities of lactate are synthesized to glycogen (<1%) compared to that obtained from glucose in both preparations (Fig.12).

In summary, these studies have shown that 1) glucose is the primary substrate for skeletal muscle glycogen synthesis, however, lactate can also provide a substantial quantity in FG and FOG muscle fibers of rats and mice, 2) the rate of glyconeogenesis in a muscle appears to be positively correlated to the percentage of fast twitch (FG + FOG) fibers in a muscle, 3) glyconeogenesis is increased when the external pH is reduced (ie. pH 6.5), and 4) there appears to be no regulation of glyconeogenesis by hormones that stimulate glycogenesis in

muscle or gluconeogenesis in liver.

Section 4 LACTATE TRANSPORT IN SKELETAL MUSCLE.

In many mammalian cells monocarboxylic acid transport is mediated by a specific transport system (Balkovetz, Leibach et al. 1988; Fafournoux, Demigne et al. 1985; Spencer and Lehninger 1976; Trosper and Philipson 1987). Skeletal muscle, as one of the primary tissues responsible for lactate production and utilization (Roth and Brooks 1990; Buchalter, Crain et al. 1989), is a strong candidate for possessing such a transport system. There would be considerable teleological advantage associated with rapid lactate transfer across muscle membranes, such as the dissipation of an intracellular lactacidosis when glycolytic flux is high, or the rapid uptake of lactate for glycogen restoration or oxidation when systemic lactate is elevated. Therefore in the following section studies aimed at identifying the mechanism of transmembrane lactate movement in skeletal muscle are presented.

4.1 Lactate transport by skeletal muscle sarcolemmal vesicles.

4.1.1 Introduction

The plasma membrane of skeletal muscle fulfills a crucial role in mediating the metabolic, contractile and electrophysiological functions of muscle cells. A

primary function of this membrane is the transport of ions into and out of the cell to maintain ionic gradients necessary for cell function. The existence of specific channels or carriers has been demonstrated for the transport of many different ions in skeletal muscle cells (Clausen 1986; Miller 1983). Despite the central role of the lactate ion in skeletal muscle metabolism, the mechanisms involved in lactate transport are still incompletely characterized. At physiological pH, lactate is almost completely dissociated and in this ionized state would be expected to traverse the hydrophobic membrane matrix quite slowly. Clearly, a membrane- bound transport system for the lactate ion would be advantageous to the muscle cell.

The existence of a specific transport system for L-lactate has been implicated as being important for L-lactate transport in muscle cells (Koch, Webster et al. 1981; Watt, MacLennan et al. 1988; Juel and Wibrand 1989). To date, the studies investigating La transport in skeletal muscle have been carried out using the perfused rat hindquarter (Watt, MacLennan et al. 1988) or isolated intact muscle prepararations (Juel and Wibrand 1989; Mainwood and Worsley-Brown 1975; Mason 1987). However, detailed characterization of the transport mechanism has been hampered by the inability to control intracellular lactate concentrations and metabolism within the tissues, or to accurately measure true initial rates of unidirectional fluxes. Therefore, despite the usefulness of previous studies (Juel and Wibrand 1989; Mainwood and Worsley-Brown 1975; Mason 1987) in providing insight into the characteristics of lactate uptake in intact tissue a more detailed kinetic study is necessary to confirm the

existence of a membrane carrier for lactate transport into skeletal muscle.

To accurately characterize the kinetic properties of these transporters a membrane vesicle preparation has the advantage of allowing transport experiments in which both the intra- and extra- vesicular milieu is controlled, in the absence of cellular metabolism, thus allowing the accurate determination of unidirectional fluxes. This approach has proved succesful in the kinetic characterization of glucose transport into skeletal muscle (Grimditch, Barnard et al. 1985; King, Hirshman et al. 1989) and recently for L-lactate transport into cardiac muscle (Trosper and Philipson 1987). Therefore, the aim of the present study was to accurately characterize L-lactate transport by highly purified skeletal muscle sarcolemmal vesicles, using monocarboxylic acid inhibitors, a protein -SH group modifier, an inorganic anion exchange inhibitor, and proton gradients as probes to identify the nature of the transport .

Methods.

Male Sprague - Dawley rats, weighing 225-250g, were used in this study. Animals were maintained in an air-conditioned room on a 12h light: dark cycle and fed a diet of Purina chow and water ad libitum.

A sarcolemmal vesicle fraction was extracted from rat hindlimb skeletal muscles according to the method of Ramlal et al. (see appendix 5, Ramlal, Rastog et al. 1989)). Briefly, rat hindlimb muscles (~50g) were minced in a

buffer of 10mM $NaHCO_3$, 0.25M sucrose, 5mM sodium azide. The tissue was subsequently homogenized using a polytron at a setting of 6 for 5 seconds, centrifuged at 1200 g for 10 min., rehomogenized and recentrifuged. The resulting supernatant (crude extract) was centrifuged (9000g for 10 min.) to obtain a mitochondria-free and nuclei free supernatant which was then centrifuged at 190,000 g for 90 min. The resulting pellet from this centrifugation step was resuspended in buffer and placed at the bottom of a discontinuous sucrose density gradient (25,30 and 35% sucrose (wt/wt). These sucrose gradients were then subjected to isopycnic ultracentrifugation at 150,000 g for 16h. The distinct membrane bands at the sucrose interfaces were diluted in 50mM Tris buffer (pH 7.4) and then recentrifuged at 190,000 g for 90 min to obtain sucrose-free membrane pellets. The pellet was then resuspended in a 50mM Tris, 100mM NaCl buffer (pH 7.4) to a final concentration of 50 ug protein/ 10ul. The membrane suspension was then snap frozen in liquid nitrogen and stored at -80^0C until used for uptake studies.

Protein was determined by the Coomassie brilliant blue method described by Bradford (Bradford 1976) using bovine serum albumin as standard. The purity of the membrane preparation was assessed by measuring the activities of the membrane marker 5' nucleotidase. The specific 5' nucleotidase activities of the crude homogenate and plasma membrane fractions was assayed as described by Klip et al. (see appendix 6, Klip and Walker 1983).

To ascertain that the membranes were in vesicular form and to assess the contamination by other cellular organelles, the SL membrane fraction (corresponding to the sucrose gradient 25% fraction) was examined by electron microscopy. The SL fraction were pelleted at 190,000 g for 90 min.. The

membrane pellet was fixed by cross linking with 2.5% glutaraldehyde,1.0% paraformaldehyde in 0.1M Cacodylate buffer (pH 7.4) for 2h.The pellet was washed with buffer and dehydrated in a graded alcohol series and loosened from the side of the centrifuge tube .The pellet was then infiltrated with Taab (Marivac) resin in the centrifuge tube, transferred to aluminum dishes for final embedding and polymerization at 60°C for 48 h. Blocks were cut out and sections cut with a Drammer knife on a Rackert OMU3 ultratome. Sections were stained with uranyl acetate and lead citrate and viewed in a Zeiss 10B electron microscope.

Lactate transport studies: The uptake medium consisted of 50mM Tris, 100mM NaCl (pH 7.4) except in the experiments in which external pH (pHe) was manipulated, in which case 50mM MOPS was substituted for Tris at the lower pH values. La transport measurements were made at room temperature (21°C ± 1°C) using a rapid filtration technique (Trosper and Philipson 1987). L-lactate flux (J_{lact}) was measured as the uptake of L-[U- ^{14}C] lactate. The transport experiments were performed as described by Trosper and Philipson (Trosper and Philipson 1987) for cardiac sarcolemmal vesicles. Briefly, an aliquot of sarcolemmal vesicles containing 45 ug of membrane protein was placed on the side of an Eppendorf tube containing isosmotic tracer uptake medium, containing 0.5 uCi of L-[U-^{14}C] lactate, in a total reaction volume of 50ul. The vesicle preparation and uptake media were equilibrated at room temperature for 1 h at 20^{0}C before the uptake experiments. In all the experiments the final concentrations of osmotically active solutes were (in mM)

Tris or Mops 50; NaCl 100; L-lactate 0.15 or 1mM. When the uptake measurements were made at the various lactate concentrations (0.15-80mM) a reciprocal decrease in NaCl was used to maintain the same total isosmotic buffer strength. The reaction tube was vortexed to initiate transport into the vesicles. After the appropriate time period 1ml of ice cold buffered isosmotic KCl stop solution was pipetted into the tube. One ml of the reaction medium containing the vesicles was then rapidly filtered using a 0.45 μM cellulose membrane filter and washed with 2 volumes (3ml each) of buffered stop solution under a vacuum of 25 in. Hg using a Millipore vacuum filtration system. After filtration the filters were placed in scintillation vials and dissolved in 600ul of ethylene glycol monomethyl ether. Aquasol (New England Nuclear) was added and the vials were counted for radioactivity (^{14}C) in a liquid scintillation counter.

The data points were corrected for the amount of L- [U^{14}C] Lactate bound to the membranes at time 0. This non-specific binding was determined by adding 45 μg of membrane protein to a reaction tube to which the stop solution had already been added, with subsequent treatment of the sample the same as for the other reactions. Initial studies were carried out to determine optimal conditions to study the kinetic entry of lactate into the vesicles. A time course for L-lactate transport was used to establish the extent to which uptake was linear in relation to time for initial rate experiments.

D-lactate, pyruvate and alpha - cyano -4 hydroxycinnamate were added to the uptake media in a concentration which would give a final concentration of

10mM, 10mM and 5mM, respectively, after adding the vesicle suspension. Where appropriate, the membrane vesicles were preincubated with the protein modifier N- ethylmaleimide (20mM) or the inorganic anion exchange inhibitor SITS (1mM) for 1h at room temperature (21°C) before the transport experiments. N-methylglucamine (titrated with HCl) was used to replace Na^+ in the Na^+ substitution experiments.

In the studies in which the external pH (pHe) was altered, the uptake medium pH was altered so that when the vesicle suspension (pH 7.4) was added the final pHe (after vortexing) was titrated to the desired pHe (pH 6-8).

Results

Table 1 indicates the protein yield and purity of sarcolemmal (SL) membranes isolated from rat hindquarter muscles. The marker enzyme data shows a 25 fold purification in the membrane marker 5' nucleotidase in the 25 % plasma membrane fraction compared to the crude muscle homogenate.

Table 1. Characterization of membrane fractions isolated from rat hindlimb skeletal muscle.

Fraction	Protein yield	5'-Nucleotidase	PI
	(mg/g tissue)	(nmol/min/mg protein)	
Crude homogenate	26.0 + 0.061	14.75 + 3.7	----
Plasma membranes (25% fraction)	0.03 + 0.005	364.2 + 95.3	25

Results are the mean + SE of four independent membrane preparations. PI- purification index.

Electron microscopy of the SL fraction (Fig 1) revealed that the membranes contained in this fraction were primarily vesicular, and were free of mitochondria and rough endoplasmic reticulum.

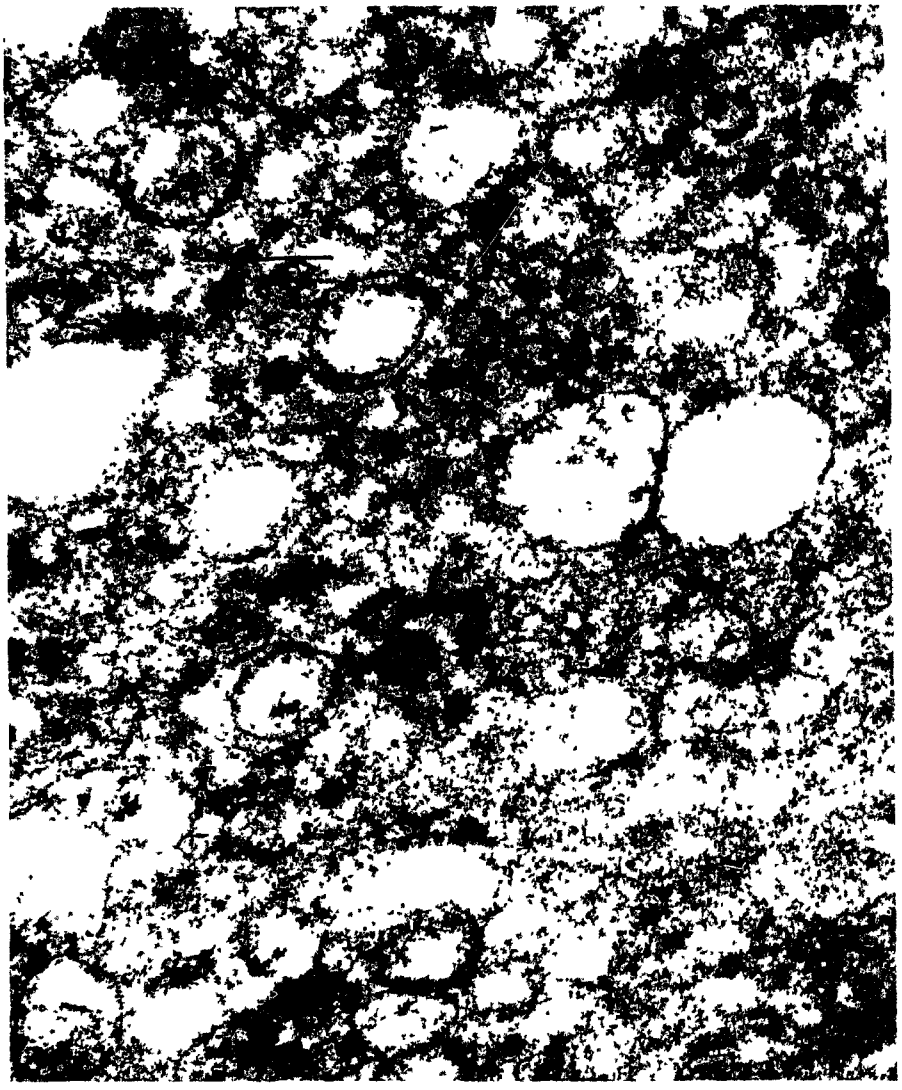


Fig. 1 Electron micrograph of muscle sarcolemmal membrane vesicles. The 25% fraction comprising the purified membrane preparation was fixed and stained as descibed under Methods, and viewed by transmission electron microscopy (x46,090). The scale bar = 0.4 μm.

Figure 2 demonstrates the uptake of L-lactate (L-La) as a function of time (3-45 s) by skeletal muscle SL vesicles. These data demonstrate that the uptake is linear up to ~ 20s. All the time course measurements were performed at an external La concentration of 1mM. At the longest time intervals < 0.65% of the total L-La present had been sequestered by the vesicles and therefore the progressive decrease in uptake at the extended time points is not due to the depletion of L-La in the extra-vesicular medium. Therefore the time interval used for all subsequent kinetic studies to measure initial rate velocities of lactate transport was 5s. The data collected at this time point are expressed as nmol/mg protein/5s.

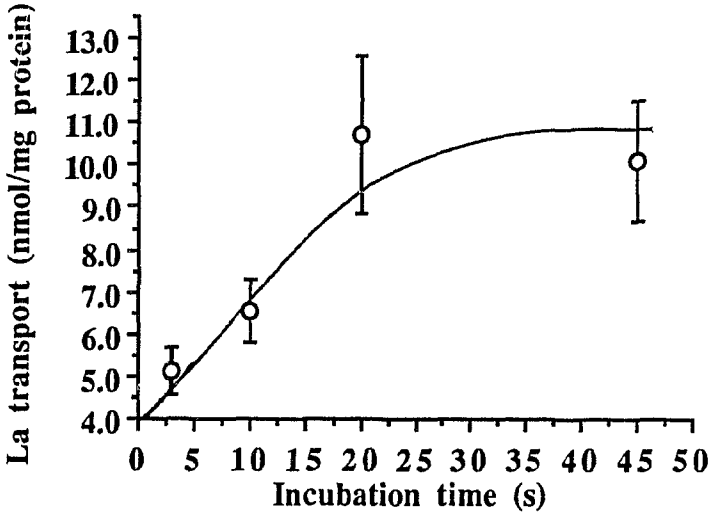


Fig. 2. L-lactate transport into sarcolemmal vesicles as a function of time at room temperature (21^{0}C). 1mM L-lactate in the extra-vesicular medium. Data points are means ± SE of triplicate determinations for 4 experiment

Kinetics of the lactate transporter: The dependence of L-la transport velocity on the extra-vesicular lactate concentration is illustrated in Fig 3. The hyperbolic nature of this curve reveals that La transport is a saturable function of the increasing La concentration. Linear transformation (line fitted by linear regression analysis) of these data into a Lineweaver- Burke plot allows an estimation of the Michaelis Menten parameters, yielding a Km of ~ 4.01 mM (see Fig 3).

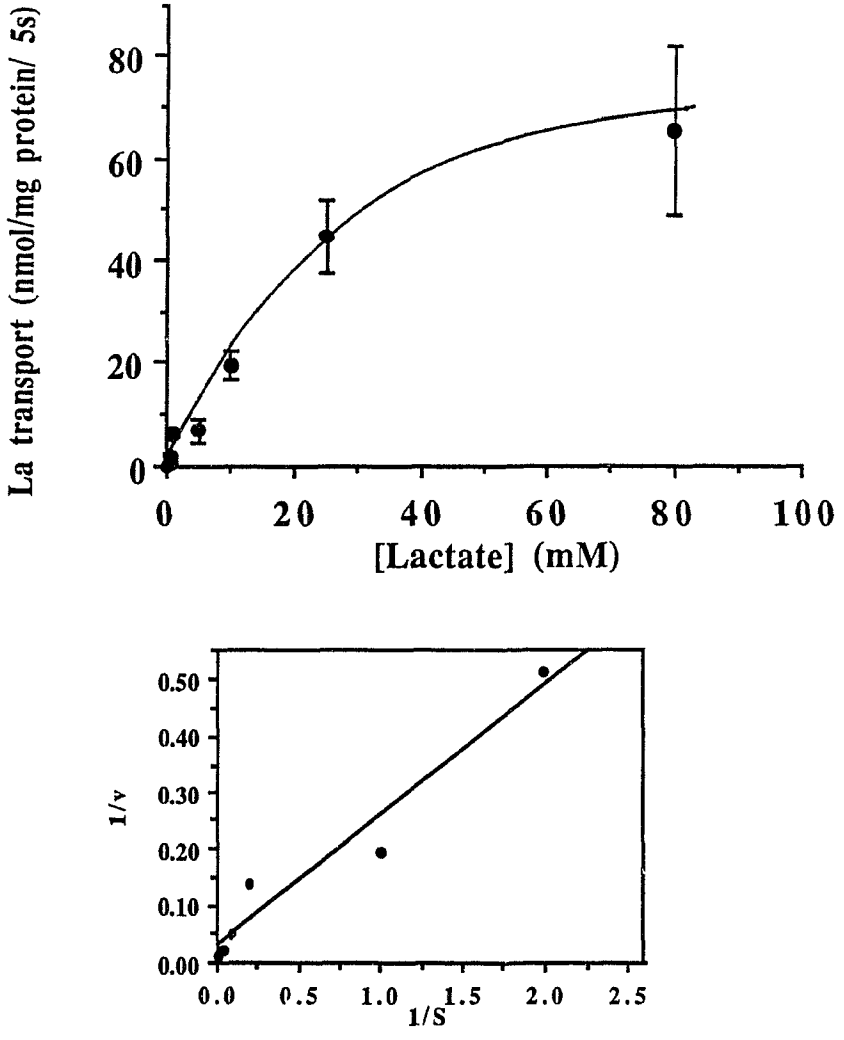


Fig. 3. Dependence of L-lactate transport on external L-lactate concentration (upper graph)Values are means ±SE of 4 or 5 experiments. Lower graph Lineweaver Burke transformation of the data. The slope, was fitted by linear regression and gives an apparent Km for L-lactate of ~4.01mM.

Effects of extra-vesicular pH on J $_{lact}$: When the internal pH (pHi) of the vesicles was 7.4, a change in the external pH (pHe) had marked effects on J_{lact} (Fig 4). Essentially the rate of J lact was accelerated by decreasing external pH (Fig 4). Also when pHe was >7.4 J_{lact} was reduced, indicating that lactate entry follows an inwardly directed proton gradient. If the data are expressed as a function of the external hydrogen ion concentration (Fig. 5), the plot indicates that the lactate transport system is most sensitive to a change in the hydrogen ion concentration in the pH range 8.0-6.5.

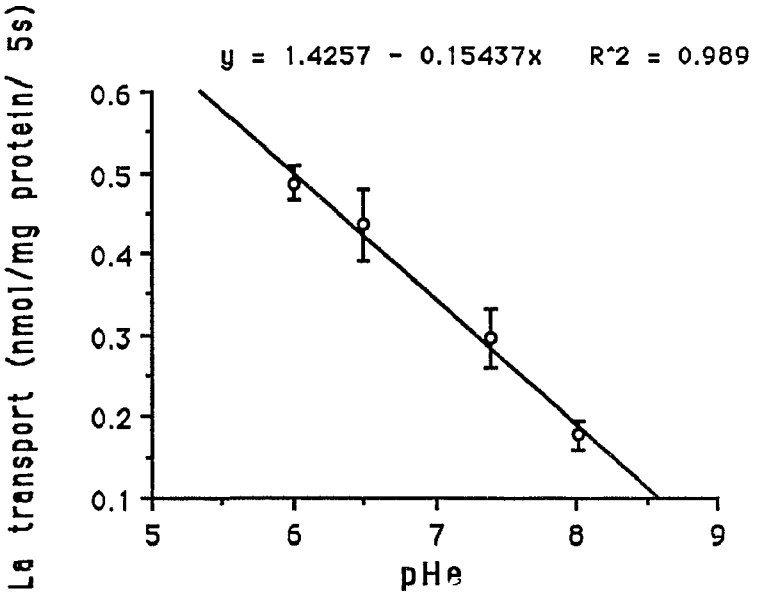


Fig. 4. Initial rate of L-lactate transport as a function of external pH. Internal pH is 7.4. Values are means ± SE of triplicate determinations for 4 experiments with different vesicle preparations.

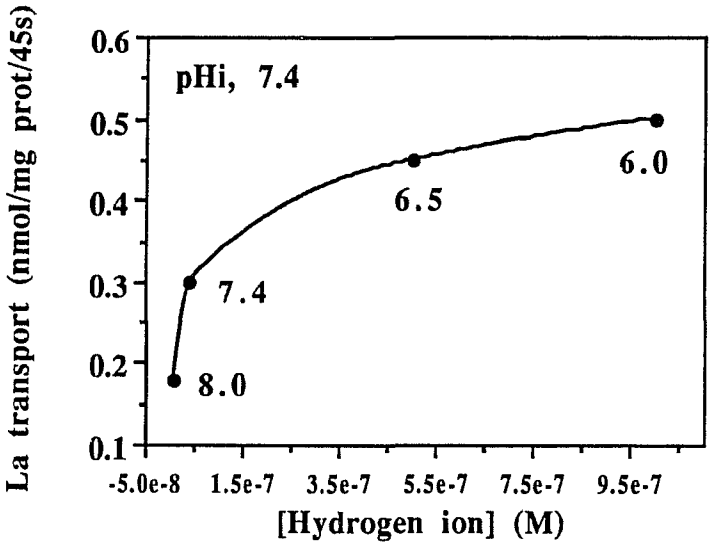


Fig. 5. Initial rate of L-lactate transport as a function of external hydrogen ion concentration. Internal pH is 7.4.External pH is indicated next to the data point. Values are means ± SE of triplicate determinations for 4 experiments with different vesicle preparations.

Effects of inhibitors on J $_{lact}$.: In order to further characterize lactate transport, the effects of various monocarboxylate anions were tested on J $_{lact}$ (Table 2). Pyruvate and alpha-cyano-4 -hydroxycinnamate at concentrations 66 and 33 times greater than the concentration of L- lactate in the extravesicular medium markedly inhibited J $_{lact}$ ($p<0.05$) (see Table 2), whereas D-lactate had no effect on J $_{lact}$. The sulphydryl group modifier N-ethylmaleimide also caused a

marked inhibition of J_{lact} ($p<0.05$) (see Table 3) when the vesicles were preincubated with this modifier for 1h. Pre-incubation with the inorganic anion exchange inhibitor SITS, was without effect on J_{lact}. When Na^+ in the reaction medium was substituted by N- methylglucamine titrated with HCl no effect was observed on J_{lact} compared to controls which had 100mM NaCl in the intra-, and extra-vesicular media (Table 3).

Table 2 . Effects of monocarboxylate inhibitors on inital rate of L- lactate uptake.

Inhibitor	Ratio La: Inhib	Uptake nmol/mg/5s	% inhibition	Significance
Control	-	0.191 ± 0.022	-	-
Pyruvate (10mM)	1: 66	0.037 ± 0.009	81	p<.05
a-cyano-h (5mM)	1: 33	0.032 ± 0.013	83	p<.05
D- Lactate (10mM)	1:66	0.146 ± 0.014	-	N.S.

Note: % inhibition is not reported if the effect is not statistically significant (p>.05)

Table 3 . Effects of protein modifiers on the initial rate of L-lactate uptake

Inhibitor	Uptake nmol/mg/5s	% inhibition of initial uptake	Significance
Control	0.191 ± 0.022	-	-
N-ethylmaleimide (20mM)	0.027 ± 0.007	86	$p<.05$
SITS (1mM)	0.195 ± 0.047	-	N.S
Na^+ substitution	0.152 ± 0.029	-	N.S.

Note: % inhibition is not reported if the effect is not statistically significant ($p>.05$)

Discussion

The predominant means by which L-lactate crosses the plasma membrane of skeletal muscle is via a facilitated carrier system. Evidence supporting this conclusion includes saturation by L-lactate, stereoselectivity for the L and not the D- lactate isomer, inhibition by other monocarboxylic acids and inhibition by a protein modifying agent. The data suggest that this carrier mediated system may account for ~ 80% of J_{lact} at low external concentrations of lactate (0.15mM). These lines of evidence suggest that a lactate transporting protein is present in the SL membrane which has similar properties to the transporter described for erythrocytes (Deuticke, Rickert et al. 1978; Deuticke, Beyer et al. 1982), cardiac muscle (Trosper and Philipson 1987), hepatocytes (Fafournoux, Demigne et al. 1985) and tumour cells (Spencer and Lehninger 1976). The present study confirms and extends previous evidence based on intact muscle experiments that a lactate transport system is present in skeletal muscle membranes (Juel and Wibrand 1989; Watt, MacLennan et al. 1988).

While this thesis was in preparation Roth and Brooks (Roth and Brooks 1990a; Roth and Brooks 1990b) published data on lactate and pyruvate transport in isolated skeletal muscle sarcolemmal vesicles. These studies show considerable similarity to the present study, in that L-lactate transport was found to be a saturable, stereoselective process which is sensitive to a pH gradient, inhibited by other monocarboxylates, and inhibited by protein modifying agents. However, the kinetic parameters differ considerably between the two

studies (Table 5). This may be a result of methodological differences in the transport studies and/or differences in the isolation procedures used. The membrane isolation method used by Roth and Brooks (Roth and Brooks 1990; Roth and Brooks 1990), which is essentially the method of Grimditch et al. (Grimditch, Barnard et al. 1985) differs considerably from the method of Klip et al.(Klip and Walker 1983) used in the present studies. In fact, differences in glucose transport studies have been attributed to differences in these two preparations (Hirshman, Goodyear et al. 1990). However, if one considers the physiological range of lactate to which a muscle is exposed, both intracellularly (Table 4) and extracellularly (Table 4), the kinetic measurements in the present study seem more appropriate for such a concentration range and also agree with the Km values for lactate transport reported in intact muscles (Km =3.5mM, Juel and Wibrand 1989).

Table 4. The range of lactate concentrations in rat skeletal muscles

	Lactate concentrations			
PARAMETER	**RED G**	**WHITE G**	**SOLEUS**	**EXTRACELL.**
Rest (umol/g ww)	**~1**	**~1**	**~0.75**	**-**
" (mM)	**~1.2**	**~1.25**	**~0.94**	**1**
Electrical Stimulation (umol/g ww)	**45**	**15**	**8.5**	**-**
" (mM)	**56**	**19**	**10**	**25**

Note; G - gastrocnemius, Extracell. - lactate concentration in the extracellular space, ww- wet weight, Electrical stimulation refers to the maximal reported values for lactate concentrations after electrical stimulation (Dudley and Terjung 1985; Meyer and Terjung 1979). The mM intracellular concentrations refer to the concentration in the intracellular water, and were calculated from the published values in umol/ g ww (Dudley and Terjung 1985; Meyer and Terjung 1979). These calculations were based on the assumption that 18% of wet weight is comprised of intracellular constituents and the remaining 82% is intracellular water (Turinsky 1987).

The very rapid phase of lactate transport between approximately 1 and 20 mM lactate indicates that the transport system has a high affinity for the substrate and is sensitive to small fluctuations in lactate concentration in the normal physiological range. Moreover, the similarities in the present study and those of Roth and Brooks (Roth and Brooks 1990a; Roth and Brooks 1990b) (Table 5) confirm the presence of a lactate carrier in skeletal muscle with considerable agreement over the nature of the carrier.

Table 5. Comparison of lactate transport parameters in isolated sarcolemmal vesicles.

PARAMETER	Roth & Brooks (1990)	Present study
Saturable	YES	YES
Stereoselective	YES	YES
Pyruvate inhibition	71%	81%
N- ethylmaleimide	86%	86%
pH sensitivity	YES	YES
Anion exchange	(DIDS) NO	(SITS) NO
Km	40.0 mM	4.01mM

Key: Percentages indicate the % inhibition of the initial rate of lactate transport. Anion exchange refers to the involvement of the inorganic anion exchanger in the transport of lactate, as determined by the inhibitors SITS and DIDS. Concentration of pyruvate in the inhibition studies was 10mM in both cases.

The inhibition of L-lactate transport by pyruvate and alpha -cyano -4 - hydroxycinnamate indicates that the transporter is probably a general monocarboxylate carrier which has also been suggested for the lactate carrier in cardiac (Trosper and Philipson 1987) and liver cells (Fafournoux, Demigne et al. 1985). Further evidence of a specific monocarboxylate carrier is atested by the failure of SITS, an inhibitior of the inorganic anion exchanger, to affect J_{lact}, thus eliminating the possibility that the general anion exchanger is the predominant mode of lactate entry. Also, the transport of L-lactate does not appear to be dependent on the cotransport of Na^+ and the lactate ion, because complete substitution of both the intra- and extra- vesicular Na^+ with N-methylglucamine had no effect on J_{lact} (Table 3).

One important finding of this study is that an inwardly directed proton gradient markedly stimulates the vectorial flux of L-lactate (see Fig. 4 and 5). The dependence of the transporter on a proton gradient is not without precedence as studies with other tissues have also shown a marked increase in lactate transport when confronted with inwardly directed lactate and proton gradients. (Balkovetz, Leibach et al. 1988). This mode of proton coupled symport for various organic solutes is being recognized as a recurring motif of regulation by many mammalian transport systems (Balkovetz, Leibach et al. 1988).

An alternative interpretation of the observation that acidic external pH enhances lactate transport could also be ascribed to changes in the protonated

form of lactate (HLa). In order to evaluate whether lactate entry is related to the external concentration of the non-dissociated lactic acid (HLa) rather than the lactate ion, the rates of lactate uptake (J_{lact}) were plotted against calculated values of non-dissociated lactic acid (see Appendix 2), based on a pK_a of 3.86 at room temperature, for two different manipulations :

a) constant pH (7.4) and varied concentrations of total lactate (0.5, 1,10, 25 mM);

b) constant L-La (1mM) and varied external pH (8.0; 7.4;6.5; 6.0).

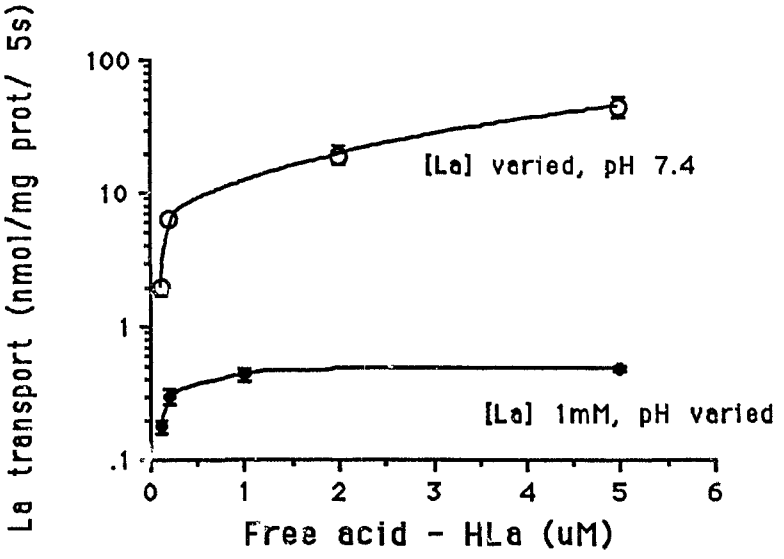


Fig. 6. Relation between external undissociated form of L-lactic acid and the rate of transport. Note this is a semi - logarithmic plot to allow comparison between the two treatments. Variations of undissociated form were obtained by two ways: constant pH (7.4) and increasing concentration of total lactate (0.5, 1, 10, 25 mM); constant concentration of lactate (1mM) with changes in the external pH (8.0, 7.4, 6.5, 6.0).Values are means ± SE for 4 experiments.

If the non-dissociated form was the only permeant form then both manipulations should yield the same J_{lact} for a given concentration of non-dissociated lactic acid (HLa). The discrepancy in the uptake curves for the two manipulations (Fig 5) shows that J_{lact} was much greater when changes in the acid form (HLa) corresponded to changes in the total lactate concentration, and

therefore the ionic form. This discrepancy indicates that lactate uptake is not simply related to the external concentration of undissociated lactic acid, and therefore, the uptake of lactate is more closely related to changes in the ionic form of L-lactate than the protonated form (HLa).

In summary, in the present study an isolated SL vesicle preparation was used to investigate L-lactate transport in skeletal muscle. This study confirms the existence of a facilitated diffusion system for L-lactate transport in the sarcolemmal membrane of skeletal muscle cells. The lactate carrier in skeletal muscle appears to possess similar properties to the carrier described for several other tissue types.

4.2 Lactate uptake in intact skeletal muscle

4.2.1 Introduction

Since skeletal muscle lactate metabolism is a primary determinant of whole body lactate dynamics (Mazzeo, Brooks et al. 1986; Jorfeldt 1970; Brooks and Gaesser 1980; Buchalter, Crain et al. 1989; Roth and Brooks 1990), there would be a teleologogical advantage in having a specific transport system mediating rapid L-lactate traversal of the muscle membrane to efficiently control and regulate intracellular concentration . Although the role of carrier-mediated transport processes in the movement of L-lactate have been described in detail for several tissue types (Balkovetz, Leibach et al. 1988; Edlund and Halestrap 1988; Mann, Zlokovic et al. 1985; Spencer and Lehninger 1976) the evidence suggesting the presence of a carrier for L-lactate in the sarcolemmal membrane of skeletal muscle cells is beginning to accumulate (Roth and Brooks 1990a; Roth and Brooks 1990b). Also, studies in isolated muscle (Juel and Wibrand 1989; Koch, Webster et al. 1981; Mainwood and Renaud 1985; Mainwood and Worsley-Brown 1975; Mason, Mainwood et al. 1986) and in the perfused rat hindquarter (Watt, MacLennan et al. 1988) suggest the presence of a putative carrier for L-lactate in skleletal muscle.

The measurement of unidirectional flux for kinetic analysis is most easily and accurately accomplished in an isolated membrane vesicle preparation (see section 4.1). However, while this elegant method allows the study of transport systems in the natural membrane matrix in the absence of cell metabolism, it

also divorces transport processes from the metabolic processes that they serve, and thus, studies using purified membranes eliminate a potential level of regulation, ie. the coupling of mass solute transport with cell metabolism. In addition, because the preparation of purified isolated vesicles is a lengthy procedure (~24 hrs), and does not leave the intracellular signalling mechanisms intact, it does not easily allow insight as to how an acute alteration in the metabolic or hormonal milieu of the cell might alter the vectorial flux of solutes. Therefore, a combined approach would be useful in investigating a transport system of interest ; ie. to use both an isolated vesicle preparation and an intact cell preparation,which show similar behaviour with respect to a particular transport system, in order to begin to study not only the basic characteristics of a particular transport system but also how acute changes in the cellular metabolic / hormonal status might regulate transport processes.

In the present studies the objectives were twofold a) to carefully quantitate the transport kinetics of unidirectional L-lactate transport in isolated skeletal muscle sarcolemmal vesicles, and b) to relate accurate kinetic information to L-lactate uptake studies in an intact cell, soleus muscle strip preparation which has been used for numerous metabolic studies (Bonen, Elder et al. 1988; Newsholme, Leighton et al. 1986; Crettaz, Horton et al. 1983; Espinal, Dohm et al. 1983).

4.2.2 Methods

Male Sprague - Dawley rats, weighing 225-250g, were used for the sarcolemmal vesicle preparation. Animals were maintained in an air-conditioned room on a 12h light: dark cycle and fed a diet of Purina chow and water ad libitum.

*Sarcolemmal vesicle preparation:*A sarcolemmal vesicle fraction was extracted from rat hindlimb skeletal muscles according to the method of Ramlal et al. (Ramlal, Rastog et al. 1989). The preparation of the membrane vesicles and the transport studies are described in detail in section 4.1.2. The sarcolemmal vesicle transport data used in this section are the same data as reported in section 4.2. However, in the context of this section these data are used to provide a reference comparison for lactate uptake characteristics in the isolated soleus muscle strip preparation .

Muscle strip preparation: Male Sprague- Dawley rats (50-70g) were anaesthetised using sodium pentobarbitol (50mg/kg ip). The soleus muscle was exposed and an incision was made at the proximal tendon with a pair of fine forceps. A ligature was placed around a thin strip of the soleus and the strip was mounted on a stainless steel clip at approximately resting length and tension.

*Lactate uptake studies:*After dissection, the muscle strips were incubated for 15 min in a gassed (95% O_2, 5% CO_2), buffer (KHBB) (see appendix 9 for molar composition), containing fat free bovine serum albumin (4%) and supplemented with 5mM glucose. The muscles were then transferred to the final incubation vials which contained KHBB, 4% BSA and 1uCi of L-[U^{14}C]-La,

various concentrations of unlabelled L-lactate or inhibitors were added as appropriate (any solvents used for the inhibitors were added to the respective control muscles). The pH of the buffer (pHe) was always 7.4, except for the experiments in which pHe was manipulated (pHe was varied in the range from 6.0-8.0). Initial experiments revealed that the pH of the buffer was stable for the short time periods used for the experimental incubations. The incubation vials were well gassed with 95% O_2 5% CO_2 and sealed for the appropriate time period. Incubations were carried out at 37°C in a shaker water bath at 50 cycles /min.

Preliminary experiments were carried out to determine lactate uptake as a function of time, to establish optimal conditions for uptake studies. Another set of experiments were also performed to estimate the extracellular space using ^3H sorbitol as the extracellular space marker. All uptake data were corrected for the contribution of lactate in the extracellular space to total tissue lactate uptake. Further uptake incubations were carried out at 45 seconds and the uptake of lactate expressed as nmol·mg protein^{-1}· 45 s^{-1}.

Inhibitor studies: In these studies the effects of various inhibitors on lactate uptake were assessed. In the monocarboxylate inhibitor experiments the inhibitors were added directly to the uptake medium, the external lactate concentration for all inhibitor experiments was 1mM lactate, the concentrations of the inhibitors were: pyruvate (10mM), D-lactate (10mM), Alanine (10mM), alpha-cyano-4-hydroxycinnamate (15mM). In the studies in which protein modification was assessed the inhibitors were added to the buffer in the preincubation period (30min.) only, and the concentrations were: N-

ethylmaleimide (10mM), cytochalasin B(10μM), 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulphonic acid (SITS) (1mM).

Muscle extract and metabolite assays. After the incubation period the muscle was rapidly removed from the buffer, immersed in ice cold saline, blotted and frozen in liquid nitrogen. The frozen muscle sample was later boiled for 5min. in 0.3ml of 1N NaOH, and the homogeneous extract neutralized with 0.3ml 1N HCl. The muscle extract was then divided into two separate aliquots; 400 ul for liquid scintillation counting and 200 ul for protein measurement. In some initial experiments the experimental incubations were carried out without the presence of the radioactive substrate and these muscles were used for the analytical determination of endogenous muscle metabolites. For these purposes, muscle samples were lyophilized for five hours at -50°C, and extraction was carried out for the muscle metabolites as follows; a 5-10 mg piece of lyophilized muscle was extracted with 150ul of 3M $HClO_4$ for 10 min, neutralized with 250ul of 2M $KHCO_3$. The neutralized extract was then analyzed enzymatically for pyruvate and lactate (Bergmeyer 1965). Muscle glycogen was assayed on a separate piece of muscle according to the method of Passoneau and Lauderdale (Passonneau and Lauderdale 1974). This method avoids the problem of losing ' acid labile' glycogen during the metabolite extraction (Jansson 1981). Protein was determined by the method of Bradford (see appendix 8, Bradford 1976) using bovine serum albumin as the standard.

Evaluation of lactate metabolism in uptake studies: Incubations were carried out in sealed vials with stoppers containing a plastic centre well (Kontes).The centre well contained 0.3 ml of a CO_2 trapping agent (1M Hyamine hydroxide). At the end of the appropriate incubation period, 0.5ml of 0.5M acetic acid was injected into the flask. The vials were then incubated for 30 min at 37°C to trap the liberated CO_2 (Pearce and Connett 1980)(Fafournoux, Demigne et al. 1985). After the incubation, the hyamine hydroxide containing the $^{14}CO_2$ was quantitated using liquid scintillation spectrometry. Identically treated flasks which contained no muscle were used as blanks.

To assess the amount of lactate that might have been utilised for glyconeogenesis (La->glycogen), the amount of L-[U-^{14}C] La that was incorporated into glycogen was determined. This was accomplished by precipitating the glycogen and determining the ^{14}C -label content in glycogen, as previously reported (see section 3).

Statistical analysis Analysis of variance was used to determine whether the treatment exerted a significant effect on the parameters measured. All data are reported as means ± SE. Significance was established at $p<.05$.

4.2.3 Results

The characteristics of the soleus muscle strips, in terms of number of fibers in each muscle strip, fiber type composition, muscle strip cross-sectional areas, andthe diameters of muscle strips are reported in Table 1. Analysis of single

fibers showed that the mean fiber diameter was 19±1 μm (n=12) and the mean fiber area was 0.5± 0.03 mm^2 (n=12).

Table 1. Descriptive characteristics of soleus muscle strips.

Parameter	Strip Sections	
Diameter (μm)	(n=8)	444±18
Area (mm^2)	(n=8)	366±23
# of fibers/section	(n=23)	239±20
Weight (mg)	(n=10)	4.2±0.2
Total protein (μg)	(n=10)	841±33
%FOG	(n=23)	19
%ST	(n=23)	81

Strip Sections refers to a transverse section of one muscle strip. FOG - fast oxidative glycolytic, ST- slow twitch. Fiber types were determined by myosin ATPase and succinate dehydrogenase histochemical stains. Values are means ±SE.

Metabolite levels in isolated muscle strips: No significant changes were observed in the levels of endogenous glycogen, lactate or pyruvate in the muscle strips over the period of pre-incubation and incubation (Fig 1).

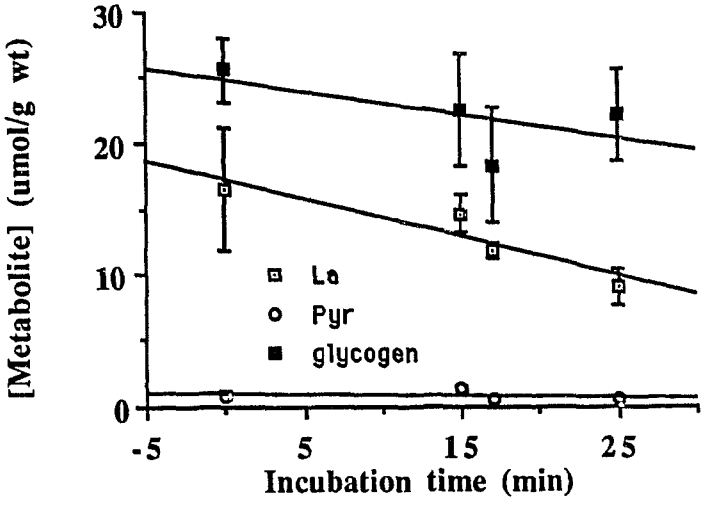


Fig 1. Metabolite levels (La-lactate, Pyr- pyruvate) in muscle strips as a function of incubation time. Lactate and pyruvate are expressed as µmol/g dry weight. Glycogen is expressed as µmol/g wet weight. Values are means ±SE. N=5-7 muscles for each data point.

Lactate metabolism in muscle strips: Measurement of glyconeogenesis in the muscle strips revealed that in the present incubations, a very small amount of the label was recovered in glycogen (7.5 fmol/mg protein/5min in glycogen, compared to a lacate uptake under the same conditions of 1.5 nmol/mg protein /5 min), this supports our previous reports that glyconeogenesis is a small contributor to glycogen synthesis in the soleus muscle, especially over a short time period (see section 3). The recovery of radioactivity in CO_2 was detected and amounted to ~ 10% of total ^{14}C uptake after 45s.The appearance of

radioactivity in CO_2 remained at $\leq$ 10% with increasing concentrations of external lactate. Fig. 2 indicates the oxidation of lactate as a function of increasing concentrations of external lactate. L-lactate oxidation increases in a linear manner with increasing concentrations of external lactate (Fig. 2). Thus, there was some minimal interference from the oxidation of lactate in these short term uptake studies but this interference was limited, and was considered negligible in these studies as done by others (Fafournoux, Demigne et al. 1985; Watt, MacLennan et al. 1988; Juel and Wibrand 1989).

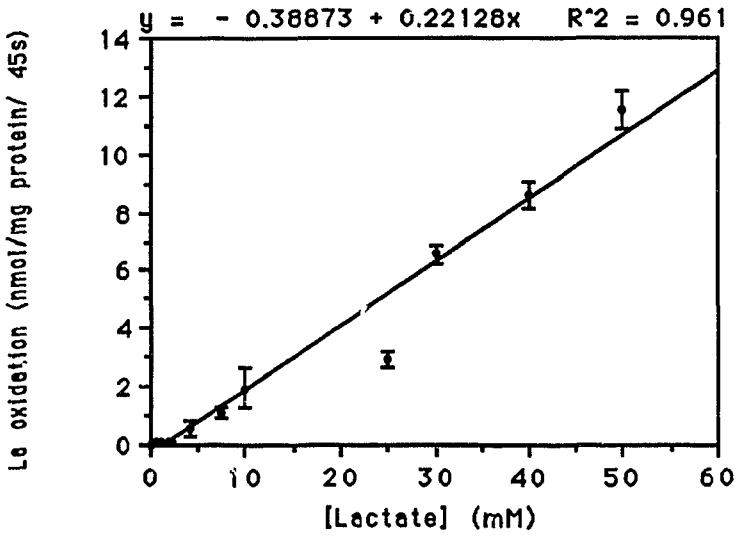


Fig.2 The oxidation of lactate as a function of the external concentration of lactate. Line is fitted by linear regression (Brace 1977).Values are means ±SE, n=5-10 muscle strips for each point.

Time course of L-lactate transport in vesicles and muscle strips. Fig. 3 shows the uptake of L-lactate (1mM) as a function of increasing incubation time for isolated soleus muscle strips. The rate of entry was very rapid in the vesicle preparation (see section 4.1.3) compared to the muscle strips (Fig. 3). However, for both preparations uptake declined with time, leading to steady state conditions after ~ 20s for the vesicles and 300 s for the muscle strips (Fig. 3). In order to measure uptake at the initial rates when uptake was still linear, incubation periods of 5s and 45s were chosen for further experiments in the vesicle and muscle strip experiments, respectively.

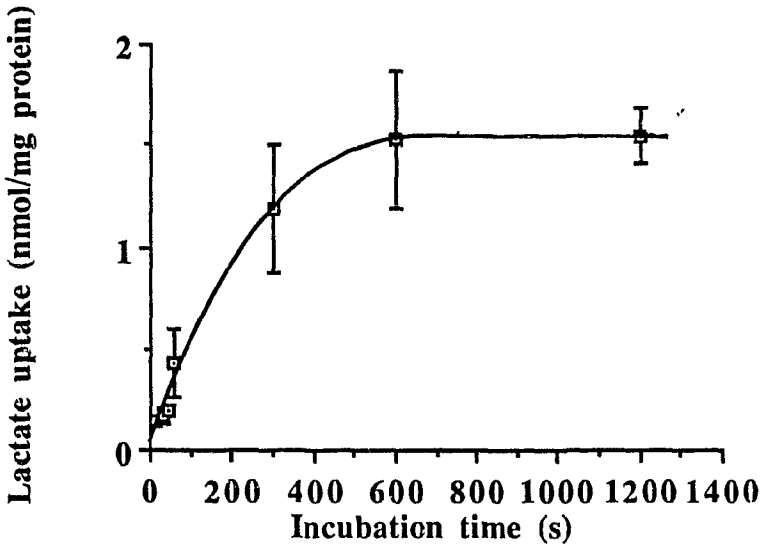


Fig. 3 Lactate uptake as a function of incubation time (s). Values are means ± SE, n=5-10 muscles for each data point.

Effect of external lactate concentration on La uptake: The dependence of L-lactate uptake (muscle strips) on the external lactate concentration is shown in Fig 4. To facilitate comparison between the uptake curves the data for each preparation were expressed as a percentage of the maximal uptake rate and plotted on the same graph (Fig. 5). Both uptake curves show some saturation, Fig.5 illustrates the similarity between the two preparations with respect to the linear part of the curve and also the lactate concentration at which the uptake begins to saturate. Transformation of the vesicle data to a Lineweaver Burke plot yields a Km of ~4mM (see section 4.1.3).

The concentration curves for both preparations do not completely saturate over the concentration range observed and this may be related to a non-saturable diffusive component or to another transport sytem for L-lactate with a very low affinity for the substrate. The rate of this component of the uptake curve was determined by regression analysis of the upper concentration points, as previously described by Christensen (Christensen 1975), and was found to be ~0.38 nmol·mg prot $^{-1}$· 5s^{-1}· mM^{-1} for the vesicles and ~0.37 nmol · mg prot^{-1}· 45s^{-1}·mM^{-1} for the muscle strips.

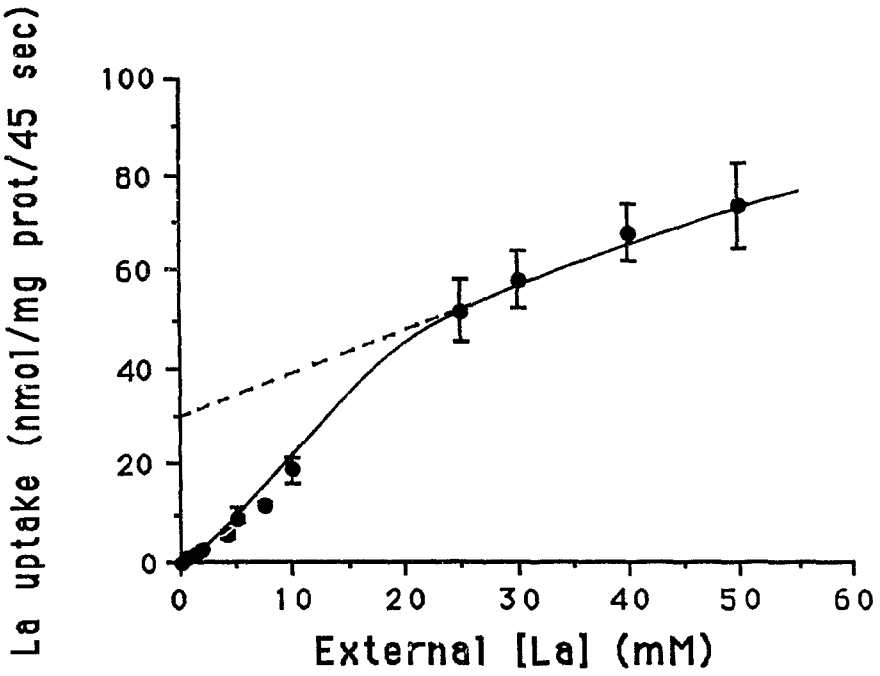


Fig. 4. The effects of the external concentration of lactate (La)(mM) on the uptake of lactate into isolated soleus muscle strips. Values are means ± SE, n=7-13 muscles at each data point. The extended dashed line is the line of best fit for the upper concentration points determined by simple linear regression and is assumed to represent the non-saturable component of the uptake curve as previously described elsewhere (Christensen 1975).

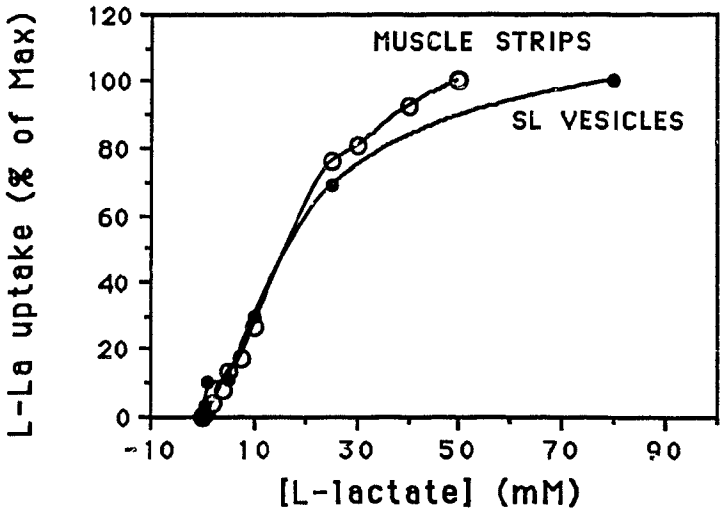


Fig. 5. Comparison of the uptake of lactate into sarcolemmal vesicles (SL vesicles) versus muscle strips. Data are expressed as a % of max.

Effects of external pH (pHe) on lactate transport When the internal pH (pHi) was 7.4 for the vesicles or muscle strips, a decrease in the pHe linearly accelerated the rate of uptake of L-lactate (Fig. 6). A reciprocal decrease in lactate uptake was seen if the pHe was >7.4. These data indicate that lactate travels along an inwardly directed proton gradient.

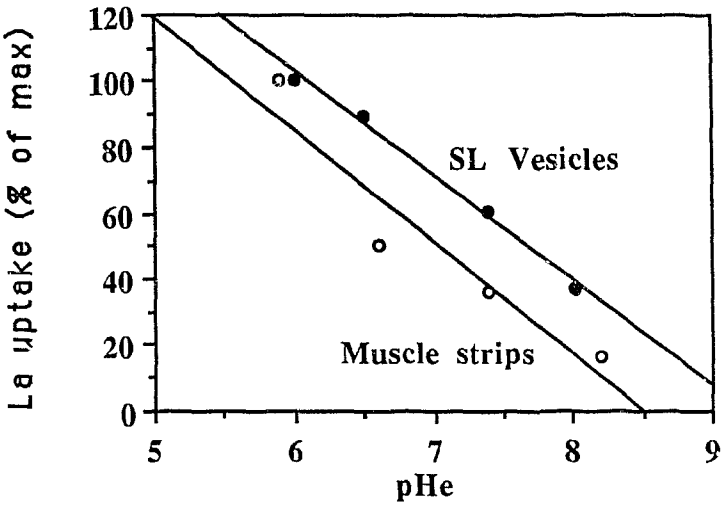


Fig. 6 Effects of external pH (pHe) on lactate uptake into isolated sarcolemmal vesicles (SL vesicles) and muscle strips. Data are expressed as a % of the maximal rate of transport observed at pHe 6.0 (pH 6.0 =100%).

Effects of inhibitors on lactate uptake: The effects of several inhibitor compounds were tested on L-lactate uptake. Pyruvate (10mM) markedly inhibited L-lactate uptake when it was present in the incubation medium at a concentration 20 times that of L-lactate ($p>.05$, Fig. 7). D- La (10mM) had no effect on lactate uptake ($p<.05$, Fig. 7). The aromatic monocarboxylate, alpha- cyano-4-hydroxycinnamate had a small inhibitory effect on L-lact ate uptake, but this effect was not significatly different (Fig. 7). The protein modifier N-ethylmaleimide caused a marked inhibition of L-lactate uptake when

the muscle strips were preincubated with this compound ($p<.05$, Fig 8). Pre-incubation of the muscle strips with an inorganic anion exchange inhibitor, SITS (1mM), did not have an effect on lactate uptake ($p>.05$, Fig.8). Reducing the incubation temperature to 22°C inhibited lactate uptake ($p<.05$, Fig.8).

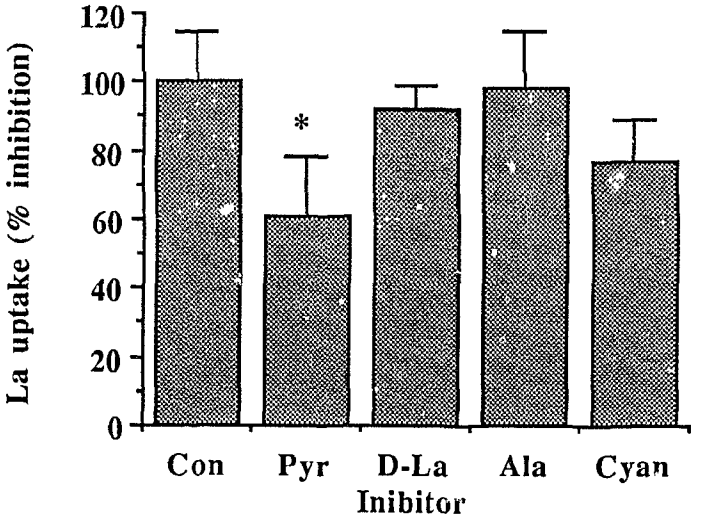


Fig. 7 Effects of inhibitors on lactate uptake by soleus muscle strips expressed as a % of the uptake at pH 7.4, 1mM lactate. Con-control; Pyr-pyruvate, D-La D-lactate; Ala-alanine; Cyan- alpha cyano-4-hydroxycinnamate (see methods for concentrations) Values are means ±SE, n=7-10 muscles at each data point.

** significantly different to the control ($p<.05$)*

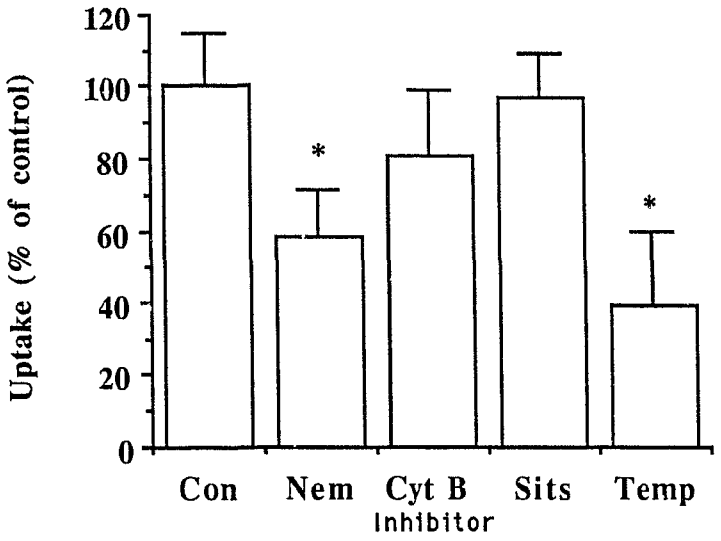


*Fig. 8 Effects of protein inhibitors on lactate uptake by isolated soleus muscle strips expressed as a % of the uptake at pH 7.4, 1mM lactate. Con- control; Nem- N-ethylmaleimide; CytB-cytochalasin B; Temp- reduced temperature (22°). Values are means ±SE, n=7-10 muscles at each data point. * significantly different to the control (p<.05)*

4.2.4 Discussion

Previous investigations have suggested that lactate transport is mediated by two components in skeletal muscle cells: a carrier mediated system and a non-saturable, diffusive process (Koch, Webster et al. 1981; Watt, MacLennan et al. 1988). The present study supports this hypothesis. Detailed kinetic data

from isolated sarcolemmal vesicle studies corroborate the contention that a saturable, stereospecific, carrier for L-lactate exists in skeletal muscle plasma membranes. Lineweaver-Burke transformation of the concentration-dependent L-lactate transport into the SL vesicles yielded a Km value of ~4mM. This is in close agreement with the Km value of 3.5mM recently reported for mouse skeletal muscle by Juel and Wibrand (Juel and Wibrand 1989). In addition, a non-saturable diffusive component of the transmembrane lactate movement is present in the isolated membrane vesicles and also in the isolated muscle strip preparations.

Further proof of the existence of a membrane carrier for L-lactate is provided by the effects of various inhibitor compounds on L-lactate uptake (Fig. 7). The inhibition of L-lactate uptake by the presence of pyruvate suggests that the carrier may be a general monocarboxylate carrier, although alpha-cyano-4-hydroxycinnamate had a much less potent effect on skeletal muscle lactate transport in the strip preparation than in the membrane vesicles, only inhibiting L-lactate uptake by 21% in the strips (Fig 7) compared to 83% in the vesicles (see section 4.1.3). This small inhibition by alpha-cyano-4-hydroxycinnamate has also been reported for other intact skeletal muscle preparations (Watt, MacLennan et al. 1988).The sulphydryl group modifier, N-ethylmaleimide, had a marked effect on reducing L-lactate uptake providing persuasive evidence that a membrane protein is involved in the lactate flux (Fig. 8). The possibility arises that lactate could be transported by the inorganic anion exchanger. However, this hypothesis is refuted by the observation that the inorganic anion exchange inhibitor, SITS, had no effect on lactate uptake (Fig8).

In order to make direct comparison between the uptake values in muscle strip and vesicle experiments it was assumed that ~12% of cell wet weight is protein and ~2% of the total protein is sarcolemmal membrane protein (Frank, Philipson et al. 1984). Using these assumptions it was calculated that under similar uptake conditions (pH 7.4, 1mM L-lactate) the uptake rates were 2.75 nmol · mg protein^{-1}· min^{-1} in isolated intact muscle strips and 3.54 nmol · mg protein^{-1}· min^{-1} in the SL vesicles. These calculations show reasonable agreement between the vesicles and muscle strips in the absolute rates of uptake.

There is considerable complementarity when the trends in the data for lactate uptake are compared for the membrane and intact muscle preparations. For example, Fig. 5 shows that when the transport data are expressed as a percentage of the maximal transport rates for each preparation, the concentration dependent uptake curves show similar trends with the initial linear portion of the curves for each prepartion having similar slopes, and the concentration at which the curves begin to show saturation being similar for both preparations.In addition, the uptake data as a function of changing external pH also show similar trends (Fig 6). These observations indicate that the uptake measurements made in the intact muscle strips, if performed under rapid uptake conditions, give an acceptable index of membrane transport.

Intact cells from various tissues, including muscle, have been extensively used for transport studies (Fafournoux, Demigne et al. 1985; Edlund and

Halestrap 1988; Juel and Wibrand 1989; Wallberg-Henriksson and Holloszy 1984). however these studies are often criticized as being in error because of metabolism of the transported substrate and the difficulty in measuring true initial rate kinetics. In subsequent attempts to minimize these problems transport has been studied in intact cell systems using various metabolic inhibitors (Fafournoux, Demigne et al. 1985), while this approach has been useful it is also problematic from a 'physiologic' perspective. Therefore, the combined approach of verifying intact cell uptake measurements with membrane vesicle studies to determine the similarity of the observed transport behaviour has potential benefits. If the comparison between the preparations show similar trends and respond to manipulations in the same direction, then physiological manipulations of the intact cell may be performed, which are not possible in the vesicle preparation, and a useful index of membrane transport can be still be attained. This approach may prove useful in the investigation of how acute changes in the cell's hormonal or metabolic status might alter cell membrane L-lactate transport, or provide insight into how the separate processes of membrane transport and intracellular metabolism are co-ordinated.

In both the SL vesicle and muscle strip preparations we have consistently observed that an inwardly directed proton gradient markedly stimulates the vectorial flux of L-lactate (see Fig 6). The observation that lactate movement follows a proton gradient has also been reported for intact muscle (Aickin and Thomas 1977; Juel 1988) and in human muscle after exercise (Juel, Bangsbo et al. 1990). However, it has been reported that lactate and proton efflux are not stoichiometrically related (Benade and Heisler 1978; Juel, Bangsbo et al. 1990),

implying the existence of other mechanisms for proton extrusion in muscle (Juel 1988). The possibility that lactate transport may be related to the concentration of protonated lactate (HLa) rather than the ionic form deserves some consideration even though lactic acid is almost completely in the dissociated form at normal physiological pH. In fact, the possibility that the protonated form is the permeant form would explain the observation that acidic external pH enhances lactate transport. In order to evaluate whether lactate entry is related to the external concentration of the non-dissociated lactic acid (HLa) rather than the lactate ion, the rates of lactate uptake for the muscle strip preparation was plotted against calculated values of non-dissociated lactic acid, based on a pK_a of 3.86, for two different manipulations :

a) constant pH (7.4) and varied concentrations of total lactate (0.5;1;10;25 mM);

b) constant L- lactate (1mM) and varied external pH (8.0; 7.4;6.5; 6.0).

If the non-dissociated form is the only permeant form then both manipulations should yield the same uptake for a given concentration of non-dissociated lactic acid (HLa). The discrepancy in the uptake curves for the two manipulations (Fig 9) shows that lactate transport was much greater when changes in the acid form (HLa) corresponded to changes in the total lactate concentration, and therefore the ionic form. The dissimilar uptake data for similar concentrations of external undissociated lactate, indicates that lactate uptake is not simply related to the external concentration of undissociated lactic acid, and therefore, the

uptake of lactate is more closely related to changes in the ionic form of L-lactate. These data from the muscle strip preparation confirm similar observations in the isolated membrane vesicles (section 4.1.3).

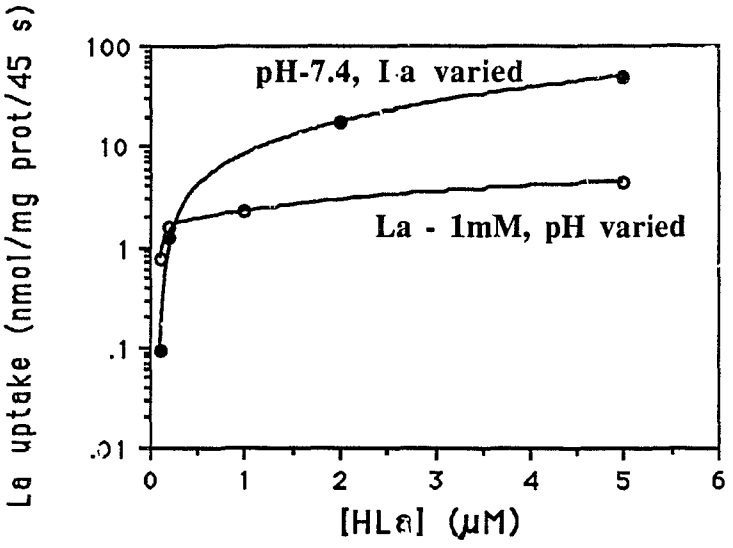


Fig. 9. Relation between external undissociated form of L-lactic acid and the rate of lactate uptake. Note this is a semi- logarithmic plot to facilitate comparison. Variations of the undissociated form were obtained by two ways : constant pH (7.4) and increasing concentration of total lactate (0.5, 1,10,25mM); constant concentration of lactate (1mM)with changes in the external pH (8.0, 7.4, 6.5, 6.0). Values are means ± SE for 4 experiments.

An alternative explanation for the pH sensitivity of the lactate transporter is that the H^+ induces a conformational change in the transporter resulting in an increase in the intrinsic activity. It is also possible that the H^+ concentration causes an enhanced recruitment of inactive transporters to the membrane. Because of the extremely rapid 'acute' effect of a pH change on lactate transport it seems unlikely that the stimulation of lactate transport is mediated via *de novo* synthesis of the membrane carrier protein.

Because skeletal muscle has the capacity for massive alterations in glycolyic flux, and therefore lactate production (Meyer and Terjung 1979; Spriet, Soderlund et al. 1987), an intriguing question concerns the capacity for lactate transport compared to the capacity for lactate production in skeletal muscle. The maximal rate of lactate production in skeletal muscle has been reported to be ~0.25 umol$\cdot g^{-1}\cdot s^{-1}$ (Meyer and Terjung 1979). By converting the vesicle derived transport data on lactate transport to the same units, the maximal rate of lactate transport is ~0.03 umol $\cdot g^{-1}\cdot s^{-1}$. Assuming that the transport rates are symmetrical (maximal inward transport $\simeq$ maximal outward transport) then the maximal rate of lactate production is ~8 times the maximal lactate transporting capacity of intact skeletal muscle. It is therefore clear from this comparison that at times when muscle glycolytic flux is maximal the membrane transport of lactate would be limiting for lactate dissipation. This supports the numerous studies in the literature which have reported the onset of an intracellular lactic acidosis in skeletal muscle when the glycolytic flux is maximal (Mainwood and Renaud 1985; Fitts and Holloszy 1976).

Considerable homogeneity seems to exist between the properties of the

lactate transporters in several tissue types. An L-lactate carrier with pH-dependency and sensitivity to similar monocarboxylate and protein inhibitors has been postulated for Ehrlich ascites cells (Spencer and Lehninger 1976), hepatocytes (Fafournoux, Demigne et al. 1985; Edlund and Halestrap 1988) , cardiac cells (Trosper and Philipson 1987), erythrocytes (Deuticke, Rickert et al. 1978), placental brush border membranes (Balkovetz, Leibach et al. 1988), and intact skeletal muscle (Watt, MacLennan et al. 1988) (Table 3 compares the characteristics of lactate transport in these tissues).

Several attempts have been made to identify the lactate transporting protein in several tissues. A protein of molecular size 40-50 kD was found to be labelled by a radioactive analog of DIDS (4,4'- diisothiocyanato- 2,2' - dihydrostilbenedisulfonate) in rabbit erythrocyte ghosts , DIDS inhibited lactate transport in the same preparation (Jennings and Adams-Lackey 1982). In a subsequent study, Donovan and Jennings (Donovan and Jennings 1985) reported a decrease in the labelling of this protein by [^{3}H] H_2 DIDS (4,4'-diisothiocyanato- 2,2' - dihydrostilbenedisulfonate) when other lactate transport inhibitors were included in the incubation. However, the specificity of these inhibitors has been questioned (Poole and Halestrap 1988)(Poole, Cranmer et al. 1990). A lactate binding protein has been purportedly isolated from rat hepatocyte plasma membranes with an apparent M_r of 40 Kd, determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE)(Welch, Metcalfe et al. 1984). Whether this protein is the lactate carrier protein has yet to be confirmed. Poole and Halestrap (Poole and Halestrap 1988) have been able to reconstitute solubilized

Table 3. Summary of lactate transport characteristics in different tissues

Organ	Mr (Kd)	Carrier	Active	Non-Ionic	Anion exchange	H^+ dep	Na^+ dep	Monocarb Inhibitors	Protein Inhibitors
Liver Edlund (1988)	40	+P	NR	+	-	+	NR	+ a,p	+
Skeletal Muscle Watt (1989)	NR	+P	NR	+	-	+	NR	+a,p	+
Erythrocytes Deuticke (1982)	40-50	+P	NR	+	-	+	NR	+a,p	+
Cardiac cells Trosper (1989)	NR	+P	NR	+	-	+	-	+a,p	+
Kidney Brush border Brand (1981)	NR	+P	E	+	NR	-	+	NR	NR
Kidney Basal lateral Stewart (1984)	NR	+P	E	+	NR	+	-	NR	NR
Placenta Balkovetz (1989)	NR	+P	NR	+	NR	+	-	+p	+
Tumour cells Spencer (1976)	NR	+P	E	NR	NR	+	NR	+a,p	+

KEY: NR- not reported; + positive effect; - no effect; P predominant at physiological concentrations; E- electroneutral A- active; a- inhibited by alpha 1,4 hydroxycinnamate; p- inhibited by pyruvate; Mr- relative molecular weight (SDS page); dep- dependence; Protein inhibitor- transport is inhibited by protein modifying agents; Monocarb inhibitor- inhibited by monocarboxylic acid derivatives.

rat and rabbit erythrocyte membrane proteins into liposomes, these proteoliposomes were shown to have an accelerated rate of monocarboxylate transport, indicating that the lactate transport protein can be reconstituted and retain its transport capacity. A subsequent report from the same group has recently documented substantial differences in the affinity of the lactate carrier from cardiac myocytes and erythrocytes for various monocarboxylates (myocytes 10 fold higher than erythrocytes) (Poole, Cranmer et al. 1990), possibly implying the presence of distinct carriers in these tissues. One feasible explanation for these observations is that there is a family of related tissue specific lactate carrier proteins which have different affinities for the substrate. Further molecular characterisation of the lactate transport carrier protein will help in elucidating the extent of the homology between different tissues.

In summary, the characterisation of L-lactate transport into isolated sarcolemmal vesicles and muscle strips revealed that both preparations yielded similar conclusions regarding the transmembrane movement of L-lactate, thus indicating that the use of the muscle strip may have some utility as a means of obtaining a useful index of L-lactate uptake in an intact cell system.

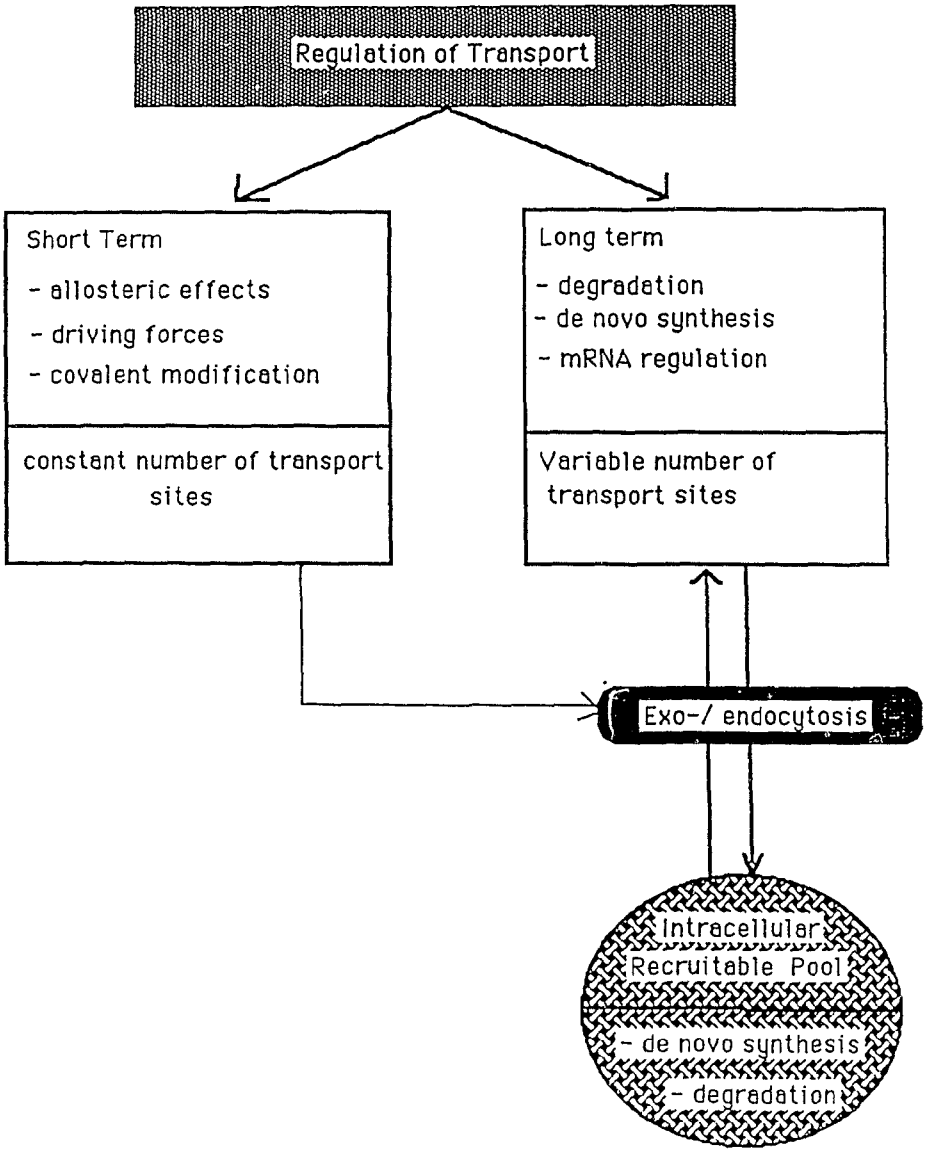
4.3 The effects of contractile activity on lactate transport in skeletal muscle.

In the preceding section the characteristics of lactate transport in skeletal muscle was addressed. From a physiologic perspective it is now interesting to consider whether this transport process is adaptable and can be regulated by functional alterations in the activity of the muscle. If one considers the regulation of membrane transport it may be arbitrarily divided into short term and long term regulation. The mechanisms which have been identified to accomplish these acute and chronic types of regulation are shown in Fig. 1. One perturbation which is known to influence both short term and long term regulation of the membrane transport of various ions and glucose in skeletal muscle is acute and chronic contractile activity (King, Hirshman et al. 1989; Sternlicht, Barnard et al. 1989; Dohm, Sinha et al. 1987; Lindiger, Heigenhauser et al. 1987). In the following section the influence of acute and chronic contractile activity on the uptake of lactate in skeletal muscle will be addressed.

4.3.1 Introduction

A number of cellular processes, such as membrane phospholipid content (Donaldson, Goldberg et al. 1987), calcium dynamics (Vergara, Tsien et al. 1985), intracellular signalling systems (Booth 1988) and membrane ion pump activity (Clausen and 1986), are altered due to an acute bout of contractile

Figure.1


Regulation of Transport
Short Term
- allosteric effects
- driving forces
- covalent modification
constant number of transport sites
Long term
- degradation
- de novo synthesis
- mRNA regulation
Variable number of transport sites
Exo-/ endocytosis
Intracellular Recruitable Pool
- de novo synthesis
- degradation

activity. Also, many studies have shown a more rapid disappearance of lactate from the circulation during mild exercise. Since lactate transport is mediated by a transport protein in muscle, and since muscle is the major determinant of whole body lactate dynamics (Buchalter, Crain et al. 1989), it is possible that lactate removal in the post-exercise period is due to an increase in the uptake of lactate.

Recently, several studies have shown that muscle glucose uptake is increased either by exercise or contraction induced by electrical stimulation, in the absence of insulin (Wallberg-Henriksson 1987; Ivy and Holloszy 1981; Garthwaite and Holloszy 1982), and this effect can persist for several hours after the contraction period (Ivy and Holloszy 1981). This enhanced glucose uptake seems to be mediated by an increase in the plasma membrane glucose transporter number and possibly by an increase in the intrinsic activity of the transporter (Goodyear, Hirshman et al. 1990, for review see Bonen, McDermott et al. 1990b). The transmembrane movement of lactate also appears to be mediated by a carrier protein (present studies, section 4, Roth and Brooks 1990a,b). Thus, in much the same way that glucose transport is stimulated by contraction, it may be hypothesized that contractile activity will stimulate lactate uptake.

Therefore, the primary purpose of the present study was to determine if a bout of contractile activity which enhances glucose uptake in muscle also enhances the uptake of lactate.

4.3.2 Methods

Muscle stimulation protocol: Male Sprague- Dawley rats (50-70g) were anaesthetised using sodium pentobarbitol (50mg/kg ip). Upon induction of surgical anesthesia the sciatic nerve distal to the greater trochanter of both legs was exposed and a ligature placed proximal to the stimulation site. The ligature was tied and the sciatic nerve proximal to the ligature was cut. The sciatic nerve distal to the ligature was then laid across the electrodes which were placed 1 cm apart. The sciatic nerve in the contralateral, non-stimulated leg was treated identically.

The hindquarter muscles of one leg innervated by the sciatic nerve were electrically stimulated to contract with 300 ms trains at a supramaximal 10-V intensity and a 1 Hz frequency. Trains consisted of 3 separate impulses in 300 ms, each 0.3 ms in duration. The stimulation period was 30min. in duration. Maintenance of anaesthesia during this period was assessed by monitoring respiratory rate and foot pinch reflexes. At the end of the stimulation protocol the muscle strips from the isolated soleus muscles from the stimulated and non-stimulated legs were removed and incubated to determine lactate or glucose uptake.

Muscle strip preparation: The soleus muscle was exposed and an incision was made at the proximal tendon with a fine pair of forceps. A ligature was placed around a thin strip (see section 4.2.3 for muscle strip characterization) of the soleus and the strip was mounted on a stainless steel clip at approximately resting length and tension.

Lactate uptake studies: After dissection, the muscle strips were pre-incubated for 15 min in a gassed (95% O_2, 5% CO_2), buffer (Krebs-Henseleit bicarbonate buffer (KHBB), containing fat free bovine serum albumin (4%) and supplemented with 5mM glucose) The muscles were then transferred to the incubation vials which contained KHBB, 4% BSA and 1uCi of L-[U^{14}C]-lactate, various concentrations of unlabelled L-lactate were added as appropriate, uptake experiments were carried out with 1, 5, and 50 mM lactate in the incubation buffer. The pH of the buffer (pHe) was always 7.4. Initial experiments revealed that the pH of the buffer was stable for the short time periods used for the experimental incubations. The incubation vials were well gassed with 95% O_2 5% CO_2 and sealed for the appropriate time period, based on the time course experiments the time period chosen was 45s because the uptake was linear in the range of 5-300s. Incubations were carried out at 37°C in a shaker water bath at 50 cycles /min. Preliminary experiments were carried out to determine La uptake as a function of time, to establish optimal conditions for uptake studies (see section 4.2.3). Another set of experiments were also performed to estimate the extracellular space using ^{3}H sorbitol as the extracellular space marker. All uptake data are corrected for the contribution of lactate in the extracellular space to total tissue lactate uptake. Further uptake incubations were carried out at 45 seconds and the uptake of lactate expressed as nmol·mg protein^{-1}· 45 s^{-1}.

After the incubation period the muscle was rapidly removed from the buffer, immersed in ice cold saline, blotted and frozen in liquid nitrogen. The frozen

muscle sample was later boiled for 5min. in 0.3ml of 1N NaOH, and the homogeneous extract neutralized with 0.3ml 1N HCl. The muscle extract was then divided into two separate aliquots; 400 ul for liquid scintillation counting to determine the ^{14}C incorporation into the muscle, and 200 ul for protein measurement. The use of total protein as a reference base is the most appropriate for expressing metabolic parameters in muscle (Kelso, Hodgson et al. 1987).

Evaluation of L-lactate metabolism in uptake studies: Incubations were carried out in sealed vials with stoppers containing a plastic centre well (Kontes).The centre well contained 0.3 ml of a CO_2 trapping agent (1M Hyamine hydroxide). At the end of the appropriate incubation period, 0.5ml of acetic acid was injected into the flask. The vials were then incubated for 30 min at 37°C to trap the liberated CO_2 from the incubation buffer (Pearce and Connett 1980). After the incubation, the hyamine hydroxide containing the $^{14}CO_2$ was quantitated using liquid scintillation spectrometry. Identically treated flasks which contained no muscle were used as blanks to determine the background radioactivity.

To assess the amount of lactate that might have been utilised for glyconeogenesis (lactate conversion to glycogen) the amount of L-[U-^{14}C] La that was incorporated into glycogen was determined. This was accomplished by precipitating the glycogen and determining the ^{14}C -label content in glycogen, as previously described (appendix 7, Chan and Exton 1976.).

Muscle extract and metabolite assays: In order to assess whether any changes in the intramuscular concentrations of metabolites, especially lactate, occurred due to the stimulation protocol used, some initial experiments were carried out without the presence of the radioactive substrate, and these muscles were used for the analytical determination of accumulated, intramuscular, glycolytic intermediates (see appendix 1 for assay details).

Glucose uptake studies: In order to detect if the electrical stimulation of the hindlimb was having an effect on one of the parameters which is known to change with a bout of contractile activity, the effects of the same 30 min. contractile period on glucose uptake was measured. These studies were performed in an analogous manner to the studies for lactate uptake, except 1μCi of ^{3}H deoxy-D-glucose, and 1mM unlabelled deoxy-D-glucose (see appendix 4 for technical details) was included in place of the lactate in the incubation medium.

Statistical analysis: For the lactate uptake studies an analysis of variance was used to determine whether the treatment exerted a significant effect on lactate uptake. In the glucose uptake studies determined at one concentration (1mM) a Student t test was used to determine the difference between the control and stimulated group. All data are reported as means $\pm$ SE. Significance was established at $p<.05$.

4.3.3 Results and Discussion

The respiratory rate of the animals during the electrical stimulation period is shown in Fig.1. These data indicate that the level of anaesthesia was consistent and was maintained during the experimental period.

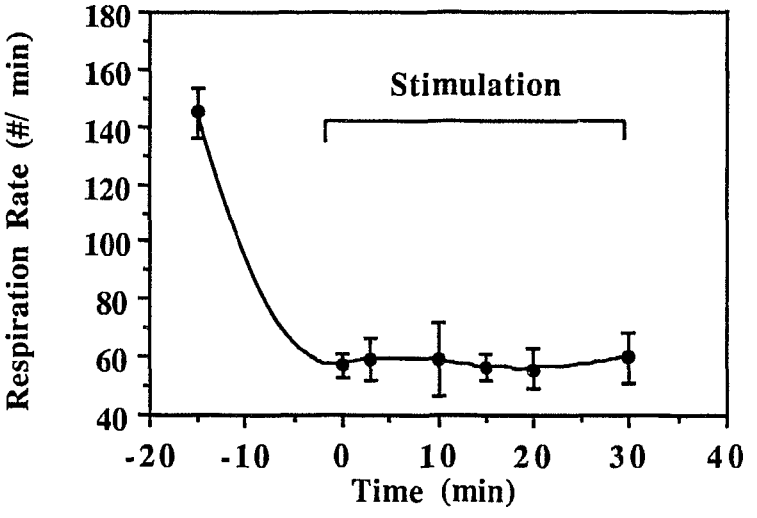


Fig.1. The respiratory rate of rats (50-70g) under sodium pentobarbitol induced anaesthesia (50mg/kg ip). The -15 min value is before the anaesthesia was induced. 0-30 min. values were measured during electrical stimulation of one hindlimb via the sciatic nerve. Values are means ± SE, n=5 rats.

Metabolite levels in isolated muscle strips: A marked decrease in the glycogen stores occurred in the stimulated hindlimb muscles compared to the contralateral non-stimulated muscles ($p<.05$)(Table 1). The electrical stimulation protocol was specifically selected to keep lactate concentrations to a minimum,

so as to avoid a gradient problem for the uptake studies. The small increase in lactate over resting levels (Table 1, Table 2 shows mM concentrations in the intracellular water) indicates that this protocol was successful (ie. lactate concentrations were approximately doubled by the stimulation, but this is a relatively small increase when one considers that this muscle can increase lactate concentrations 10-15 fold with electrical stimulation, and the increase in lactate only accounts for approximately 2.6% of the glycogen which is degraded in the 30 min. stimulation period) (Table 1 and 2). If there had been a large increase in the endogenous lactate concentrations due to the stimulation it would have made the interpretation of the uptake measurements difficult because the lactate concentration gradient would be different between the control and stimulated muscles. Therefore these experiments were performed at various concentrations of lactate (1,5,50mM) to ensure that a lactate gradient into the muscle was established in both the control and the stimulated muscle strips (see Table 2 for intracellular lactate concentrations).

Table 1. Effects of contraction induced by electrical stimulation on muscle metabolite concentrations (umol/g dry weight).

	umol/ g dry weight		
Treatment	Lactate	Pyruvate	Glycogen
Control	5.4 ±1.1	0.77 ±0.2	155.0 ±38
Stimulated	11.8 ±5.8	0.87 ±0.3	42.3* ±12

Values are means ±SE, n=5 muscles /data point. significantly different to the control ($p<0.05$)*

Table 2. Effects of contraction induced by electrical stimulation on muscle metabolite concentrations (mM intracellular water).

	mM (intracellular water)	
Treatment	Lactate	Pyruvate
Control	1.3 ±0.27	0.19 ±0.05
Stimulated	2.9 ±1.4	0.21 ±0.07

Footnote. These conversions were based on the fact that 78% of total cell mass is intracellular water. Calculations are based on the data in Table 1.

L-lactate metabolism in muscle strips: Measurement of glyconeogenesis in the muscle strips revealed that in the present incubations, a small but significant amount of label was recovered in glycogen (7.5 fmol/mg protein/ 5 min), this supports previous reports that glyconeogenesis is a small contributor to glycogen synthesis in the soleus muscle, especially over a short time period (Bonen, McDermott et al. 1990c). Therefore the amount of lactate converted to glycogen in these uptake studies is not meaningful compared to the total flux of lactate into the tissues (eg. under identical conditions the uptake of lactate is ~1.5 nmol/mg protein and of this only 7.5 fmol/mg protein/5min would be converted to glycogen in the same period). The recovery of radioactivity in CO_2 was detected and amounted to ~ 10% of total ^{14}C uptake in 45s.The appearance of radioactivity in CO_2 remained at $\leq$ 10% with increasing concentrations of external lactate. Thus, there was some minimal interference from metabolism in these short term uptake studies and this was treated as experimental error, as reported by others (Fafournoux, Demigne et al. 1985; Juel and Wibrand 1989; Watt, MacLennan et al. 1988; Koch, Webster et al. 1981).

Glucose uptake studies: Glucose uptake in the stimulated and non-stimulated muscles is shown in Fig.2. These data indicate that glucose uptake is enhanced as a result of 30 min.of contractile activity elicited by electrical stimulation. These data are consistent with numerous reports in the literature suggesting that an acute bout of contractile activity enhances glucose uptake in muscle for an

extended period after the contractile activity has ceased (Ivy and Holloszy 1981; Wallberg-Henriksson 1987).

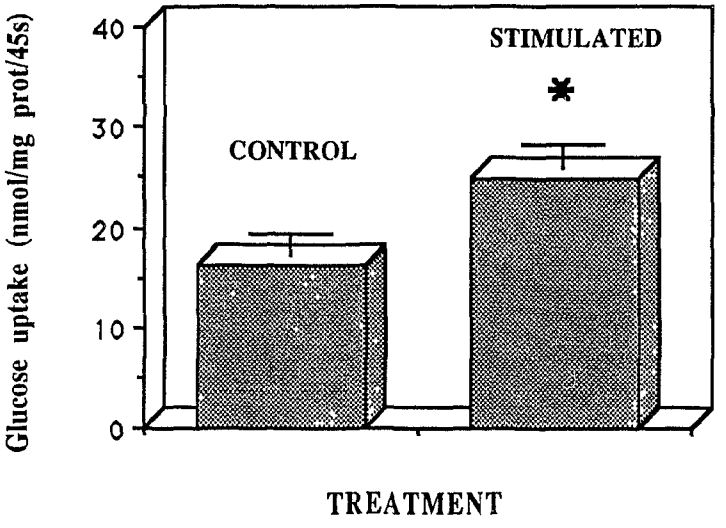


Fig. 2 The effects of electrical stimulation on the uptake of deoxy-glucose in skeletal muscle strips. Hindlimb muscles from one leg were electrically stimulated via the sciatic nerve for 30 min. After the stimulation period the stimulated and contralateral non-stimulated soleus muscles were stripped and incubated in vitro for glucose uptake measurements. Values are means ±SE, n=8. significantly different to control (t-test, p<0.05).*

Lactate uptake studies: Lactate uptake in the stimulated and non-stimulated muscles is shown in Fig.3. These data indicate that at three different external lactate concentrations (1,5,50 mM) there was no difference in lactate uptake

between the stimulated and non-stimulated conditions. These data clearly indicate that the stimulatory effect of contractile activity on the uptake of glucose in muscle is not observed for lactate uptake.

Different mechanisms associated with the glucose and lactate transport processes may account for the present observations. It is established that the exercise-induced enhancement of glucose uptake is mediated by a recruitment of more transporters to the plasma membrane (Sternlicht, Barnard et al. 1989) or possibly by an increase in the affinity of the glucose transporter for glucose (Goodyear, Hirshman et al. 1990).

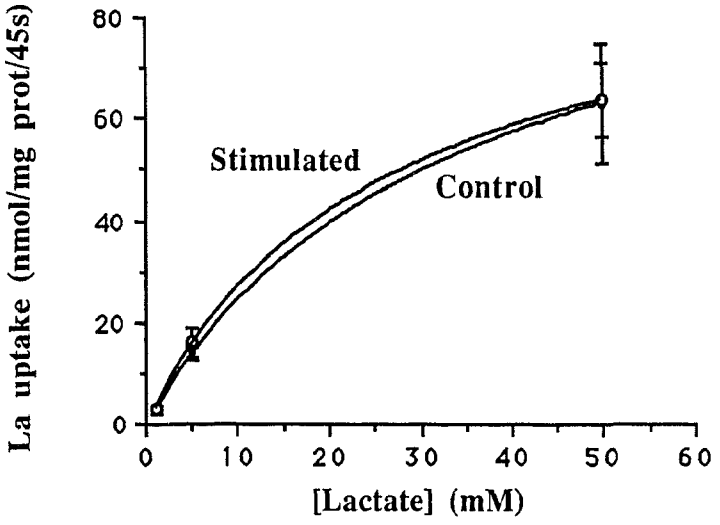


Fig. 3 The effects of electrical stimulation on the uptake of L-lactate in skeletal muscle strips. Hindlimb muscles from one leg were electrically stimulated via the sciatic nerve for 30 min. After the stimulation period the stimulated and contralateral non-stimulated soleus muscles were stripped and incubated in vitro for lactate uptake measurements. L-lactate uptake was determined in control and stimulated muscles at three external concentrations of lactate (1, 5, and 50 mM).

In terms of the overall regulation of substrate fluxes the present studies could reflect fundamental differences in the way lactate and glucose movement is controlled in muscle cells. This may be due to the fact that glucose concentrations in the post-absorptive state remain almost invariant in the circulation, and therefore in order to regulate glucose uptake in peripheral tissues several complex mechanisms have evolved to tightly regulate uptake (ie. insulin stimulation and contractile activity invoked enhancement of glucose uptake, Hirshman, Goodyear et al. 1990; Kern, Tapscott et al. 1989; Kern, Tapscott et al. 1989). However, the situation is somewhat different for lactate because there can be large concentration shifts in both the intracellular and extracellular compartments, and therefore the primary determinant of lactate flux may be the driving force of the concentration gradient in combination with the pH gradient in the post-exercise period, as opposed to changes in the availability or affinity of the transporter per se. Evidence for this suggestion is implicit in the previously described studies (section 4.1) showing that lactate transport is highly sensitive to changes in substrate concentration and pHe.

In summary, these studies show that contractile activity induced by electrical stimulation does not induce a persistent increase in lactate uptake in skeletal muscle in the post-exercise period. These data are in contrast to the enhanced glucose uptake observed in skeletal muscle after a similar bout of contractile activity.

4.4 Effects of chronic contractile activity on lactate transport.

4.4.1 Introduction

Marked phenotypic changes occur in skeletal muscle as a result of chronic endurance training (Holloszy and Coyle 1984; Henriksson 1977; Mole, Oscai et al. 1971). At the molecular level, these changes include alterations in: beta-receptor density (Buckenmeyer, Goldfarb et al. 1990), enzyme levels (Hickson, Heusner et al. 1976), insulin binding (Bonen, Clune et al. 1986), mitochondrial proliferation (Holloszy 1967, Holloszy, Oscai et al. 1970; Holloszy and Coyle 1984), cellular signalling mechanisms (Buckenmeyer, Goldfarb et al. 1990; Booth 1988; Donaldson, Goldberg et al. 1987), translocation of proteins to the sarcolemma (Dohm, Sinha et al. 1987) and, as yet, many unidentified alterations in the transcription of various genes. This molecular plasticity underlies considerable functional alteration in the muscle's capacity to perform work after a training program.

One important physiological correlate of these training adaptations is that lactate accumulation in skeletal muscle at any work rate is reduced after a period of endurance training (Favier, Constable et al. 1986; Donovan and Brooks 1983; Proscurshim, Russo et al. 1989). One explanation for this phenomenon is the increased oxidative capacity of the muscle and a reduction in lactate production (Favier, Constable et al. 1986). On the other hand, it has been argued that the production of lactate is unaffected by training, but the clearance rate (the ratio of lactate removal to lactate concentration) is altered (Donovan and Brooks 1983;

Donovan and Pagliassotti 1989). A more recent study has furthered the case for this 'enhanced clearance' hypothesis, reporting that endurance trained rats have an enhanced capacity to dissipate an exogenously infused lactate load compared to sedentary rats (Donovan and Pagliassotti 1990). The mechanisms which mediate the enhanced efficiency for lactate removal in trained animals have not been elucidated. The clearance rate could potentially be altered by 1) enhanced clearance in other organs such as the heart, liver and kidney, 2) increased uptake in muscle for glyconeogenesis, and 3) removal by contracting oxidative fibers (Donovan and Pagliassotti 1990). In reality probably all of these factors contribute to the enhanced lactate clearance observed after a period of training. The muscle membrane transport of lactate can be seen to be a fundamental aspect of such clearance, both in the need for lactate efflux from muscle which is producing lactate, and also for lactate influx into less active muscle for oxidation and/or glyconeogenesis.

There is a growing body of evidence suggesting the presence of a saturable lactate carrier in the membrane matrix which may be important for the movement of lactate at physiological concentrations (present studies, section 4, Roth and Brooks 1990a,b; Juel and Wibrand 1989). Transmembrane lactate flux may well be an important unconsidered aspect of the training response in skeletal muscle because lactate efflux in muscle is known to be greatly exceeded by the rate of lactate production when glycolysis is accelerated (See section 4). Therefore, an alteration in the capacity of the membrane to increase the rate of lactate transport would result in a reduced intracellular concentration for a given rate of lactate production. Thus, in view of the foregoing discussion, it was hypothesized that the lactate transporting capacity of skeletal muscle would be increased as a result of endurance training.

4.4.2 Methods.

Male Sprague - Dawley rats, weighing 225-250g, were used in this study. Animals were maintained in an air-conditioned room on a 12h light: dark cycle and fed a diet of Purina chow and water ad libitum. Animals were randomly assigned to one of two groups, control (n=10) or endurance trained (n=14).The endurance training protocol used in the present study has been used in previous studies from this laboratory, and was found to increase succinate dehydrogenase activity (SDH) activity in the hindlimb muscles (Bonen, Clune et al. 1986).

Endurance-trained animals were run on a motorized treadmill 4 days/week up to 25m/min on a 15% grade for five weeks. The duration of each running period was progressively increased from 30 min./day in week 1 (20m/min,9% grade); 45min/day in weeks 2 and 3 (20m/min, 9% grade); and 60 min/day in weeks 4 and 5 (25m/min, 9 and 15% grades, respectively). In week 6 the training protocol was continued (identical to that of week 5). To control for the possible 'acute' residual effects of the last training bout on membrane transport, both the trained and sedentary animals performed a standardized exercise bout, running for 15min. at a speed of 15m/min on an 8% grade 24 hours before they were killed. At the appropriate time rats were killed by an overdose injection (intraperitoneal) of pentobarbitol sodium (85mg/kg ip). On induction of surgical anesthesia (~2min) and before the animal died, the hindlimb muscles were rapidly dissected, frozen in liquid nitrogen, and stored at -80°C prior to the isolation of the sarcolemmal vesicles. Skeletal muscle sarcolemmal vesicles were obtained from rat hindlimb skeletal muscles according to the method of Ramlal et al.(Ramlal, Rastog et al.

1989) as previously described (section 4.1.2). Lactate transport measurements were carried out as previously described (section 4.1.2). Transport experiments were performed at two external concentrations of lactate (1mM and 50 mM) with the internal and extravesicular pH maintained at 7.4.

Statistical analysis: A repeated measures Anova (2way (Time -weeks; Treatment - training or control)) was used to determine the effects of training on body weight . A Student t test was used to determine whether the treatment (Tr, Control) exerted a significant effect on the parameter measured (La transport). All data are reported as means $\pm$SE. Significance was established at $p < .05$.

4.4.3 Results and discussion

Body weights were different between the trained and sedentary animals (Anova, $p<0.0001$) and an interactive effect ($p<0.0001$) between time and the treatments was also observed, indicating that the differences in weight between the two groups were greater towards the end of the study (Fig.1). This reduced weight gain of the trained animals compared to the sedentary (control) group is typically observed in studies with male rats (Bonen, Clune et al. 1986).

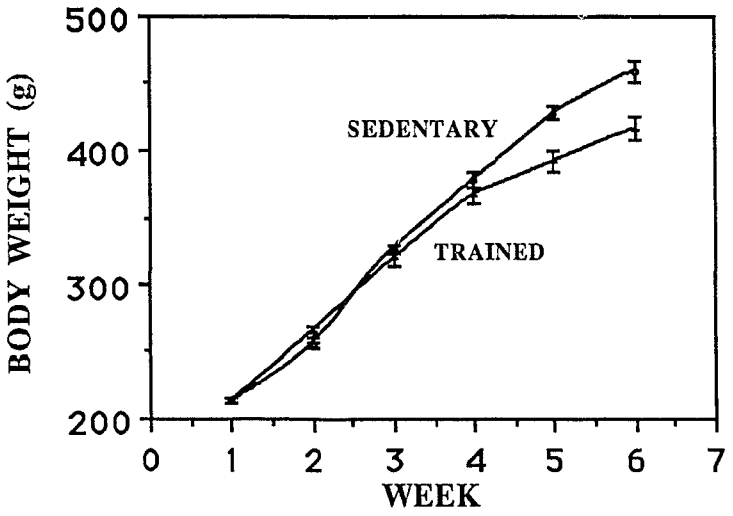


Fig.1 The body weights (g) of sedentary (n=10) and trained animals (n=14) during a 6 week training program (see methods for details). Values are means ± SE. There was a significant effect (p<0.001) of treatment (ie. training) on the weight gain with time (ANOVA).

When the transport experiments were carried out at an external concentration of 1mM lactate (pH 7.4) there was a difference between the capacity of the membranes from the trained rats to transport lactate compared to the sedentary rats ($p<0.05$) (Fig.2A). However, when the experiments were carried out with 50 mM lactate in the external medium there was no difference in the lactate transporting capacity of the membrane vesicles (Fig.2B).

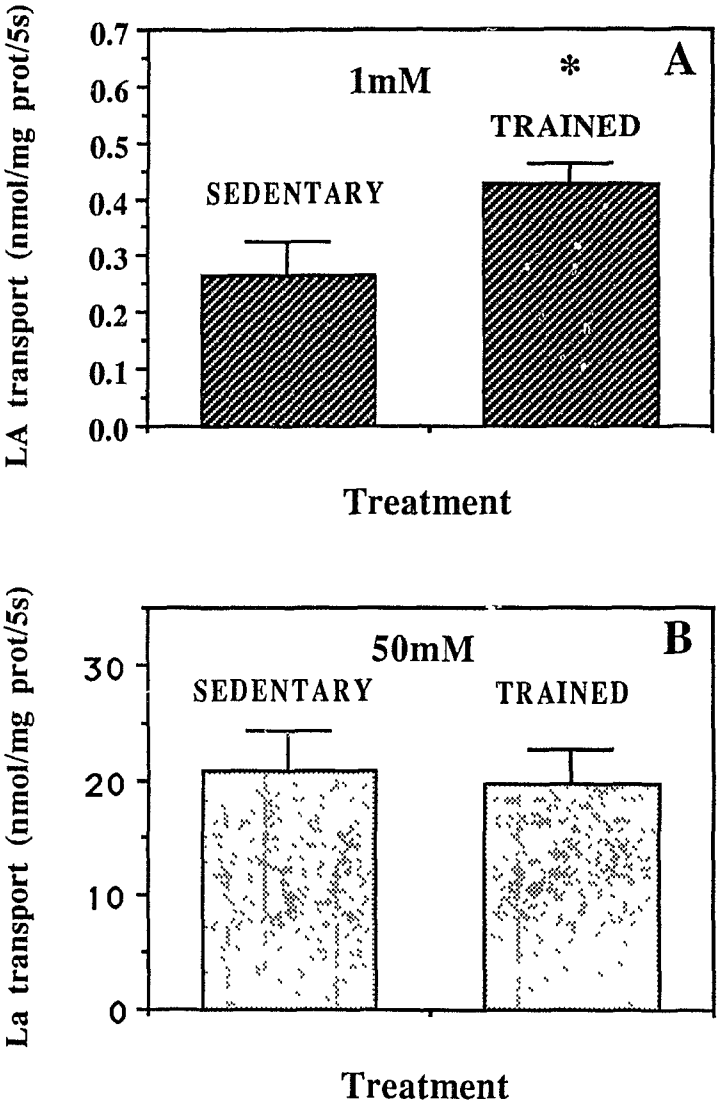


Fig.2. The effects of endurance training (6 weeks) on lactate transport in isolated sarcolemmal vesicles. A: Transport measurements taken at external concentration of 1mM L-lactate at pH 7.4 B: Transport measurements taken at external concentration of 50 mM L-lactate.Values are means of triplicates for 3-4 experiments.

These observations may be explained by the possibility that lactate transport kinetics are altered following a period of training. Our observations are consistent with the possibility that the Km value for lactate transport is reduced in trained muscle, indicating a higher affinity of the transporter for the substrate (Fig.3) . The fact that lactate transport was not altered at high external concentrations of lactate might be explained by a lack of change in the maximal transport capacity (Vmax) of the transport system due to endurance training (Fig3).

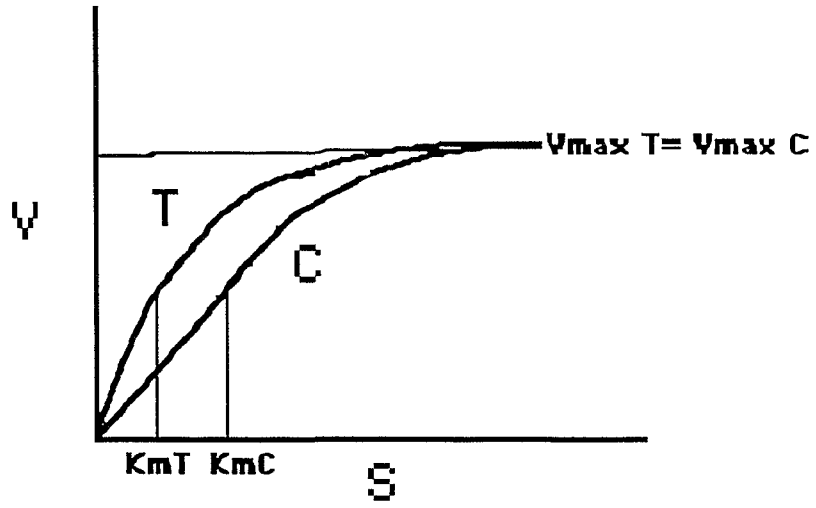


Fig.3 Hypothetical plot of the changes in lactate transport in trained and sedentary rat muscle membranes based on Michaelis Menten kinetics.

An alternative, but more complex, explanation of these data is that several (>1) lactate transporting proteins exist in the muscle membrane, each with a different affinity for the substrate. If this is the case then it is possible that the relative amount of one protein isoform compared to the amount of another may change due to endurance training, and in this manner, assuming different affinities of the carrier isoforms for lactate, alter the 'total' transport kinetics of lactate. Indeed, it has been postulated that a family of lactate transporting proteins may exist which may be tissue specific and possibly differentially regulated due to the markedly different lactate transport kinetics in erythrocytes and myocytes (Poole, Cranmer et al. 1990). Whether there are multiple isoforms of the lactate transporting protein in skeletal muscle remains to be investigated.

If the kinetics of lactate transport are altered due to endurance training and this is mediated primarily by an enhanced ability to transport lactate, then this may be important for physiological regulation of whole body lactate dynamics. An increase in the trained animal's ability to achieve a higher rate of lactate transport for a given lactate concentration could translate into an increased capacity for lactate removal from the cell when lactate production is high intracellularly. Or conversely, an enhanced ability to remove lactate from the circulation into the tissues for oxidation and/or glyconeogenesis when systemic lactate concentrations are elevated. Interestingly, Donovan and Pagliassotti (Donovan and Pagliassotti 1989; Donovan and Pagliassotti 1990) recently reported that endurance training enhanced the efficiency of lactate removal. This conclusion was based on experiments in which lactate was exogenously infused in increasing quantities into trained and control animals and the lactate clearance was measured. These data indicate that with

progressively increasing rates of lactate infusion, the systemic lactate concentrations of the sedentary rats increased at a rate approximately twofold greater than the concentration in the endurance trained rats, indicating a much improved ability of the trained rats to dissipate a lactate load. The removal rates in these studies (Donovan and Pagliassotti 1990) were saturable with respect to increasing concentrations of exogenously infused lactate. When they expressed their data as a Lineweaver-Burke plot it was found that the 'Vmax' of the system was not changed with training, but there was a dramatic reduction in the 'Km' for this process from 12mM to 4mM, indicating an increase in the rate of lactate disposal but no change in the maximal capacity of the system due to training. Of course, the enhanced clearance of lactate due to training in these studies could be attributed to an alteration in the lactate 'handling ' capacity of a number of different organs (liver, cardiac muscle,skeletal muscle, blood). However, it is interesting that the changes occurring at the level of the skeletal muscle membrane (present study) are mirrored by changes at the whole body level for altered lactate dynamics due to endurance training. The muscles' lactate transporting capacity may therefore be an important determinant of whole body lactate kinetics because skeletal muscle, due to its total mass and rich perfusion, is the primary consumer of lactate (Brooks and Gaesser 1980; Jorfeldt 1970).

In this study both the sedentary and the endurance trained rats were acutely exercised 24 hrs before they were killed (see Methods). This approach was used to provide an experimental control for any residual effects of the last exercise bout. This may be of some concern because acute exercise can have profound effects on the membrane transport of solutes and ions (Wallberg-Henriksson 1987; Lindiger, Heigenhauser et al. 1987). For example, glucose transport in muscle is enhanced

for a prolonged period after cessation of the contractile stimulus after a single bout of contractile activity (Ivy and Holloszy 1981). In fact, the effects of training on glucose transport in muscle have been attributed to the effect of the last training bout in several studies (Ivy and Holloszy 1981). Therefore, such a residual effect of exercise indicates that considerable care is necessary to dissociate the effects of training from the effects of an acute bout of activity. Therefore, the increased rate of lactate transport in trained animals represent a 'true' training-induced effect on lactate transport and not a residual effect from the last exercise bout.

In summary, the capacity of the muscle membrane to transport lactate is enhanced as a result of endurance training. However, this is only observed at a concentration of lactate that is considerably lower than the maximal transport capacity for this substrate. It is speculated that this alteration in lactate transporting capacity may reflect an alteration in the Km but not the Vmax of the lactate transport system in skeletal muscle membranes.

Section 5 SUMMARY

At the outset of this thesis it was stated that several aspects of mammalian skeletal muscle lactate physiology would be addressed, focusing on the idea that lactate is an important intermediary metabolite, as opposed to a fatigue-inducing end product.

In the initial studies the widely believed premise that glycogenolysis and lactate production only occur when a muscle is vigorously contracting was tested. In these studies it was found that a reduction in muscle glycogen and a subsequent accumulation of lactate occurred in skeletal muscle which was not performing contractile work. Using a rigorous muscle EMG monitoring system, it was shown that the activity of these 'non-exercising' muscles was less than the activity seen under normal resting conditions. In addition, even though lactate accumulated in these muscles, the glycogen decrement could not be explained by the accumulation of glycolytic intermediates in the muscle. It is therefore postulated that the mobilized glycogen is converted into lactate units which efflux from the muscle to join the oxidizable/gluconeogenic carbon pool in the circulation. The conventional view would be that lactate effluxed into the circulation would be cleared by the liver for hepatic gluconeogenesis, however, the contribution of lactate as an oxidative or glyconeogenic substrate source for other muscles must also be considered (Talmadge, Scheide et al. 1989; Jorfeldt 1970; Mazzeo, Brooks et al. 1986).

Lactate conversion to glycogen (glyconeogenesis) in skeletal muscle has been a topic of some debate among muscle physiologists for many years, firstly as to whether this process actually occurs in mammalian muscle, and secondly whether it is a significant metabolic process in muscle (Johnson and Bagby 1988; Stevenson, Mitchell et al. 1987). Quantitative evidence provided in the present studies reveals that glyconeogenesis does occur at different rates in the three biochemically distinct types of skeletal muscle (FG>FOG> SO), confirming previous reports (McLane and Holloszy 1979). In addition, direct comparisons of the use of lactate as a precursor for glycogen restoration compared to glucose were made in the present studies. It was found that glucose was a far more preferable substrate for glycogen synthesis than lactate in the normal physiological range of substrate concentrations.Only in the FT muscle is lactate a physiologically meaningful precursor for muscle glycogen synthesis.

Hormones which affect carbohydrate metabolism in muscle and gluconeogenesis in liver did not affect glyconeogenesis in muscle. It was observed that glyconeogenesis in muscle was enhanced when external pH was acidic (pH 6.5). Interestingly, in the present studies, lactate transport was also stimulated by acidic external pH. It is therefore appealing to consider the possibility that delivery of lactate into the cell may be an important regulatory step in the glyconeogenic pathway. Thus, the remaining challenge is to ascertain whether there is any other physiological regulation of glyconeogenesis in muscle and also to delineate the enzymatic pathway involved in lactate conversion to glycogen.

Because of the known detrimental physiologic effects of lactate accumulation in any tissue compartment (Heisler 1989; Izutsu 1972) it appeared possible that the transmembrane flux of the hydrophilic lactate ion might be a crucial aspect of lactate metabolism, particularly in skeletal muscle because of the large shifts in glycolytic flux and lactate production which can occur. In view of the above considerations the permeability of skeletal muscle to the lactate ion was investigated.

When these studies were undertaken very little information was available concerning the flux of the lactate ion across membranes in the absence of metabolizing conditions, which obscure the interpretation of initial rate measurements. Therefore, lactate transport in purified plasma membrane vesicles was characterized. These studies revealed that the mechanism of lactate transport in muscle displayed the classic criteria for a carrier mediated transport system, showing saturability with respect to L-lactate, stereospecificity, inhibition by other monocarboxylic acids and by a protein modifying compound, and marked pH sensitivity. Lactate uptake studies carried out on intact muscle strips displayed similar characteristics to the isolated membrane studies with regard to the mechanism of lactate traversal of the muscle cell membrane. The similar nature of the lactate uptake characteristics in the isolated membrane vesicles and the intact muscle strips demonstrates the utility of the muscle strip preparation for obtaining an index of lactate transport in an intact cell preparation.

Despite the diversity in function that exists in hepatocytes, erythrocytes, cardiac cells, and skeletal muscle, the nature of lactate transport in these tissues shows a remarkable degree of similarity, possibly suggesting the presence of a single lactate transporting protein or a family of related, tissue-specific proteins responsible for lactate transport. Molecular characterization of the lactate transport protein should elucidate these questions.

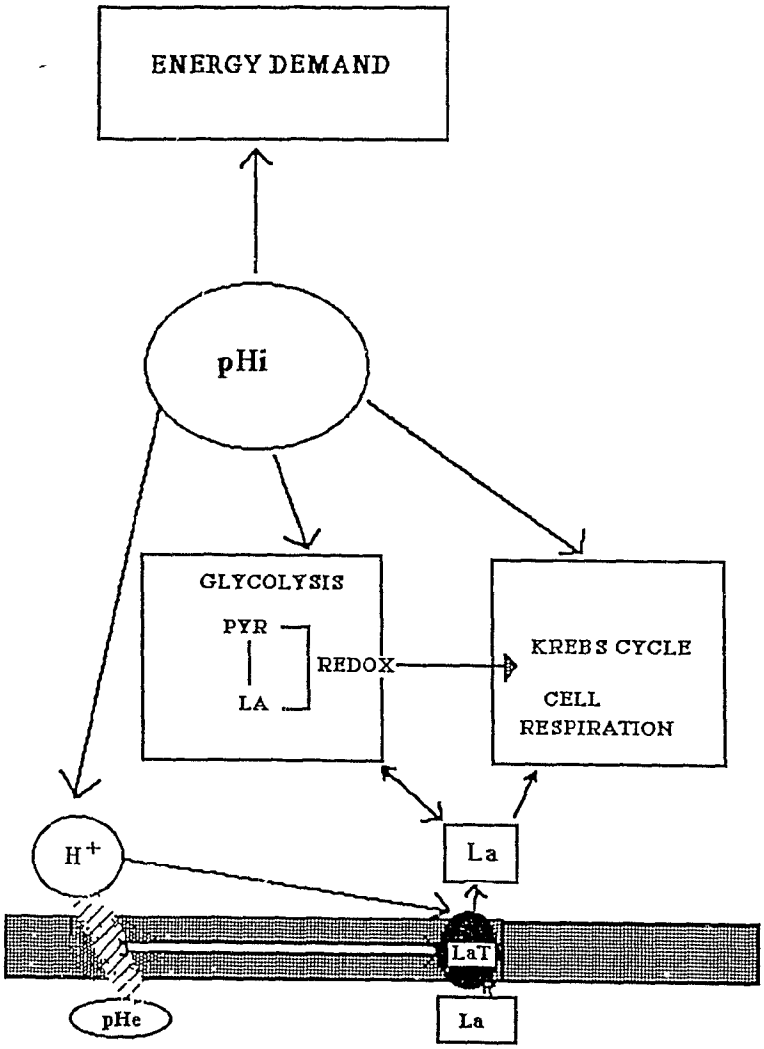
The next important question to be answered is that of the manifold nature of pH in regulating a multitude of metabolic events. With the increasing appreciation of the role of pH as a metabolic signal, the complex nature of how the mass transport of solutes is co-ordinated with intracellular metabolism to maintain reasonable cellular constancy has begun to be addressed (Busa and Nuccitelli 1984; Roos and Boron 1981; Boutilier and Ferguson 1989). From the present studies it is interesting to consider the role that pH might play in the co-ordination of glycolytic flux and lactate transport.

In the present studies it has consistently been observed that a proton gradient markedly stimulates the vectorial flux of L-lactate. The dependence of lactate transport on a proton gradient is not without precedence as studies on other tissues have also also shown a marked increase in lactate flux when confronted with lactate and proton gradients (Balkovetz, Leibach et al. 1988). This mechanism of proton coupled symport for various organic solutes is being recognized as a recurring motif of regulation in several mammalian transport systems.

The question is; how does the muscle cell maintain intracellular lactate concentration within tolerable limits in the face of markedly varying rates of lactate production and transport ? Clearly, if the production (glycolysis) and transport mechanisms for lactate operate independently and in an uncoordinated manner, it is difficult to see how the intracellular lactate concentration can be homeostatically regulated. Obviously some form of 'cross talk' between the two processes would be necessary to co-ordinate their behaviour.

Interactions between intracellular pH and metabolic transitions have been observed (Trivedi and Danforth 1966). Ionization of the active sites on many ezymes has been shown to alter substrate binding and catalytic properties of the enzyme. Intracellular pH has been shown to have an important regulatory role at key loci in the glycolytic pathway, primarily by its allosteric effects on key rate limiting enzymes (Trivedi and Danforth 1966) , and a pH gradient is also a potent regulator of lactate transport (present work, Roth and Brooks 1990a,b). Therefore, a speculative hypothesis can be proposed in which both processes may be regulated by the pH status of the intracellular and extracellular milieu to control the production (glycolysis) and efflux/influx mechanisms simultaneously in a co-ordinated manner (Fig. 1).

Figure.1


ENERGY DEMAND
pHi
GLYCOLYSIS
PYR
LA
REDOX
KREBS CYCLE
CELL
RESPIRATION
H+
La
LaT
pHe
La

If one pursues this hypothesis the following scenario can be considered; the muscle cell is contracting vigorously and glycolytic flux is high, the intracellular concentrations of lactate and H^+ are increasing exponentially to levels which would endanger cell integrity. One could then predict that two mechanisms would be simultaneously mobilized by the 'pH signal' to inhibit a further build up of these potentially hazardous metabolic by-products : a) the decrease in pHi due to the increased H^+ would inhibit key glycolytic enzymes to sharply attenuate the production of lactate and H^+, and b) the outwardly directed pH gradient would simultaneously stimulate the lactate transport system to efflux lactate and H^+ into the extracellular compartment . Thus, a return towards constancy of the intracellular milieu is achieved, by the co-ordinated effects of pH on reducing lactate production and increasing lactate efflux. As our appreciation of the true complexity of how metabolism is regulated advances, the interdependence of solute flux and metabolism must be considered and the regulatory signals delineated.

In closing, the present studies were aimed at identifying the nature of lactate as an intermediary metabolite in skeletal muscle cells. In particular, the idea that lactate can be produced when anaerobic metabolism is not predominant is rapidly gaining credence, and is supported in this work. The metabolic role that lactate might play under these conditions may well be dissociated from its role in muscle fatigue. The potential of lactate as a precursor for glycogen synthesis was assessed and found to be greatest in the FT muscle, supplying approximately 25-35% of the substrate for glycogen synthesis compared to the

contribution of glucose to this process. External pH altered the glyconeogenic rate in muscle, however further physiologic regulation of glyconeogenesis by the hormonal milieu was not observed. Experiments using isolated membrane vesicles and intact muscle strips revealed that the transmembrane movement of the lactate ion appears to be mediated by a specific carrier, which shares common features with the lactate carrier from several other tissues. Finally, it is speculated that the diverse effects of pH on many aspects of cell metabolism and transport suggest that the 'pH signal' may serve to integrate cell metabolism and solute transport.

APPENDIX 1 Biochemical assays for glycolytic intermediates in muscle and blood.

These assays are based on the enzymatic assay procedures of Bergmeyer (1965). The adaptation of these assays to the analysis of muscle metabolites has been carried out in several laboratories. The following assay procedures are therefore a combination of procedures generously forwarded by the research groups of Dr. T. Graham, University of Guelph; Dr. H Green, University of Waterloo; and this laboratory.

METABOLITE EXTRACTION

A. Reagents

3.0M PCA	129.1 ml 70% PCA bring to 500 ml with water store 0-4°C 1 month
2.2M	2.2g $KHC0_3$ add 10.0ml water (make fresh daily)

B. PROCEDURE

1. Freeze dry tissue for 5hrs at -50°C.
2. Store -80°C under a vacuum if the tissue is not to be used immediately.
3. Remove tissue from vacuum.
4. Place in preweighed microcentrifuge tube and weigh.
5. Place tubes in an ethanol bath -5°C.
6. Add 150uL of precooled 3.0 M PCA.

7. Extract for 20 min vortexing several times.

8. To the supernatant add 300uL of 2.2M $KHC0_3$ and vortex until bubbling stops.

9. Centrifuge for 20 min 0°C at 1500 G.

10. Remove 400uL of the supernatant and place at -80°C to refreeze. Use this extract for the appropriate muscle metabolite assays.

Muscle Pyruvate

Lactate Dehydrogenase

Pyruvate + NADH ----------> Lactate + NAD

	Reagent	Stock Conc	Final Conc	Volume for (ML) 25	50	100
1.	Phosphate Buffer	50.0mM	50.0mM	25.00mL	50.00mL	100.00mL
2.	NADH	15.0mM	5.0uM	5.00uL	10.00uL	15.00uL
3.	LDH B&M 106 984	1250U/mL	0.06U/mL	See Procedure		

Note: Add reagents 1 to 2. Bring to volume with distilled water, adjust to pH 7.0.

PREPARATION OF DILUTE ENZYME

1. Add 10 uL of LDH (to a 12*75 mm test tube containing 2.0 mL reagent. Mix by inversion.

PREPARATION OF 50mM PHOSPHATE BUFFER pH 7.0

1. Na_2HPO_4 FW 141.96 426mg
   KH_2PO_4 FW 136.09 272mg
   Bring to 100mL with water
   pH to 7.0, make fresh daily.

PROCEDURE FOR ASSAY

1. Use 3 10*75 test tubes for each sample. Also include 3 tubes labeled STD and 3 tubes labeled BLK.

2. Add 50 uL of sample to appropriately labeled tubes.

3. Add 50 uL of water to tubes labeled BLK.

4. Add 50 uL of 10.0 uM pyruvate standard to tubes labeled STD.

5. Add 1.0 mL dilute reagent to all tubes. Vortex and wipe.

6. Read against quinine standard 5 (R1).

7. Add 10 uL of dilute LDH to all tubes. Vortex and wipe.

8. Place in dark for 20 minutes.

9. Read against quinine standard 5 (R2).

MUSCLE LACTATE

LDH

Lactate + NAD ----------> Pyruvate + NADH

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

Reagent	Stock Conc	Final Conc	Volume for (ML) 25	50	100
1. Hydrazine	1.00M	100.0mM	2.50mL	5.00mL	10.00mL
2. Glycine	1.00M	100.0mM	2.50mL	5.00mL	10.00mL
3. NAD	100.0mM	0.5mM	125.00uL	250.00uL	500.00uL
4. LDH	1250u/mL	8U/mL	See Procedure		

Note: Add reagents 1 to 3 . Bring to volume with distilled water adjust to pH 10.0

Preparation of dilute enzyme

1. Add 250 uL of LDH to a 12*75 mm test tube containing 1.0 mL reagent. Mix by inversion.

Procedure for assay

NB Due to the high concentration of lactate in perspiration, extreme care must be taken when handling pipette tips, cuvettes and test tubes.

1. Use 3 10*75 tubes for each sample. Also include 3 tubes labeled STD and 3 tubes labeled BLK.

2. Add 10.00 uL of sample to appropriately labeled tubes.

3. Add 10.00 uL of water to tubes labeled BLK.

4. Add 10.00 uL of 500.00uM lactate standard to tubes labeled STD.

5. Add 1.0mL dilute reagent to all tubes. Vortex and wipe.

6. Read against quinine standard 3 (R1).

7. Add 25 uL of dilute LDH to all tubes. Vortex and wipe.

8. Place in dark for 60 min.

9. Read against quinine standard 3 (R2).

FRUC-1,6-P, DHAP AND GYLCERALDEHYDE -3-P

Fructose-1,6-P ---(Aldolase)---> DHAP + Glyceraldehyde-P

DHAP ---(Triose-P-Isomerase)---> Glyceraldehyde-P

Glyceraldehyde -P + NAD ---(GAP-Dehydrogenase)---> 3-P Glycerate + NADH

Reagent	Stock Conc	Final Conc	Volume for (ml) 25	50	100
1. Imidazole	1.00M	50.0mM	1.25mL	2.50mL	5.00mL
2. NAD	100mM	100uM	25.00uL	50.00uL	100.00uL
3. $Na_2H_2SO_4$	1.00M	1.0mM	25.00uL	50.00uL	100.00uL
4. EDTA	200mM	1.0mM	125.00uL	250.00uL	500.00uL
5. 2-Mercapto-ethanol	14.30M	2.0mM	3.50uL	7.00uL	15.00uL
6. Glyceralde-hyde-P-DH B&M105 694	800U/mL	0.8U/mL	See Procedure		
7. Triose-P-Isomerase B&M109 762	61350U/mL	2.4U/mL	See Procedure		
8. Aldolase	90U/mL	0.02U/mL	See Procedure		

NOTE: Add reagents 1 to 5. Bring to volume with distilled water, adjust to pH 7.5

PREPARATION OF DILUTE ENZYME

1. Add 100 uL of glyceraldehyde-p-dehydrogenase (GAP-d-H) to a 12*75 mm test tube containing 1.0 mL reagent. Mix by inversion. .

2. Add 5 uL of triose-p-isomerase to a 12*75 mm test tube containing 1.0mL reagent. Mix by inversion.

3. Add 5 uL of aldolase to a 12*75 mm test tube containing 2.0 mL reagent. Mix by inversion.

PROCEDURE FOR ASSAY

1. Use 3 10*75 test tubes for each sample. Also include 3 tubes labeled STD and 3 tubes labeled BLK.

2. Add 75 uL of sample to appropriately labeled tubes.

3. Add 75 uL of water to tubes labeled BLK.

4. Add 75 uL of 2.0 uM F-1,6-P standard to tubes labeled STD.

5. Add 1.0 mL dilute reagent to all tubes. Vortex and wipe.

6. Read against quinine standard 5 (R1).

7. Add 10 uL of dilute GAP dehydrogenase to all tubes. Vortex and wipe.

8. Place in dark for 15 minutes.

9. Read against quinine standard 5 (R2).

10. Add 10 uL of dilute Triose-P-Isomerase to all tubes. Vortex and wipe.

11. Place in dark for 15 minutes.

12. Read against quinine standard 5 (R3).

13. Add 10 U of dilute aldolase to all tubes. Vortex and wipe.

14. Place in dark for 15 minutes.

15. Read against quinine standard 5 (R4).

Tecnical note. In the calculations remember that 1 mole of F-1,6-P converted to glycerol-3-phosphate oxidizes 2 mole of NADH to NAD.

MUSCLE GLUCOSE, G-6-P, G-1-P, F-6-P

Glucose + ATP ---(Hexokinase)---> Glucose-6-P + ADP

Fructose-6-P + ATP ---(Phosphoglucoisomerase)---> Glucose-6-P + ADP

Glucose-1-P + ATP ---(Phosphoglucomutase)---> Glucose-6-P + ADP

Glucose-6-P + NADP ---(G-6-P-DH)---> Glucanolactone +NADPH

Reagent	Stock Conc	Final Conc	Volume for (ML) 25	50	100
1. TrispH8.1	1.0M	50.0mM	1.25mL	2.50mL	5.0mL
2. Mg Cl_2	1.00M	1.0mM	25.00uL	50.00uL	100.00uL
3. D.T.T.	00.50M	00.5mM	25.00uL	50.00uL	100.00uL
4. ATP	300.0mM	300.0uM	25.00uL	50.00uL	100.00uL
5. NADP	50.0mM	50.0uM	25.00uL	50.00uL	100.00uL
6. EDTA	200mM	100uM	12.50uL	25.00uL	50.00uL
7. G-6-P-DH B&M 127 035	350U/mL	0.02U/mL	See Procedure		
8. P-G-M B&M 108 375	400U/mL	0.065U/mL	See Procedure		
9. P-G-I B&M 128 139	3500U/mL	0.35U/mL	See Procedure		
10. B&M 127 175	Hexokinase	280U/mL	00.14U/mL	See Procedure	

NOTE: Add reagents 1 to 6. Bring to volume with distilled water, adjust to pH 8.0
B&M Boehringer-Mannheim, all other chemicals from Sigma chemicals.

PREPARATION OF DILUTE ENZYME

1. Add 10uL of glucose-6-Pase (G-6-P ase) to a 12*75 mm test tube containing 1.5 mL reagent. Mix by inversion.

2. Add 20uL of phosphoglucomutase (PGM) to a 12*75 mm test tube containing 1.0 mL reagent. Mix by inversion.

3. Add 10uL of phosphoglucosisomerase (PGI) to a 12*75 mm test tube containing 1.0mL reagent. Mix by inversion.

4. Add 25 uL of hexokinase to a 12*75 mm test tube containing 1.0 mL.

PROCEDURE FOR ASSAY

1. Use 3 10*75 tubes for each sample. Also include 3 tubes labeled STD and 3 tubes labeled BLK.

2. Add 100.0 uL of sample to appropriately labeled tubes.

3. Add 100.0 uL of water to tubes labeled BLK.

4. Add 100.0 uL of 5.0 µM G-6-P/10.0 µM glucose standard to tubes labeled STD.

5. Add 1.0mL dilute reagent to all tubes. Vortex and wipe.

6. Read against quinine standard 5 (R1).

7. Add 10 uL of dilute G-6-P-DH to all tubes. Vortex and wipe.

8. Place in dark for 15 minutes.

9. Read against quinine standard 5 (R2).

10.Add 10 uL of dilute P-G-M to all tubes. Vortex and wipe.

11.Place in dark for 15 minutes.

12.Read against quinine standard 5 (R3).

13.Add 10 uL of dilute P-G-I to all tubes. Vortex and wipe.

14.Place in dark of 15 minutes.

15.Read against quinine standard 5 (R4).

16.Read against quinine standard 2 (R5)

17.Add 25 uL of dilute hexokinase to all tubes. Vortex and wipe.

18.Place in dark for 15 minutes.

19.Read against quinine standard 2 (R6).

CALCULATIONS FOR MUSCLE METABOLITES

Tissue weight	= 4.0 mg
Concentration of STD.	= 89.72 uM/L
Extraction concentration	= 4.0mg *1.6 mg/mL
Volumn of sample	= 10 uL
Delta STD	=115 (^STD)
Delta sample	= 68 (^sample)
Delta blank	= 52.2 (^B)

CALCULATION

$$\frac{\text{\^{}Sample-\^{}B}}{\text{\^{}STD-\^{}B}} * \text{Conc of STD (umol/L) / Extract concentration (g/L)} = \mu\text{mol/g}$$

$$\frac{68 - 52.2}{115 - 52.2} * 89.72 / 6.4 = 3.52\ \mu\text{mol/g}$$

HYDROLYSIS OF TISSUE FOR GLYCOGEN ASSAY

Reagents

2.0N HCL	To 83 mL water add 17 mL 37% HCL
2.0N NaOH	To 8 gm NaOH add 100mL water

PROCEDURE

1. Use frozen tissue or tissue residue from perchloric acid extract.
2. Add tissue to a 1.5 microfuge tube. (Not to exceed 10mg dry weight).
3. Add 0.750mL 2N HCL and flatten sample to increase surface area.
4. Record weight of each tube.
5. Place at 100°C for 2 hours. Mix after the first hour.

6. Reweigh each tube and add water to achieve initial weight.
7. Add 0.750mL 2N NaOH and mix well.
8. Store -90°C until analysis.

MUSCLE GLYCOGEN

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

$$\text{Glucose-6-P + NADP} \xrightarrow{\text{G-6-P-DH}} \text{Glucanolactone + NADPH}$$

Reagent	Stock Conc	Final Conc	Volume for (ml) 25	50	100
1. Tris pH 8.1	1.00M	50.0mM	1.25mL	2.50mL	5.00mL
2. Mg C12	1.00M	1.0mM	25.00uL	50.00uL	100.00uL
3. D.T.T.	00.50M	00.5mM	25.00uL	50.00uL	100.00uL
4. ATP	300.0mM	300.0uM	25.00uL	50.00uL	00.00uL
5. NADP	50.0mM	50.0uM	25.00uL	50.00uL	100.00uL
6. G-6-P-DH B&M 127 035	350U/mL	00.02U/mL	2.50uL	5.00uL	10.00uL
7. Hexokinase B&M 127 175	280U/mL	00.14U/mL	See Procedure		

NOTE: Add reagents 1 to 5. Bring to volume with distilled water, adjust to pH 8.1 and add reagents 6.

PREPARATION OF DILUTE ENZYME

1. Add 25 uL of Hexokinase to a 12*75 mm test tube containing 1.0 mL reagent. Mix by inversion.

PROCEDURE FOR ASSAY

1. Use 3 10*75 tubes for each sample. Also include 3 tubes labeled STD and 3 tubes labeled BLK.
2. Add 10.00 uL of sample to appropriately labeled tubes.
3. Add 10.00 uL of water to tubes labeled BLK.
4. Add 10.00 uL of 500.00 uM glucose standard to tubes labeled STD.
5. Add 1.0 mL dilute reagent to all tubes. Vortex and wipe.
6. Read against quinine standard 2 (R1).
7. Add 25 uL of dilute hexokinase to all tubes. Vortex and wipe.
8. Place in dark for 60 minutes.
9. Read against quinine standard 2 (R2).

CALCULATIONS FOR MUSCLE GLYCOGEN

Tissue weight = 4.0 mg

Concentration of STD. = 507.0uM/L

Extract volume = 1.0 mL (750 uL 1.0N HCL + 750 uL 1.0N NaOH)

Extract concentration = 4.0 mg/1.5 mL (2.67 mg/mL or 2.67 g/L)

Volume of sample = 10 uL

Delta STD = 38.5 (^STD)

Delta sample = 83.7 (^sample)

Delta blank = 0.9 (^B)

CALCULATION

$$\frac{\text{^Sample - ^B}}{\text{^STD - ^B}} * \text{conc of STD (µmol/L) / extract concentration (gm/L)} = \text{µmol/gm}$$

$$\frac{83.7 - 0.9}{38.5 - 0.9} * 507.0 / 2.67 = 279.12 \text{ uM glucosyl units/gm tissue}$$

BLOOD METABOLITE SAMPLING

INITIAL PREPARATION OF BLOOD

1. Add 0.5 ml (500 ul) of cold 0.6M Perchloric Acid into a microfuge tube, keep on ice.

2. Add 0.1 ml (100ul) of whole blood. Mix and cool on ice.

3. Centrifuge at 15,000 for 2 min. in a refrigerated Eppendorf microfuge (0-4°C).

4. Add 0.25 ml (250ul) of 1.25M $KHC0_3$ (made fresh daily) and let sit for 10 min. at 0-4°C.

5. Centrifuge at 1500g for 2 min. in a refrigerated Eppendorf microfuge (0-4°C).

6. Remove supernatant and store frozen in an appropriately labeled microfuge tube.

STABILITY OF FROZEN SUPERNATANT

1. Pyruvate / 14 days / will polymerize on prolonged storage.

2. Lactate / 3 months.

3. Glucose / 3 months.

4. Glycerol / 3 months.

5. Alanine / 3 months.

6. B-hydroxybutyrate / 14 days

REAGENTS

1. 1.25M KHC03	1.25 gm KHC03 in a 16•100mm tube. Add 10 mL water, mix well (fresh daily).
2. 0.6M PCA	51.5 mL 70% Perchloric Acid. Bring to 1000 mL, store 0°C two months.

BLOOD GLUCOSE

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

$$\text{Glucose -6-P + NAD} \xrightarrow{\text{G-6-P-DH}} \text{Glucanolactone + NADPH}$$

Reagent	Stock Conc	Final Conc	Volume for (ML) 25	50	100
1. Tris pH 8.1	1.00M	50.0mM	1.25mL	2.50mL	5.00mL
2. Mg Cl_2	1.00M	1.0mM	25.00uL	50.00uL	100.00uL
3. D.T.T.	00.50M	00.5mM	25.00uL	50.00uL	100.00uL
4. ATP	300.0mM	300.0uM	25.00uL	50.00uL	100.00uL
5. NADP	50.0mM	50.0uM	25.00uL	50.00uL	100.00uL
6. G-6-P-DH BM 127 035	350U/mL	00.02U/mL	2.50uL	5.00uL	10.00uL
7. Hexokinase BM 127 175	280U/mL	00.14U/mL	See Procedure		

NOTE: Add reagents 1 to 5. Bring to volume with distilled water, adjust to pH 8.1 and add reagent 6.

PREPARATION OF DILUTE ENZYME

1. Add 25 uL of Hexokinase to a 12*75 mm test tube containing 1.0 mL reagent. Mix by inversion.

PROCEDURE FOR ASSAY

1. Use 3 10*75 tubes for each sample. Also include 3 tubes labeled STD and 3 tubes labeled BLK.

2. Add 25.00 uL of sample to appropriately labeled tubes.

3. Add 25.00 uL of water to tubes labeled BLK.

4. Add 25.00 uL of 500.00 uM Glucose standard to tubes labeled STD.

5. Add 1.0 mL dilute reagent to all tubes. Vortex and wipe.

6. Read against quinine standard 2 (R1).

7. Add 25 uL of dilute Hexokinase to all tubes. Vortex and wipe.

8. Place in dark for 60 minutes.

9. Read against quinine standard 2 (R2).

BLOOD METABOLITE CALCULATIONS

1. Volume of whole blood
   = 100 uL

2. Volume of PCA = 500 uL

3. Volume of KHC03 = 250 uL

4. Total Volume = 850 uL

5. Dilution of Blood = 8.5:1

6. ^R Sample = R2-R1=10

7. ^R Standard = R2-R1=15

8. ^R Blank = R2-R1=2

9. Conc of Standard = 0.555mM/L

CALCULATION

$$\frac{(\text{^R Sample}) - (\text{^R Blank})}{(\text{^R Standard}) - (\text{^R Blank})} * \text{Conc. Standard (mM)} * \text{Dilution} = \text{Conc. mM}$$

$$\frac{10 - 2}{15 - 2} * 0.555 * 8.5 = 2.9\text{mM}$$

APPENDIX 2 Calculations.

Calculations for tissue uptake of substrate

Convert CPM's to DPM's by efficiency factor, eg. if counting efficiency is 40% then;

$$\frac{\text{CPM/mg protein}}{1} * \frac{100}{40} = \text{DPM/ mg protein} \qquad (1)$$

$$\frac{\text{DPM/ mg protein}}{\text{DPM's in buffer}} * \frac{\text{mmoles of substrate in buffer}}{1} = \text{mmoles / mg protein} \qquad (2)$$

Calculation for correcting the intracellular substrate concentration for the extracellular space contribution.

$$\text{Tissue space (ml / mg protein)} = \frac{\text{muscle [S] (mmol/mg prot)}}{\text{buffer [S] (mmol/ ml)}} \qquad (3)$$

Where, [S] is the concentration of the substrate or extracellular space marker.

A tissue space (T) is calculated for both the substrate (S) and the extracellular space marker (ESM).

$$\text{Intracellular space (ml/mg protein)} = TS - TESM \qquad (4)$$

Where TS is the tissue space for the substrate and TESM is the tissue space for the extracellular space marker.

To calculate the uptake of a substrate correcting for the extracellular space for different concentrations of substrate;

$$\text{Intracellular [S]} \text{ (nmol/ml int water)} = \frac{\text{TS (ul/mg protein)} * \text{buffer [S] (nmol/ml)}}{\text{(Total tissue water (ul/mg prot) - TES (ul/mg prot)}} \quad (5)$$

Where; S is the substrate concentration, TS is the tissue substrate space, [S] is the buffer substrate concentration; TES is the tissue extracellular space; and Total tissue water is calculated by the Σ of ; Intracellular water content + extracellular water content (ul/mg protein).

To convert substrate concentrations from nmol/ml intracellular water into nmol/mg protein. Use values from equations 5 and 3 in this calculation.

$$\frac{\text{nmol}}{\text{ml}} * \frac{\text{ml}}{\text{mg prot}} = \text{Intracellular concentration (nmol/mg protein)} \quad (6)$$

Calculation for glyconeogenesis/ glycogenesis

$$\frac{\text{DPM in MGP/ mg protein}}{\text{DPM in buffer}} * \frac{\text{mmol GS in buffer}}{1} = \text{mmol GS / mg protein)} \quad (7)$$

Where; MGP is the muscle glycogen pool; GS is the glyconeogenic / glycogenic substrate

Glyconeogenic/ glycogenic rate calculation

$$\frac{\text{DPM in MGP/mg protein}}{\text{DPM in buffer}} * \frac{\text{mmol GS in buffer}}{1} * \frac{1}{\text{IT (min)}} = \text{mmol GS / mg prot / min} \quad (8)$$

Where; MGP is the muscle glycogen pool; GS is the glyconeogenic substrate; IT is the incubation time.

Calculation of free acid concentrations using the Hendersson-Hasselbach equation

$$pH = pK_a + \log \frac{[A]}{[HA]} \quad (9)$$

eg. If pH = 5.5 and pK_a of the acid 4.76

$$\text{Log} \frac{[A]}{} = 5.5\text{-}4.76$$

$$\text{Log}\frac{[A]}{[HA]} = .74$$

$$\frac{\text{moles/l A}}{\text{moles/l HA}} = 5.5$$

Therefore, the ratio of anion to acid is 5.5, ie. 5.5M A^- to 1M HA and therefore, in solution a molar concentration of the acid would be 84.6% dissociated under the stated conditions.

For lactic acid (pK_a3.86) at pH 7.4 (20°C) the ratio (using the above calculation) would be
3467 A^- : 1 HA, therefore lactic acid would be 99.97 % dissociated.

% Dissociation of lactic acid at 20°C, with varied pH

pH	8.0	7.4	6.5	6.0
% dissociated	99.99	99.97	99.77	99.28

APPENDIX 3 COMPUTER PROGRAMS.

Computer program in BASIC to calculate muscle metabolite concentrations from fluorometric assay procedures.

Note, although this program refers to glycogen or lactate other metabolite concentrations from the enzymatic fluorometric assay procedures can be determined using the same program

```
PRINT "Program to determine the metabolite concentrations"
PRINT "for the Flourometric assays.
PRINT "
PRINT "
PRINT
INPUT "  How many replicates of each sample ";rep
PRINT
PRINT "  Do you want this program to calculate the extract concentrations"
PRINT "  for you (after you enter all the necessary weights) for each sample ?"
INPUT "  Otherwise, you must! (y/n) ";dil$
PRINT
PRINT
PRINT
INPUT " Will you be wanting a printout (y/n) ";prin$
IF prin$="y"THEN INPUT "What was the date of the assay (dd/mm/yy) ";dat$
CLS
INPUT "How many blanks were there ";numbl
FOR i=1 TO numbl
  PRINT
  PRINT "Blank # ";i
  INPUT "Initial reading for blank = ";bli(i)
  INPUT "Final reading for blank = ";blf(i)
  delta(i)= blf(i)- bli(i)
  tot=tot+delta(i)
  delta=0
  NEXT i
E=tot/numbl
PRINT "Therefore the mean of the delta blanks was ";E
```

```
PRINT:PRINT
INPUT "press return to continue ";ret$
10 CLS:count=0
PRINT "****************INputting Standard
Data*****************************"
PRINT
PRINT " 1. What is the substrate being measured :"
INPUT "      a) glycogen      or      b)lactate      (a/b) ";met$
IF met$="a" THEN strate$="Glycogen Assay":GOTO 15
IF met$="b" THEN strate$="Lactate Assay":GOTO 15
GOTO 10
15 PRINT
INPUT " 2. Concentration of standard (umol/l) ";D
PRINT
INPUT " 3. How many standards were there ";stan
FOR j=1 TO stan
PRINT:PRINT "Standard #";j
INPUT "Initial reading ";X(j)
INPUT "Final reading ";Y(j)
NEXT j
IF prin$<>"y" THEN 20
LPRINT "******************Metabolite concentrations for a
";strate$;"**********************"
LPRINT "    date of experiment : ";dat$
LPRINT "    Standard concentration = ";D;" umol/l"
LPRINT " ","----------------------------------------"
LPRINT " ","|Standard and Blank readings:","|"
LPRINT " ","|","initial","final","|"
FOR i=1 TO stan
LPRINT " ","|std #";i,X(i),Y(i),"|"
NEXT i
FOR i=1 TO numbl
LPRINT " ","|blk #";i,bli(i),blf(i),"|"
NEXT i
LPRINT " ","----------------------------------------"
LPRINT
LPRINT "Sample","Dilution of","Initial","Final","Concentration"
```

```
LPRINT "   #","muscle (g/l)","reading","reading","(umol/g ww)"
LPRINT "-------------------------------------------------------------------------
"
20 CLS:count=count+1
PRINT "******************INputting
Data*******************************"
PRINT "[NB. If you want to quit, enter 9999]"
PRINT " ","Sample # ";count:PRINT
IF dil$<>"y" THEN 25
PRINT " 1. Calculation for dilution:
INPUT " What was the wet weight of the sample (mg) ";wet
IF wet=9999 THEN END
INPUT " What was the weight (of sample, HCl, tube) before boiling (g) ";pre
INPUT " What was the weight (of sample, HCl, tube) after boiling (g) ";post
C=wet/(1.5+(post-pre))
25 IF dil$<>"y" THEN INPUT "1. Dilution of muscle sample used in assay
(mg/ml or g/l) ";C
IF C=9999 THEN END
PRINT
FOR i=1 TO rep
PRINT "Replicate # ";i
INPUT "initial reading = ";Q(i)
IF Q=9999 THEN END
INPUT "final reading = ";R(i)
NEXT i
PRINT:LET sum=0:LET tota=0
FOR i=1 TO stan
LET N(i)=Y(i)-X(i)-E
LET sum=sum+N(i)
NEXT i
LET ave=sum/stan
FOR j=1 TO rep
LET H(j)=R(j)-Q(j)-E
LET tota=tota+H(j)
NEXT j
LET aver=tota/rep
LET X1=(aver/ave)*D/C
CLS
```

```
PRINT "******************OUTputting
Data*****************************"
PRINT " ","Sample # ";count:PRINT
PRINT " The concentration of the sample = ";X1;" umol/g ww"
PRINT
IF prin$<>"y" THEN 30
IF rep>numbl THEN LET numbl=rep: IF numbl>=stan THEN stan=numbl:GOTO
25
IF rep>stan THEN LET stan=rep:GOTO 25
25 LPRINT "(";count;")",C," 1 :";Q(1),R(1),X1
IF rep=1 THEN 30
part=rep-1
FOR i=1 TO part
j=i+1
LPRINT " "," ",j;":";Q(j),R(j)
NEXT i
30 PRINT
IF prin$<>"y" THEN INPUT "Press ENTER to continue ";ent$
GOTO 20
END
```

A BASIC program to determine tissue content of radiolabel (CPM/mg protein).

```
PRINT "This program determines CPM/ mg protein.
PRINT " what is the blank?"
INPUT A
PRINT " what is the dilution factor (df) (eg. 300 ul counted from 600ul, df=0.5)
INPUT G
4 PRINT "what is the CPM for the sample"
INPUT B
LPRINT B " cpm"
PRINT "what is the total protein content of the sample (ug)"
INPUT C
LPRINT C " ug total protein "
LET X= B-A
LET Y= X/C
LET W=Y/G
LET H= W*1000
PRINT H"cpm/mg protein"
LPRINT H "cpm/mg prot corrected for the tissue dilution factor"
GOTO 4
```

A BASIC program to determine the extracellular space contribution to substrate uptake in the perfusion and in vitro muscle incubation procedures.

```
PRINT "  This program determines the uptake of a substrate correcting for the"
PRINT "  extracellular space."
PRINT "**You will need the following data to use this program:"
PRINT "    a) sample conc. of the substrate (eg. lactate) (mmol/mg protein)"
PRINT "    b) sample conc. of the extracellular space marker (mmol/mg protein)"
PRINT " [NB. can substitute uptake/unit wt. in (a) and (b)  (mmol/mg wt) in above
calculations]"
PRINT:INPUT "What was the medium concentration of the substrate (mmol/ml)
";B:PRINT
INPUT "Will you be wanting a printout (y/n) ";prin$
IF prin$="y" THEN INPUT "What was the date of the experiment (dd/mm/yy)
";dat$
IF prin$<>"y" THEN 10
LPRINT
LPRINT "******** Determining the uptake of a substrate correcting for the
extracellular space *********"
LPRINT "  date of experiment : ";dat$
LPRINT "  {The medium concentration was ";B;" mmol/ml}
LPRINT
LPRINT "|Mus. content","|Mus. content","| ex. space","| in. space","|tot
tissue","|concentration"
LPRINT "|substrate","|ex sp marker","| ul/mg prot.","| ul/mg prot.","|  water","|
substrate"
LPRINT "|mmol/mg prot","|mmol/mg prot","| ","| ","|ul/mg prot.","|nmol/mg
prot."
LPRINT "----------------------------------------------------------------------------
-----"
10 CLS
PRINT "**********************INputting
Data*****************************"
PRINT" [NB. If you want to quit, enter 9999 as the muscle content of substrate]"
PRINT
```

```
INPUT " 1. What is the muscle content of substrate (mmol/mg protein) ";A
PRINT:IF A=9999 THEN END
PRINT " 2. What is the muscle content of extracellular space marker "
INPUT "    (mmol/mg protein) ";C
LET K=A/B
LET L=C/B
LET S=L*1000
LET M=K-L
LET N=M*1000
LET Z=N+S
LET O=B*1000000!
LET Q=L*1000
LET P=(N*O)/(Z-Q)
LET X=1/M
LET R=P/X
PRINT
PRINT "**********************OUTputting
Data*************************"
PRINT
PRINT "==> The extracellular space is ";L;" ml/mg protein"
PRINT "                                or ";S;" ul/mg protein"
PRINT
PRINT "==> The intracellular space is ";N;" ul/mg protein"
PRINT
PRINT "==> The total tissue water is ";Z;" ul/mg protein"
PRINT
PRINT "==> The concentration of substrate is ";R;" nmol/mg protein"
PRINT
PRINT
PRINT
IF prin$<>"y" THEN 20
 LPRINT USING "| ##.####^^^^ ";A,C,S,N,Z,R
20 INPUT "Press ENTER to continue ";ent$
GOTO 10
END
```

A BASIC program for determining tissue protein content from the assay of Bradford or Lowry.

```
PRINT "Program for the calculation of proteins from STD curve."
PRINT "**The following is needed to run this program:"
PRINT "   1. regression equation for the standard curve (From Cricket graph or
PRINT other) of the form y = a + bx where a is the y intercept and b is the beta
PRINT coefficient for x "
PRINT "   2. OD's for samples."
PRINT "NOTE--When determining the std curve on Cricket Graph use"
PRINT "          y axis = concentration of STDs, x axis = ODs"
PRINT "      --If any of the ODs for the samples are out of the standard"
PRINT "          range this procedure is invalid because the assay is only"
PRINT "          linear over a narrow concentration range nb- the standard curve
PRINT "should be a  straight line and fitted with an equation of the order y= a+bx
PRINT "where a is the y axis intercept and b is the beta coefficient for x."
PRINT:INPUT "Will you be wanting a printout (y/n) ";prin$
IF prin$="y" THEN INPUT "What was the date of the experiment (dd/mm/yy)
";dat$
CLS:count=0
PRINT "*******************INputting Constant
Data**********************"
PRINT
INPUT " 1. Enter the intercept value for the regression equation ";A
PRINT
INPUT " 2. Enter the beta coefficient for x from regression equation ";B
PRINT
PRINT " 3. Enter the amount of dissolved tissue homogenate"
INPUT "    used in assay (ul) ";D
PRINT
PRINT " 4. Enter the amount of solvent used to dissolve"
INPUT "    the tissue (ul) ";E
PRINT:PRINT:PRINT
LET F=E/D
PRINT " This makes the dilution factor ";F
PRINT:INPUT "Press ENTER to continue ";ent$
```

```
IF prin$<>"y" THEN 10
LPRINT "*****************Calculating proteins from STD
curve***************"
LPRINT " "," "," ","date of experiment : ";dat$
LPRINT "The intercept from the regression equation = ";A
LPRINT "The x coefficient from the regression equation = ";B
LPRINT "The amount of dissolved tissue homogenate used in assay = ";D;" ul"
LPRINT "The amount of solute used to dissolve the tissue = ";E;" ul"
LPRINT "  {Therefore, the dilution factor = ";F;"}":LPRINT
LPRINT "Sample","First OD","Second OD","  Prot per","Tot prot in"
LPRINT "  #"," "," ","vol assay(ug)","sample (ug)"
LPRINT "-------------------------------------------------------------------------"
10 CLS:count=count+1
PRINT "**********************INputting
Data*********************************"
PRINT "[NB. If you want to quit, enter 9999 as the first duplicate OD]"
PRINT "Sample # ";count
PRINT
INPUT "==> Enter the first duplicate OD for the sample ";C
IF C=9999 THEN END
PRINT
INPUT "==> Enter the second duplicate OD for the sample ";J
PRINT
PRINT "**********************OUTputting
Data*********************************"
LET K=(C+J)/2
LET Y=A+(B*K)
LET G=F*Y
PRINT " Total protein per assay volume = ";Y;" ug
PRINT
PRINT " Total protein in sample = ";G;" ug"
PRINT:PRINT
IF prin$<>"y" THEN 20
LPRINT "(";count;")",C,J,Y,G
20 INPUT "Press ENTER to continue ";en$
GOTO 10
END
```

Basic program for the calculation of substrate uptake determined radiochemically in the hindlimb perfusion or in vitro incubation procedures.

```
PRINT "Program for the determination for substrate uptake by tracers"

LET B=2.2*10^6
PRINT:PRINT:PRINT:PRINT:PRINT:PRINT:PRINT:PRINT
INPUT "Will you be wanting a printout ";prin$
IF prin$="y" THEN INPUT "What was the date of the experiment ";dat$
CLS:count=0
PRINT "******************INputting Standard
Data**********************"
PRINT
PRINT " 1. How much tracer was added to the incubation buffer"
INPUT "       (or perfusate) in uCi ";C
PRINT
INPUT " 2. How much cold 'molar' substrate in incubation buffer (mmol) ";E
PRINT
INPUT " 3. What is the counting efficiency of the tracer, if it is 40%, then the
factor to enter is  100/40=2.5) ";F
IF prin$<>"y" THEN 10
LPRINT "**********************Determination of Substrate Uptake by
Tracers**********************"
LPRINT " "," ","date of experiment : ";dat$
LPRINT "Tracer added to the incubation buffer (or perfusate) = ";C;" uCi"
LPRINT "Cold substrate in incubation buffer = ";E;" mmol"
LPRINT "The counting efficiency of the tracer = ";F
LPRINT "Sample","CPMs/mg prot."," ","Uptake tracer"," ","Uptake tracer"
LPRINT "  #","(for tissue)"," ","mmol/mg prot."," ","nmol/mg prot.
LPRINT "-------------------------------------------------------------------------------
----"
10 CLS:count=count+1
PRINT "************************INputting
Data*****************************"
```

```
PRINT "[NB. If you want to quit, enter 9999 as the CPM count]"
PRINT
PRINT "Sample # ";count
INPUT "==> How many CPMs/mg protein (for the tissue) ";A
IF A=9999 THEN END
LET D=B*C
LET G=A*F
LET H=(G/D)*(E/1)
LET I=H*10^6
PRINT:PRINT
PRINT "**********OUTputting
Data**********"
PRINT
PRINT "The uptake of the tracer = ";H;" mmol/mg protein"
PRINT "                         = ";I;" nmol/mg protein"
PRINT:PRINT
IF prin$<>"y" THEN 20
LPRINT "(";count;")",A," ",H,I
20 INPUT "Press ENTER to continue ";ent$
GOTO 10
END
```

APPENDIX 4. TECHNICAL DATA ON RADIOLABELLED SUBSTRATES

L-[U-^{14}C] LACTIC ACID, SODIUM SALT

* * *

CH_3-CH-COONa

OH

$C_3H_5NaO_3$ M.W. =112

Specific activity = 5.66 GBq/mmol (15.3mCi/mmol)
(6.5nmol/μCi)

Radiochemical purity determined by HPLC= 99.9%

DEOXY-D-GLUCOSE

* *

2-[1,2-^{3}H (N)]- $HOCH_2$ $(CHOH)_3$ CH_2 CHO

MW= 164.2

Specific activity= 30.2 Ci/mmol

SORBITOL

*

D-[1-^{3}H (N)]- $HOCH_2$ $[CHOH]_4$ CH_2OH

MW= 182.2

Specific activity= 15.5 Ci/mmol

APPENDIX 5. MUSCLE MEMBRANE PREPARATION (NOTES)

1. Prepare isolation buffer as follows:

10mM $NaHCO_3$ 0.8401 g
0.25 M sucrose 85.58 g
5 mM sodium azide 0.325 g
make up to 1 litre with dH_20(volumetric flask).

(I place 4-6 beakers with 40ml buffer each on ice to allow to cool.)

2. Anesthetize rat with approximately 0.1 ml sodium pentobarbitol per 100g rat body weight. Remove hindlimb muscles as quickly as possible. (Weigh tissue as you extract- 5g for 40 ml buffer, be certain the buffer does not warm up.)

3. Pour muscle and buffer into a petri dish, cut up the muscle into small pieces.KEEP ON ICE.

4. Divide muscle and buffer into polycarbonate tubes so that volume is evenly distrubuted. Each polycarb tube should not be filled more than half way because overflow will occur in next step.

5. Homogenize on Polytron (Model PCU serial #1921) for 6 seconds on a setting of 6. Clean tip of polytron of excess material after each tube.

6. Make sure the volume in all tubes or pairs of tubes is equal.

7. Spin at 3100 rpm (1200g) for 10 minutes.

8. Combine supernatants into a beaker. Put on ice. (Supernatant #1)

9. Add 3 ml isolation buffer to the pellet, mix and re-homogenize on polytron as above. Include also in this spin supernatant #1 divided into equal volume tubes. Spin at 3100 (1200g) rpm for 10 minutes.

10. Measure total volume of resulting supernatant (crude homogenate). Record this volume. Save approx. 1 ml for marker enzymes. Snap freeze and store in -70°C freezer. Discard the pellets.

11. Spin supernatant at 8500 rpm (9000g) for 10 minutes. Discard pellet.

12. Combine supernatant in tubes for ultracentrifuge. Top with dH_20 if not enough supernatant to fill tube. Make blanks using dH2O. ("tighten caps")

13. Spin for 1.5 hours at 39000 rpm (190,000g) if using SW41 rotor or at 39500 rpm if using 50.2 Ti rotor.

14. While waiting, make up 35%, 30% and 25% (w:w) sucrose solutions. Put on ice.

15. After the spin, discard supernatant, invert tubes to allow to drain.

16. Add appropriate amount of 35% sucrose to the tubes, depending on how many sample tubes there are, to equal a total volume of 6 ml.

17. Resuspend, scraping pellet off the bottom of the tube. Using a pasteur pipette, transfer suspension to tissue homogenizer. Add the same amount of 35% sucrose as above to the same tube, scrape any remaining pellet and transfer to homogenizer. Total amount of sample should be 12 ml.

18. Homogenize with 5 passes with the drill and pestle.

19. Transfer suspension to tube for sucrose gradient as follows:

Samples: 4.0 ml of 35% sucrose/sample
3.6 ml of 30% sucrose
3.6 ml of 25% sucrose use 25% to fill tube,

therefore you will use about 5ml 25% sucrose.
(Layer the gradients very carefully and also make sucrose blanks)

20. Spin gradients for 16 hours (overnight) at 32000 rpm (150,000g).

NEXT DAY:

21. Before turning off ultracentrifuge prepare 50mM Tris Buffer:
(0.6 g Trizma base in 100 ml dH_20 pH 7.4).

22. Examine tubes for white colored bands (mark tubes as you remove them from the holder)

23. Aspirate bands, as well as material below lower band for samples. Put in appropriately marked test tubes PM is the upper band(25%),the band at the 30% also contains plasma membranes with more contamination than the 25% band. MM is the bottom portion (35%). Clear layer on top and between PM and MM is waste.

24. Top tubes with TRIS buffer. Aspirate some material from the blank tubes and top with TRIS to balance. Mix well.

25. Spin at 39000 rpm (190,000g) for 1.5 hours. (This removes the sucrose from the samples)

26. Pour off supernatant. Reconstitute sample with 625 ul of Tris.
Mix using small pellet pestle. Snap freeze and store in -70°C freezer until transport assay.

APPENDIX 6. 5' NUCLEOTIDASE ASSAY

Marker for Plasma Membranes

Solutions:

A 50 mM AMP pH 7.0 with NaOH (adenosine 5' monophosphate)

B 0.5 M Tris-HCl pH 8.3 (Trizma base, titrate with HCl)

C 0.1 M $MgCl_2$

Substrate: Combine: A: 1 volume ...
B: 2 volume ...
C: 1 volume ...
H_2O: 5 volume ... Store frozen

Stop Solution: 10% SDS

Reagent: 4% $FeSO_4$ prepared fresh in (1% Ammonium molybdate in 1.15N H_2SO_4) store 1% solution in fridge

PROCEDURE: 1. 100 µg protein, bring up to 50µl dH_2O
2. Add 500µl substrate. Mix.
3. Incubate at 37°C for 30 minutes.
4. Add 250µl stop solution. Mix.
5. Add 750µl Reagent. Vortex. Wait 10 minutes.
6. Read at 700nm.

Note: Always run a blank with substrate but no protein and a blank with protein in water, no substrate.

Standard curve:

1mM Phosphorus (use potassium phosphate monobasic)

1.36g in 10ml dH_2O = 1M, use 1ml in 9 ml dH_2O= 0.1M Pi.

[Pi] (µmoles)	0.1M (µl) Stock	+ dH_2O (µl)
0	0	50
1.0	1	49
5.0	5	45
10.0	10	40
25.0	25	25
50.0	50	0

Appendix 7 Notes on glycogen extraction for ^{14}C label incorporation.

1. Dissolve muscle (10-20mg) in 0.3ml of 1N NaOH in screw capped tubes - boil for 5 min. agitate occasionally.
2. Add 0.3ml of 1N HCl,vortex, cool in ice bath - take 40uL for protein assay
3. In remaining tubes add 0.5ml of 30% KOH saturated with Na_2SO_4 and boil for 5mins, agitate occasionally.
4. Cool on ice and add 0.5ml of 'carrier glycogen' (40mg/ml of glycogen dissolved in 30% KOH saturated with Na_2SO_4. Vortex and transfer to centrifuge tubes.
5. add 3ml of 95% EtOH to precipitate glycogen, place in freezer at -15°C for 60 min.
6. Centrifuge for 20min. at 4000*g decant supernatant, dissolve precipitate in 1ml of H_2O and add 3ml of 95% EtOH (wash step)
7. repeat step 6 three times more (4 spins in all).
8. After 4th wash dissolve the precipitate in 1ml H_2O and transfer to plastic scintillation vial, add 10 ml of scintillation fluid (Aquasol) and count for appropriate radioactive label.

Appendix 8 Total protein assay procedure (Bradford method)

Make up stock protein solution of Bovine Serum Albumin (BSA) 1mg/ml H_2O

Concentration(BSA)(µg)	µl stock	H_2O(µl)	Coomassie blue (ml)
12.5	12.5	87.5	5
25	25	75	5
50	50	50	5
75	75	25	5
100	100	0	5
Sample	10	90	5

Vortex , wait 5mins and read on a spectrophotometer at 595nm. read all samples within 1 hr. after adding Coomassie blue.

Preparation of Coomassie blue stock solution.
0.5g of CB + methanol (make up to 250ml)
stir for 4 hrs.
add 250 ml H_2O and 500 ml H_3PO_4 let this solution stand at room temp overnight. Dilute the stock 5 fold before using. Filter the CB through Whatman grade 1 filter paper before using in the assay. (see computer program for protein determination for calculating results). The OD for the unkown sample is compared to the (linear) standard curve to determine the concentration of the sample.

Appedix 9. Physiological media

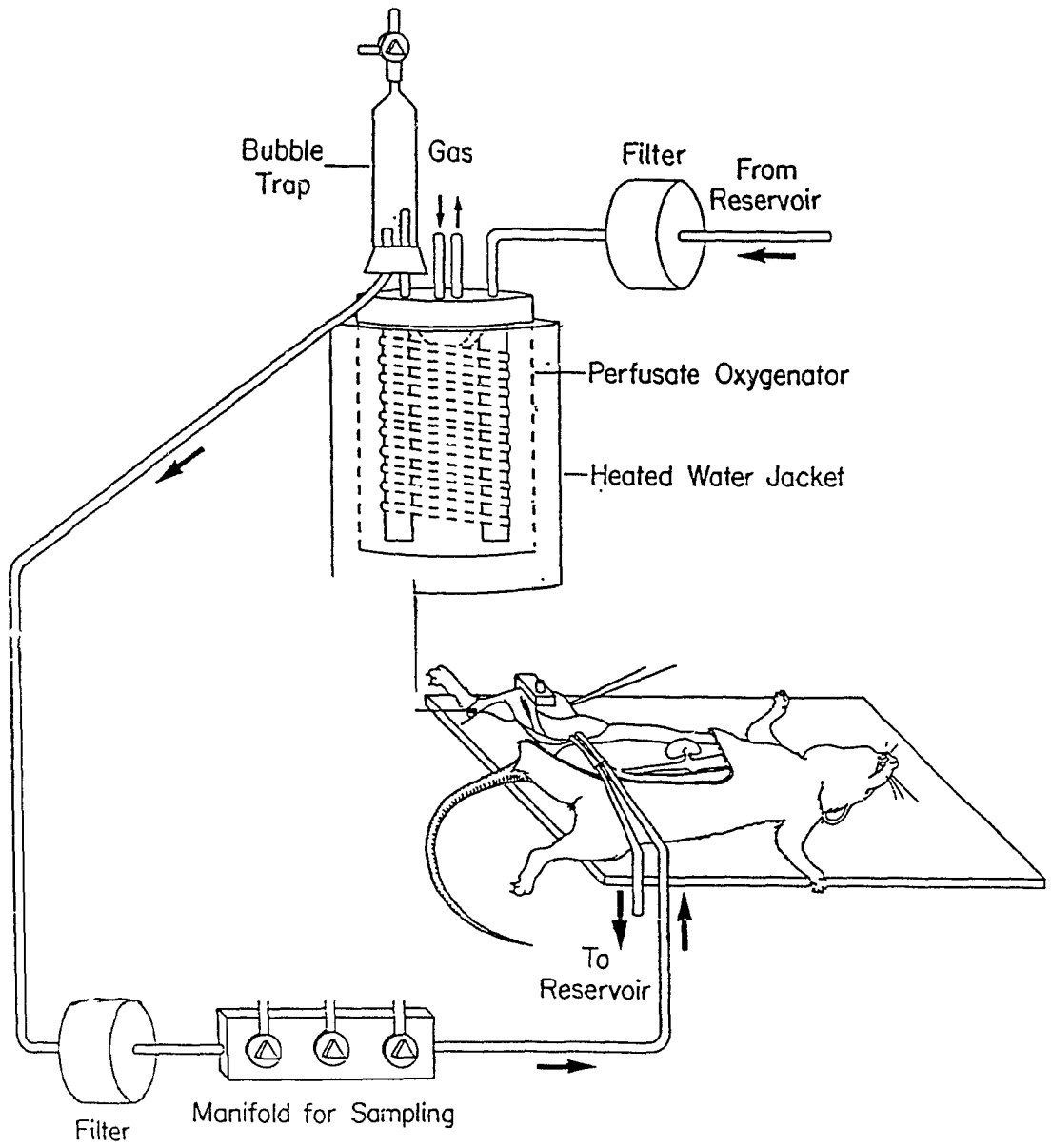
Krebs-Henseleit bicarbonate buffer.

(Solutions required)	Parts by volume
0.9% NaCl (0.154 M)	100
1.15% KCl (0.154 M)	4
1.22% Ca Cl_2 (0.11M)	3
2.11% KH_2PO_4 (0.154M)	1
3.8% $MgSO_4$ (0.154M)	1
1.3% $NaHCO_3$ ¥	21

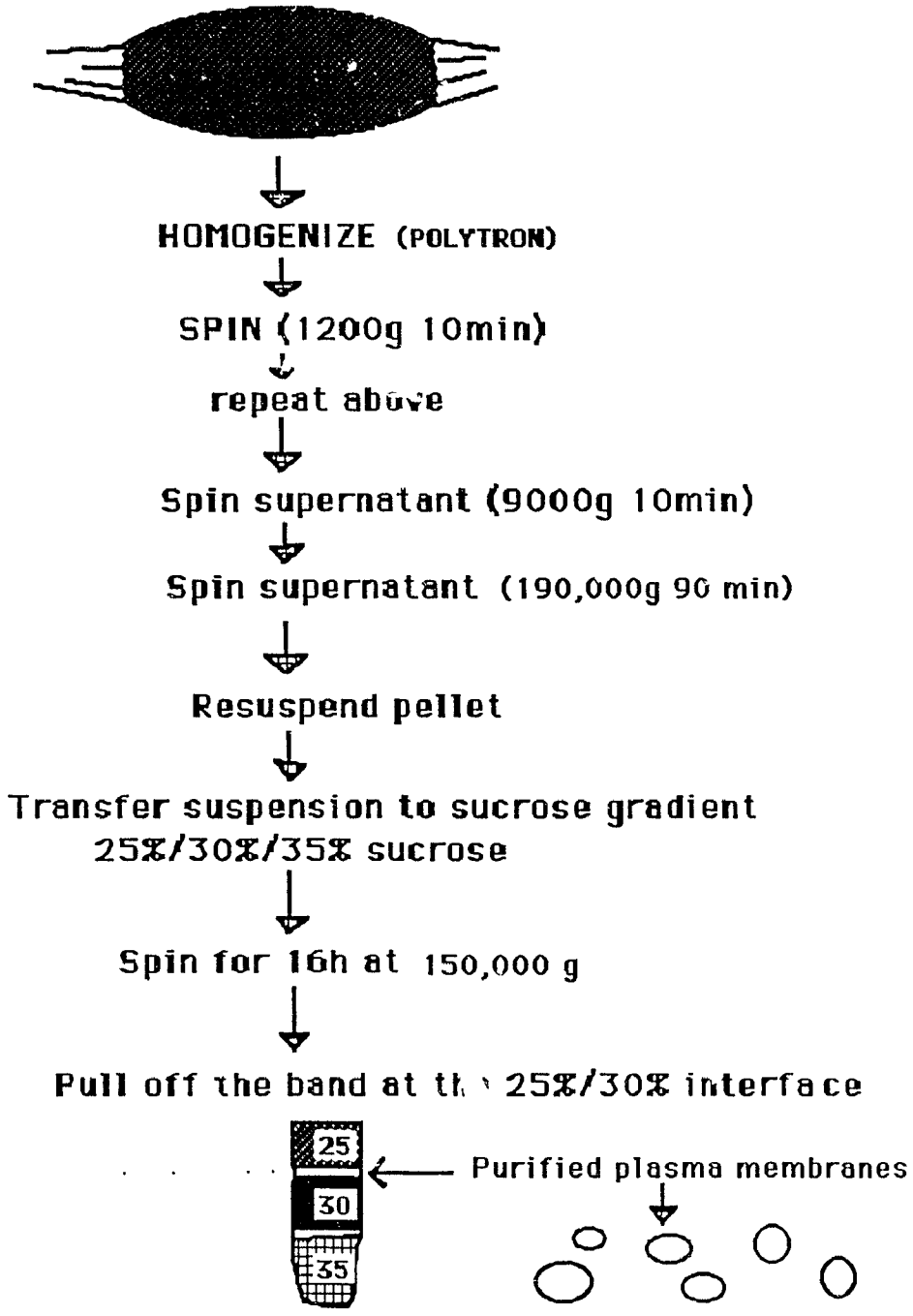
Note, ¥- gassed with 100% CO_2 for 1hr before mixing with other solutions

All solutions are approximately isotonic with serum

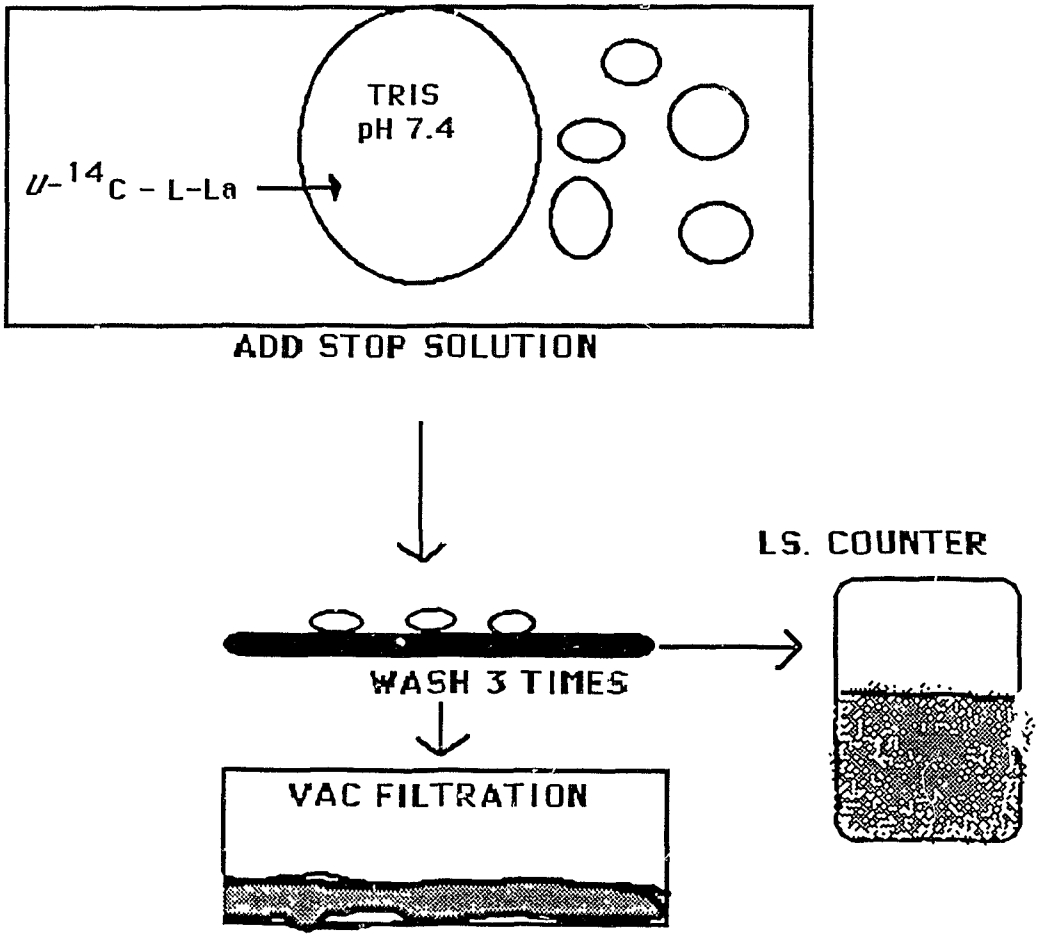
PERFUSED RAT HINDLIMB PREPARATION



MUSCLE MEMBRANE ISOLATION PROCEDURE



LACTATE TRANSPORT ASSAY IN PURIFIED SARCOLEMMAL VESICLES



Appendix A manuscript to be published in the Canadian Journal of Physiology and Pharmacology in press.by Bonen,A, Blewett, C, McDermott, JC, Elder, GCB.

A model for nonexercising hindlimb muscles in exercising animals.

Non-exercising muscles appear to be metabolically active during exercise. Animal models for this purpose have not been established. However, we have been able to teach animals to run on their forelimbs while their hindlimbs are suspended above the treadmill with no visible limb movement. To document that indeed this mode of exercise does not provoke additional muscle activity we have compared the levels of neural activation of the soleus and plantaris muscles using a computer analysis of the electromyographic interference pattern, recorded from bipolar fine wire electrodes implanted across each muscle. Via computer analyses of the electromygraphic interference patterns the frequencies and amplitudes of motor unit action potentials were obtained. The data were sampled during 20 sec of every minute of observation. Comparisons were made in four conditions: a) resting on the treadmill while bearing weight on the hindlimbs (normal rest), b) running on the treadmill (15m/min, 8% grade) on all 4 limbs (normal exercise), c) resting while the hindlimbs were suspended in a harness above the treadmill (suspended rest), and d) exercising with the forelimbs (15m/min, 8% grade) while the hindlimbs were suspended above the treadmill (suspended exercise). All four experimental conditions were carried out for 90 min each and were performed by each animal. The results clearly show that muscle activities (frequencies and amplitudes), when the hindlimbs are suspended above the treadmill, at rest or during exercise, are lower than the activities in these same muscles when the animals are at rest, supporting only their body weight. Activities in the same muscles during exercise were from 300-2000% greater than during hindlimb suspension. Despite these large differences in muscle activities, comparable quantities of glycogen are lost from these hindlimb suspended (non-exercising) muscles during exercise and when these same muscles are performing the exercise. The physiologic data indicate that suspending the hindlimbs of a rat during forelimb exercise provides a convenient animal model for the study of non-exercising muscle during exercise. In fact the soleus and plantaris activities during suspended exercise are less than during normal rest.

Introduction

Muscle metabolic rates are markedly increased by contractile activity. However, in previous work we have shown that glycogen is lost from both exercising and non-exercising muscles in humans (Bonen et al 1985). A similar glycogen loss was observed in non-exercising hindlimb muscles of rats that were taught to run on their forelimbs while the hindlimbs were suspended above the treadmill (McDermott, Elder and Bonen, 1987). Although we observed no visible movement in the suspended hindlimbs during exercise, Delp and Armstrong (1988) have questioned whether the hindlimb muscles are truly inactive, during this mode of running and whether glycogenolysis does indeed occur in non-exercising skeletal muscles. Considerable evidence does suggest that glycogenolysis in non-exercising muscles should not be surprising (see McDermott, Elder and Bonen, 1988). However, the physiological characteristics of the non-exercising muscles in the hindlimb suspended exercising model have only been evaluated qualitatively (McDermott, Elder and Bonen, 1987).

It is possible to obtain direct estimates of muscle activity patterns (e.m.g.) from implanted electrodes (Alford et al, 1987; Hnik et al 1985; Jasmin and Gardiner 1987). Procedures for the monitoring of neural activation in muscles have been provided by Nandedkar, Sanders and Stalberg (1986a, b). Their method seemed to provide an excellent means for quantifying the activity patterns of rat skeletal muscles in vivo in our exercising, hindlimb suspended animals. Therefore, we monitored the levels of neural activation of soleus and plantaris muscles in rats throughout 90 min of i) rest, ii) normal treadmill exercise, iii) suspended rest, and iv)g suspended exercise. In separate groups of animals we also measured muscle glycogen concentrations at the beginning and after 90 min of the four experimental treatments, in order to verify our earlier observations (McDermott, Elder and Bonen, 1987) that muscle glycogenolysis can occur in non-exercising muscles.

Methods

Five male rats (Long Evans; 250-300g) were used in these studies. To prevent selected skeletal muscles from exercising during treadmill exercise, a non-invasive hindlimb suspension system was used to elevate the hindlimbs, so that treadmill running could be performed by the forelimbs (see Fig.1 in ref. McDermott, Elder

and Bonen, 1987). The harness used for suspension was made of a canvas belt which was fastened around the animals' lower abdominal region. Two leg straps were then passed around the upper part of the hindlimb and attached to the main harness. An additional strap was then placed around the caudal end of the main harness to keep it stable. The rats were suspended from the posterior part of the harness with the hindlimbs 2cm above the treadmill.

Prior to the experiments all animals were familiarized with treadmill exercise while wearing the suspension harness. This was placed on the animal under light ether anaesthesia before each exercise familiarization and 30 minutes before the experimental period. Familiarization for suspended exercise involved the following procedures: a) the rats were habituated to treadmill running on 4 limbs without requiring the stimulus of an electric shock before suspended exercise was initiated, b) four familiarization sessions of 10-15min suspended exercise were conducted; the initial familiarization session occurred at 5-7m/min, 8% grade (% grade = (rise/run) * 100) and the speed was gradually increased during the next three sessions to the desired running speed of 15m/min at 8% grade.

Bipolar fine wire electrodes (Cooner stainless steel wire, AS632) were used to record the EMG activity. The electrodes were implanted while the rats were under sodium pentobarbital anesthesia (Somnotol 65mg/kg) under aseptic conditions several weeks before the experiments (good recordings have been maintained for 10 weeks, unpublished data). A lateral incision <4mm long was made to expose the mid-region of the soleus, and a medial incision exposed the lower one-third of the plantaris. Electrodes were placed across the bellies of the muscles at approximately right angles to the fibers, 2 mm apart with the recording surface 3.5 mm long. The wires were sutured to surrounding tissue to stabilize them and two loops, one proximal to the muscle and one in the neck region, were inserted to prevent direct pulling on the muscles. The wires were passed to the dorsal surface of the neck under the skin where they were soldered to female Molex plugs glued to Velcro strips which were placed around the trunk and sutured at several points. The electrodes were connected via a shielded cable to a wideband a.c. preamplifier (Grass, 7P5).

In order to prevent the shielded cable from twisting when the animal turned in its cage a special apparatus was designed. The wires in the shielded cable were passed through a hollow plexiglass rod mounted on a freely turning, low friction bearing.

These wires were soldered to 4 x 1 cm wide stainless steel rings mounted on the outside of the plexiglass rod. Any turning movement of the animal turned this device. Movement around the cage was accommodated by setting this device at the end of a plexiglass arm mounted via a second low friction bearing on a stainless steel rod mounted in the center of the cage. Against each steel ring, rested under sprung tension, an exposed strip of stainless steel wire that was connected to the amplifiers. As the ring turned contact was maintained.

The activity levels in soleus and plantaris were determined from analyses of the number of turns, and their amplitudes, recorded in the complex interference patterns in a manner comparable to that described by Nandedkar, Sanders and Stalberg (1986b). Briefly, the e.m.g. signals were low and high pass filtered between 10 and 40kHz and digitized on-line using an A-D board (AI13 Interactive Structures) mounted in an Apple IIe. An assembly language program recorded the mean number of "turns" (see below) and the mean amplitude of the turns from the soleus and plantaris muscles during a total of 20 sec sampling period per minute. The program sampled each channel for 500 msec in sequence, with a sampling rate of 2KHz. A "turn" was defined as any positive potential which had an amplitude greater than a preset threshold set above the noise level of the signal (normally <80 mV) in a recording range of 0-5 V. A fall in amplitude with a subsequent increase was interpreted as a new turn. The program recorded the frequency and mean amplitude of all turns during each 20 sec sampling period every minute throughout the 90 min experimental periods.

The activity patterns in the soleus and plantaris muscles were studied during four procedures: a) resting on the treadmill while bearing weight on the hindlimbs (normal rest), b) running on the treadmill (15m/min, 8% grade) on all 4 limbs (normal exercise), c) resting while the hindlimbs were suspended in a harness above the treadmill (suspended rest), and d) exercising with the forelimbs (15m/min, 8% grade) while the hindlimbs were suspended above the treadmill (suspended exercise). All four experimental conditions were carried out for 90 min each and were performed by each animal over several days. Muscle activity patterns (turns and their amplitudes) were monitored continuously (20sec/min) for the the entire 90 min experimental periods.

In separate groups of animals glycogen concentrations in the soleus and plantaris

muscles were measured prior to the experimental treatment (t=0) and at the end of 90 min of normal rest, 90 min normal exercise, 90 min suspended rest and 90 min suspended exercise. At the appropriate time (t=0 or t=90 min.) animals were sacrificed by an i.p. injection of sodium pentobarbitol. Upon induction of surgical anaesthesia (_ 2 min.) the soleus, and plantaris were rapidly removed, placed in ice-cold saline (0.9%), weighed, blotted, frozen in liquid nitrogen and stored at -80°C. Muscle samples were assayed for glycogen content using the method of Passoneau and Lauderdale, 1974.

The statistical comparisons of the MUAP interference patterns were made with a repeated measures analysis of variance. Glycogen concentrations were compared with t-tests.

Results

Representative raw data for one animal are shown in Fig 1. The frequencies and amplitudes of turns determined for an individual animal (Fig 2) and the group means (Fig 3) are shown for the soleus and plantaris muscles. For the sake of clarity of the presentation of the results, the data from each animal in each condition were averaged for each 5 min period. These individual 5 min averages from the 5 animals were then used to calculate the group means that have been plotted (Fig 3). However, the statistical analyses were performed on the continuous data obtained each minute throughout the four, 90 min experiments.

Frequency of Action Potentials

Soleus: The frequencies of turns in normally resting animals ranged from 6000-12000/min (Fig 2). During suspended rest and suspended exercise these frequencies decreased considerably to 2000-4000/min. This level of activity was significantly less than that observed during normal rest and normal running ($P<0.05$). The highest frequencies were observed during normal treadmill running (14000-15000/min).

Plantaris: Similar trends were observed in the plantaris to those seen in the soleus (Fig 2). The lowest frequencies occurred during suspended rest and suspended exercise, and these were quite similar (1000-2500/min). Much higher frequencies

were seen during normal rest (3500-6000/min; $P<0.05$). The highest frequencies occurred during normal running (13000-14000/min; $P<0.05$ compared to each of the other three conditions).

Amplitudes of Action Potentials.

Soleus: Amplitudes of the electromyographic interference patterns were quite similar during normal rest and suspended rest (Fig 2). During suspended exercise a consistently lower range of amplitudes were found when compared to the normal rest and suspended rest conditions ($P<0.05$). The largest amplitudes were observed during normal exercise ($P<0.05$; compared to each of the three other conditions)

Plantaris: The amplitudes in the suspended rest, suspended exercise and normal rest conditions were all quite similar (Fig 2). These were consistently lower than during normal running ($P<0.05$).

Glycogen

In a separate group of animals muscle glycogen concentrations were measured before and after 90 min of each of the experimental treatments. Slight glycogen decrements occurred during the 90 min period at rest (Table 1), but these decrements were not statistically significant ($P>0.05$), except in the soleus muscles of the animals that were suspended for 90 min at rest (t=0 vs t=90; Table 1; $P<0.05$). No differences were observed in glycogen concentrations at the end of the experiments between the normal rest (t=90) and suspended rest conditions (t=90) for either the plantaris or soleus muscles (Table 1; $P>0.05$). For further comparison purposes (see below) these 90 min data from normally resting and suspended resting muscles were therefore pooled.

Ninety minutes of normal exercise and 90 minutes of suspended exercise provoked significant decrements in muscle glycogen concentrations (Table 1; $P<0.05$). However, no differences in glycogen concentrations were present between normally exercised muscles (t=90) and when these muscles were suspended above the treadmill during exercise (t=90) (Table 1; $P>0.05$). In comparison to the glycogen concentrations after 90 min rest (pooled data of normal rest and suspended rest groups), normal exercise and suspended exercise reduced glycogen

concentrations by 45% and 41%, respectively, in the soleus, and by 58% and 55%, respectively, in the plantaris.

Discussion

The present results clearly show that muscle activities (frequencies and amplitudes), when the hindlimbs are suspended above the treadmill, at rest or during exercise, are lower than the activities in these same muscles when the animals are at rest, supporting only their body weight. Activities in the same muscles during exercise were from 300-2000% greater than during hindlimb suspension. Despite these large differences in muscle activities, comparable quantities of glycogen were lost from these hindlimb suspended (non-exercising) muscles during exercise and when these muscles were performing the exercise. The physiologic data indicate that suspending the hindlimbs of a rat during forelimb exercise provides a convenient animal model for the study of non-exercising muscle during exercise. In fact the soleus and plantaris activities during suspended exercise are less than during normal rest.

The study of muscle activity patterns via implanted electrodes have also been performed by others (Alford et al, 1987; Hnik et al 1985; Jasmin and Gardiner 1987).For the purpose of monitoring the activities in the soleus and plantaris muscles we used procedures similar to those of Nandedkar, Sanders and Stalberg (1986a,b). The quantitative analysis of the electromyographic interference patterns, as used in this study, provides a good index of the motor unit activation in skeletal muscles. In simulation studies by Nandedkar, Sanders and Stalberg, 1986a, in which electromyographic interference patterns were created from the electronic addition of motor unit action potentials, the number of "turns" increased directly with the number of motor unit action potential discharges up to about 18000/min. Above this frequency, the interference among motor unit action potentials prevents complete resolution of all "turns" (Nandedkar, Sanders and Stalberg, 1986a). The increase in mean amplitudes, as determined by simulation studies (Nandedkar, Sanders and Stalberg, 1986a) was attributable to the recruitment of large amplitude motor unit action potentials rather than the summation of several motor unit action potentials.(Nandedkar, Sanders and Stalberg, 1986a). In the context of the present studies it appears therefore that i) the observed frequencies (Fig 1 and 2) provide an adequate estimation of the motor unit action potentials in the muscle, except perhaps during the normal running when some of the frequencies began to approach the

sensitivity resolution of the technique, and ii) that the amplitude increments are disproportionately related to the presence of large amplitude motor unit action potentials rather than to an increase in amplitude in all motor unit action potentials. Presumably then, the number of "turns" observed provide the best index of the increased muscle activity in our studies, since this measure corresponds directly to the neural activation of the muscle. Therefore, the very large differences in the frequencies of the nonexercising muscles in our hindlimb suspension model, when compared to the frequencies in the exercising muscles and normally resting muscles (Fig 1 and 2), can be taken to represent large differences in muscle activities. The supposedly slight underestimation in the frequencies during normal running, (see above), is not too serious in our present studies, since there were very large differences between exercising and non-exercising muscles, which were the primary focus of this study. Also, since all animals participated in each of the four experimental conditions the data were obtained from the same area of the same muscle during the four different activities, and therefore, the relative differences among the experimental conditions reflect qualitatively meaningful differences in muscle activities during different activity modes. It is very clear in these studies that the activities in suspended hindlimb muscles, at rest and during exercise, are very much lower than in exercising hindlimb muscles.

In the present study we have again observed that similar quantities of muscle glycogen are lost from exercising and non-exercising muscle during exercise. We have reported this phenomenon previously in animals (McDermott, Elder and Bonen, 1987) and humans (Bonen et al 1985). Indeed it was this human work that prompted us to develop the present animal model. Criticism (Delp and Armstrong, 1988) of our earlier study (McDermott, Elder and Bonen, 1987) provided the impetus for the detailed description of the muscles' MUAP patterns in the present study and to repeat the measurements of glycogen. The increase in systemic epinephrine, a known stimulus for muscle glycogenolysis (Richter, 1984), provides the most logical explanation for this glycogen loss in non-exercising muscles, since glycogenolysis is not observed in exercising and non-exercising muscles of adrenalectomized rats run under identical experimental conditions (McDermott, Elder and Bonen, 1987). Our present results confirm that glycogenolysis in exercising and non-exercising muscles is quite similar despite large difference in muscle activities.t

Our observations of glycogenolysis in non-exercising muscles in animals (i.e three separate studies: McDermott, Elder and Bonen, 1987, present study, and more recent unpublished work) and in humans (Bonen et al 1985) has also been reported elsewhere. Ahlborg, Hagenfeldt and Wahren, (1975) noted, without comment, a glycogen decrement in non-exercising leg muscles of humans during single leg exercise (N=3). This was also thought to occur in arm muscles during leg exercise, due to the production of lactate by arm muscles (Ahlborg and Felig, 1982; Ahlborg, 1985). In light of these observations the question is why glycogen decrements were not observed in non-exercising muscles in the early experiments by Scandinavian workers (Bergstrom and Hultman, 1967; Pernow and Saltin 1971). One explanation may be that the method of glycogen extraction used in these studies seriously underestimated the glycogen loss (i.e. up to 25% error) in human muscle (Jansson, 1981), and therefore the accuracy of the assay procedure was compromised. In our work two different glycogen assays have been used, both of which measure total glycogen (McDermott, Elder and Bonen, 1987; present study). Our results therefore are unlikely to be to a quirk of the glycogen assay procedures such as has been identified in some earlier work (see Jansson, 1981 for details).

Another reason why our observations are "new", is that the effect of epinephrine on muscle glycogenolysis was not fully appreciated until recent work from different laboratories (Arnall et al 1986; Gorski, 1978; McDermott, Elder and Bonen 1987; Richter et al 1981, 1982; Richter 1984). Early workers did not show much interest in non-exercising muscles, because it was widely believed that epinephrine did not provoke glycogenolysis in exercising muscle (Gollnick et al 1970) . Work from this laboratory (Bonen et al 1985; McDermott, Elder and Bonen, 1987) and others (Richter et al 1981; 1982) have challenged this idea. Our non-exercising animal model supports the idea that glycogenolysis can occur in any type of muscle (Gorski, 1978; Richter 1984), whether it is exercising or not (Bonen et al 1985; McDermott, Elder and Bonen, 1987; present study), providing that epinephrine increments have been provoked either during exercise (McDermott, Elder and Bonen, 1987) or by infusion of epinephrine (Richter 1984). Absence of epinephrine reduces glycogenolysis in exercising (Arnall et al 1986; McDermott, Elder and Bonen, 1987; Richter, 1984) and non-exercising muscles (McDermott, Elder and Bonen, 1987). Therefore, it is the older work (Bergstrom and Hultman, 1967; Gollnick et al 1970; Pernow and Saltin, 1971) that seems to be at variance with the expected epinephrine-induced effects on glycogenolysis in non-exercising

skeletal muscle during exercise, when epinephrine levels are known to be increased.

We do not believe that the glycogenolysis observed in non-exercising muscles is simply a function of the suspension procedure per se. In our studies we have always included a control condition in which animals are suspended for 90 min but they are not exercising. During a 90 min rest period, whether the animals are suspended or whether they are resting normally, a decrease in muscle glycogen can occur in the soleus (McDermott, Elder and Bonen, 1987; present study, Table 1). Note however that 1) at 90 min the soleus glycogen in suspended control and control animals do not differ ($P>0.05$; Table 1), 2) that plantaris muscle glycogen is not altered in the control, hindlimb suspended animals (McDermott, Elder and Bonen, 1987; present study), and 3) that the glycogen reduction in muscles from suspended animals during 90 min of forelimb exercise markedly exceeded the glycogen losses in the muscles of suspended control and normally resting control animals.

It also seems unlikely that the hindlimb suspension might compromise the bloodflow to the hindlimb muscles. The exercise regimen (15m/min, 8% grade) is little more than a walking speed. Thus, we suspect that substrate and oxygen delivery are likely to be quite adequate for this low intensity exercise, even if blood flow is somewhat affected. At this low exercise intensity hemodynamic problems, if present at all, in the suspended, mildly exercising animals would also presumably be occurring in the control suspended animals. Yet, in these control animals the glycogen decrement in 90 min did not differ from those of normally rested animals, whereas in the suspended exercising animals a markedly greater loss of glycogen was observed. Had either glucose uptake or oxygen delivery been impaired a large glycogen loss would have been expected in control suspended animals. Moreover, animals can be suspended at rest for as long as 28 and 200 days (Bonen, Elder and Tan, 1988; Elder and McComas, 1987), with the result that muscle glycogen concentrations are increased by 6 days of suspension (Henriksen, Tischler and Johnson, 1985). This suggests that suspension does not impair glucose delivery, nor bloodflow therefore, to suspended animals at rest, and by inference is unlikely to do so in very mildly exercising, hindlimb suspended animals.

Glucocorticoids are not expected to have a role in muscle glycogenolysis. Corticosterone is not necessarily increased during hindlimb suspension, since

normal concentrations are observed 7 days after unloading the hindlimbs. (Halloran et al 1988; Popovic et al 1982). We adapted the animals to unloading (see methods) and this might have prevented an increase in glucocorticoids due to stress. However, even if the concentrations of this hormone were increased, this steroid is unlikely to affect muscle carbohydrate metabolism. This hormone reduces glucose uptake and glycogenesis only at pharmacological concentrations ($\geq 10^{-8}$ M) in mouse and rat muscles (Bonen and Tan 1989; Leighton et al 1987; Tan and Bonen, 1985). Such concentrations are unlikely to be attained in animals adapted to hindlimb suspension exercise.

The proced ures for reducing muscle activity with hindlimb suspension are simple and non-invasive. Stress to the animals is not evident when they have been properly adapted to the harness and the suspension procedure. Catecholamine levels were shown to be comparable in hindlimb suspended animals and in non-suspended animals in our earlier work (McDermott, Elder and Bonen, 1987). The glycogenolysis observed in non-exercising muscles is unlikely to be due to the suspension procedures per se. Hindlimb suspension may offer advantages over other methods that have been used to reduce muscle activity (e.g. neuromuscular blockade using curare (Glenn, Laughlin and Armstrong, 1987), denervation (Shoji, 1986: Villa-Moruzzi and Bergamini, 1983) and limb immobilization (Booth, 1982)). Prolonged reduced activity provided by hindlimb suspension has also been used for physiologic (>200 days; Elder and McComas, 1987) and metabolic studies (28 days; Bonen, Elder and Tan, 1988) in our laboratories. Therefore, the present hindlimb suspension model provides an opportunity to study the metabolism in muscles at rest and during exercise when other muscles are performing the work.

In summary, the present data show clearly that the muscle activities of the soleus and plantaris remain low when the hindlimbs are suspended above the treadmill and exercise is performed on the forelimbs. It is of considerable experimental advantage to be able to study the same muscles (soleus, plantaris) when they participate in exercise or when they do not participate in exercise while still being exposed to the metabolic and endocrine milieu of an exercising animal. We conclude that suspended exercise provides a convenient method for the study of a non-exercising muscle in an exercising animal.

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TABLE 1. Glycogen concentrations (umol glucosyl units/g wet weight) in soleus and plantaris muscles before (t=0) and after 90 min of rest, exercise, suspended rest and suspended exercise (X±SEM).

		Muscle	
Time, min	Condition	Soleus	Plantaris
0	normal rest	26.1 ± 1.8	21.0 ± 1.4
90	normal rest	23.8 ± 2.5	17.9 ± 1.5
90	suspended rest	20.7 ± 0.8	22.4 ± 2.9
90	normal exercise	12.5 ± 2.6	8.4 ± 1.6
90	suspended exercise	13.3 ± 1.8	9.0 ± 1.2

N=5 animals per group

a

$P<0.05$ suspended rest (t=90 min) vs normal rest (t=0 min)

b

$P<0.05$ exercise (t=90 min) vs corresponding rest conditions (t=90 min)

List of Figures

Fig 1.Examples of interference patterns obtained from soleus muscle during normal rest (a, b, c) and during running at 15m/min (d, e)

a) Example of a single motor unit firing at rest

b) Involvement of several motor units with the threshold level indicated by the dashed line

c) A complex interference pattern obtained during the movement about the cage is show. The continuous line represents the baseline and the dashed line the threshold level set above the signal noise level. The computer program recognizes each peak above threshold as as a turn (represented by dots above the peaks) from which to calculate the turn frequencies. Concurrently, the amplitude for each turn is determined and averaged for all turns during the data acquisition period

d) The interference pattern of a single step cycle during running at 15m/min

e) The interference pattern during several steps while running at 15m/min

The vertical amplitude bars represent 224 uV for a, b, c, e., and 258 uV for d. The horizontal time scales represent 32 ms for a, b, d, 12 ms for c, and 620 ms for e.

Fig 2. Frequency and amplitude of motor unit action potentials (MUAP) in the soleus muscle of an individual, representative animal during normal rest, normal exercise (15m/min, 8% grade) suspended rest and suspended exercise (15m/min, 8% grade). All data shown were obtained throughout the complete 90 min of each experiment.

Fig 3. Mean Frequencies and amplitudes of motor unit action potentials (MUAP) in soleus and plantaris muscles during normal rest, normal exercise (15m/min, 8% grade), suspended rest and suspended exercise (15m/min, 8% grade). (N=5 animals, each of which participated in all experiments. For clarity of presentation of the data, the results from each animal in each condition were averaged over 5 min periods, and these individual 5 min averages from the 5 animals were then used to calculate the group means that have been plotted. Error bars have also been deleted to retain clarity in this figure)

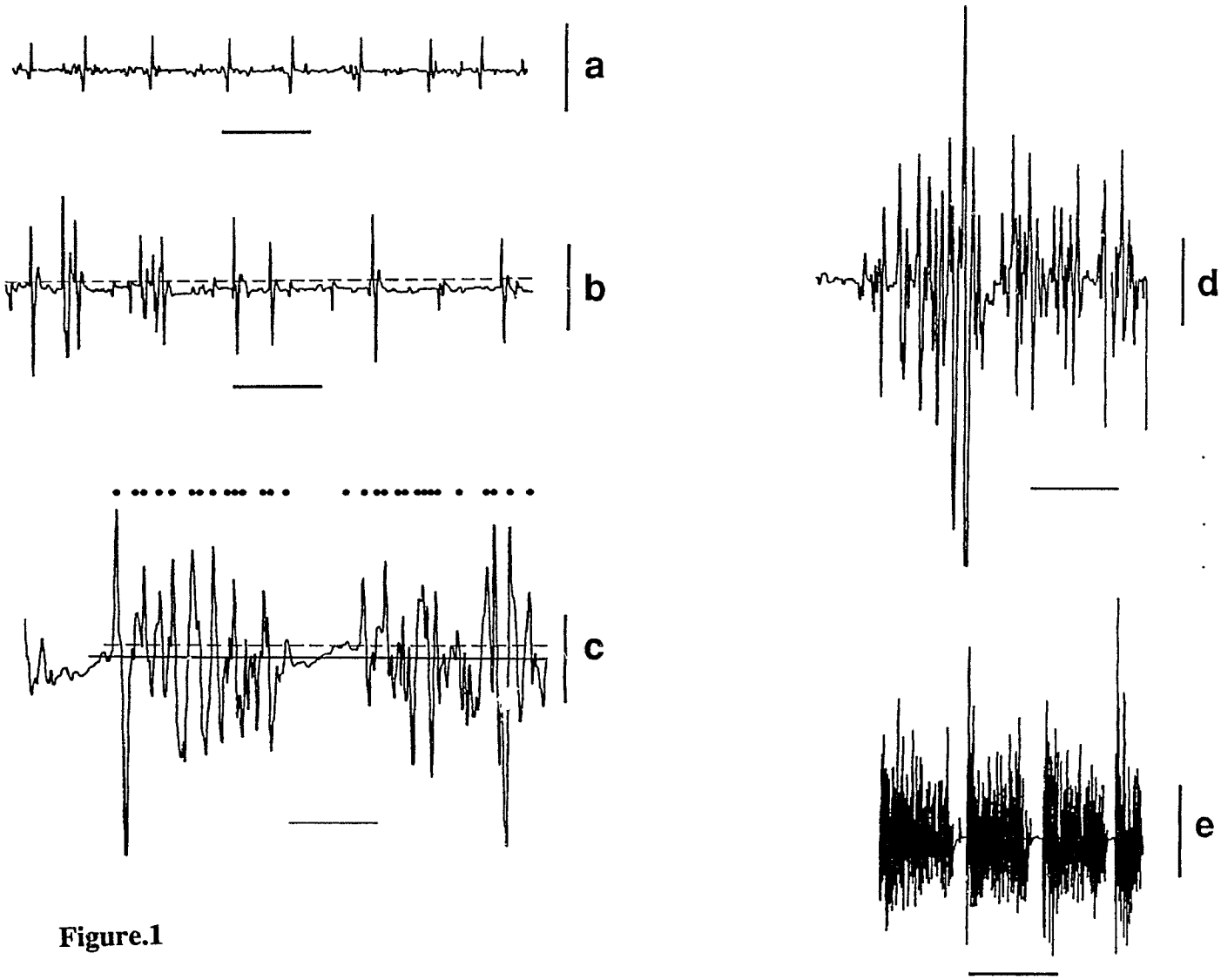
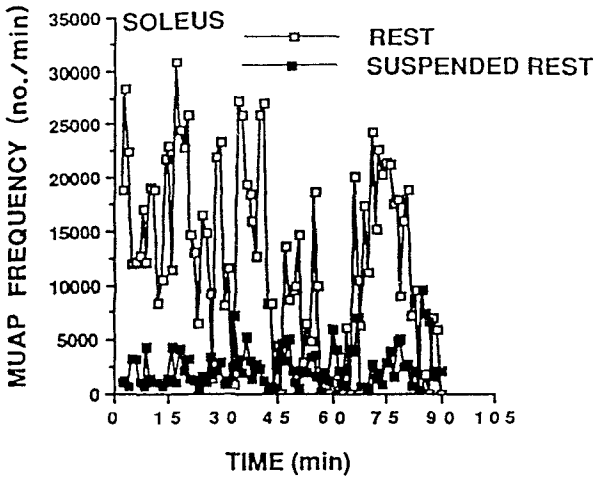


Figure.1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SUSPENDED REST
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20000
15000
10000
5000
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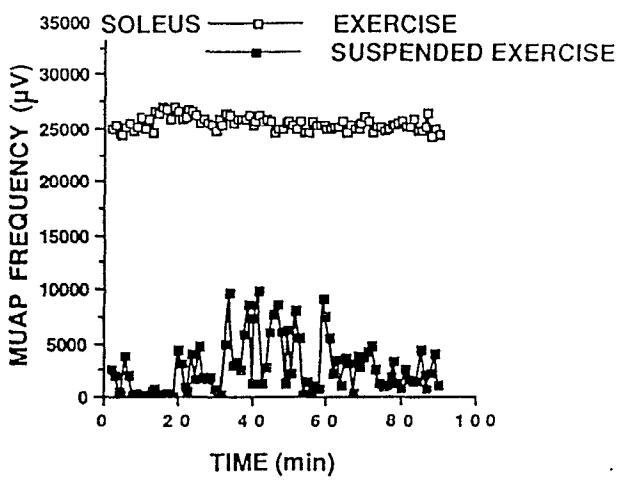
TIME (min)


SOLEUS
EXERCISE
SUSPENDED EXERCISE
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25000
20000
15000
10000
5000
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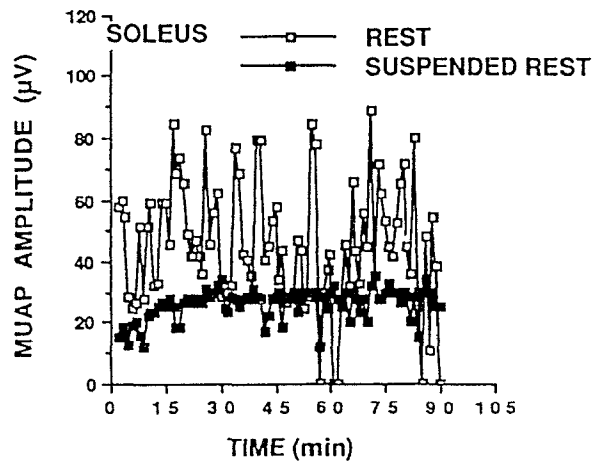
TIME (min)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REST
SUSPENDED REST
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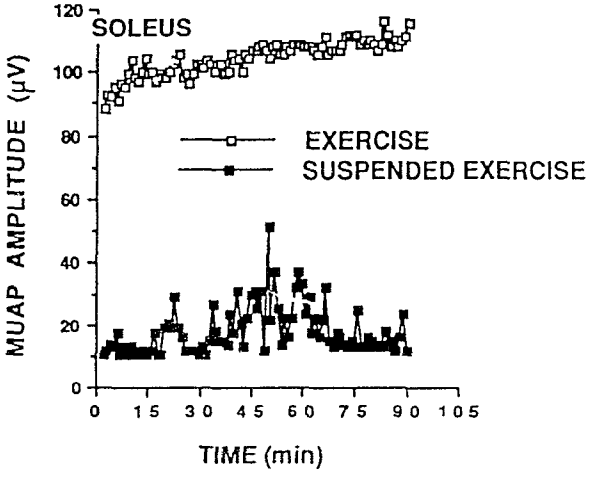
80
60
40
20
0
0 15 30 45 60 75 90 105
TIME (min)


SOLEUS
EXERCISE
SUSPENDED EXERCISE
MUAP AMPLITUDE (μV)
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100
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60
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0 15 30 45 60 75 90 105
TIME (min)

Figure.2

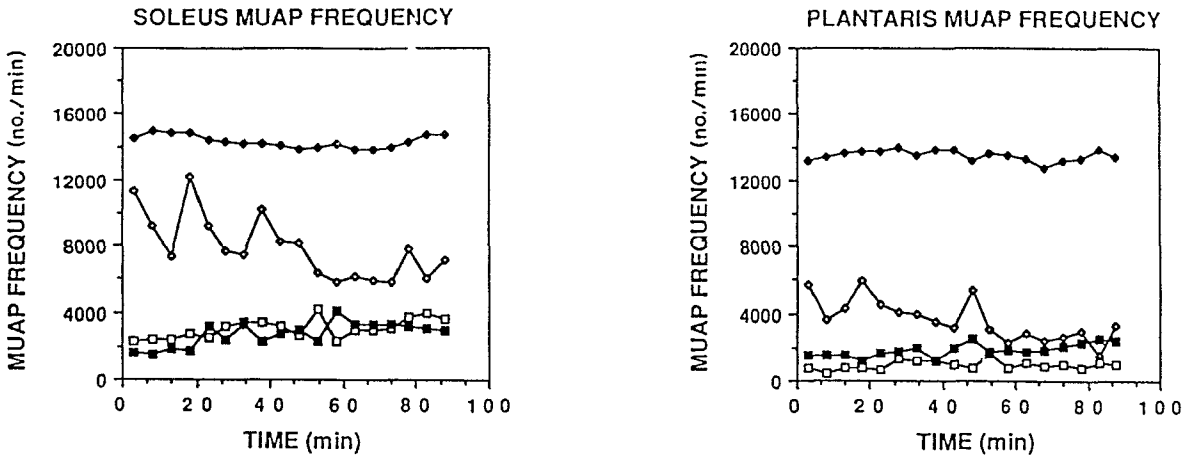
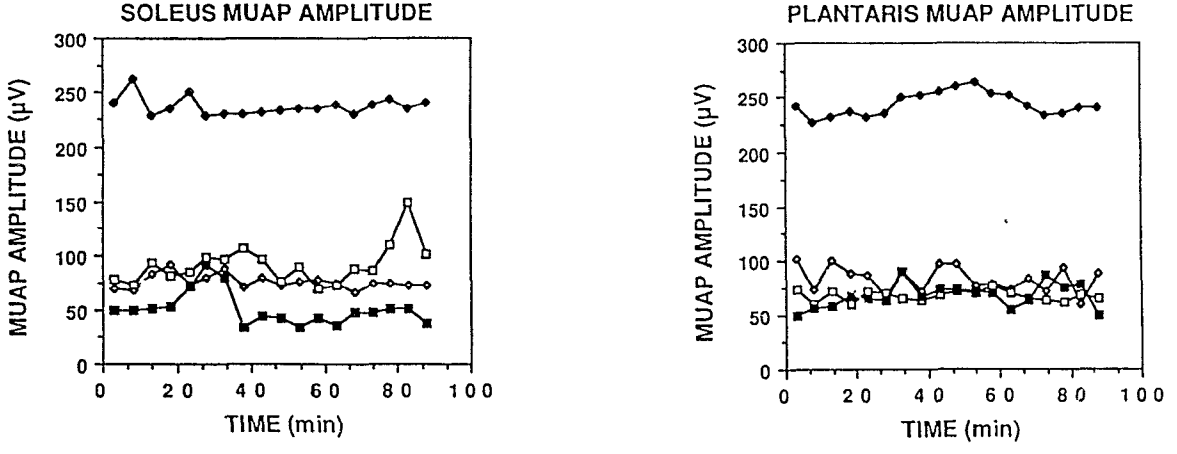

SOLEUS MUAP FREQUENCY
MUAP FREQUENCY (no./min)
20000
16000
12000
8000
4000
0
0 20 40 60 80 100
TIME (min)
PLANTARIS MUAP FREQUENCY
MUAP FREQUENCY (no./min)
20000
16000
12000
8000
4000
0
0 20 40 60 80 100
TIME (min)
REST
SUSPENDED REST
EXERCISE
SUSPENDED EXERCISE

Figure.3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MUAP AMPLITUDE (μV)
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250
200
150
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0
0 20 40 60 80 100
TIME (min)
PLANTARIS MUAP AMPLITUDE
MUAP AMPLITUDE (μV)
300
250
200
150
100
50
0
0 20 40 60 80 100
TIME (min)

APPENDIX 12. A manuscript submitted for publication in the Can. J. Phyiol. Pharmacol. as part of a published symposium. This manuscript is the written form of an invited presentation from a symposium on muscle metabolism given at the Canadian Association of Sport Science AGM Sept. 1990.

LACTATE METABOLISM AND TRANSPORT IN SKELETAL MUSCLE

Introduction

Over the last decade considerable research has been focussed on the production of lactic acid in skeletal muscle, specifically, as a fatigue-inducing metabolite during anaerobiosis (Gollnick et al. 1986; Gladden 1989). More recently there has been a surge of interest in the metabolic fate of lactate, and a re-evaluation of the role of lactate as an intermediary metabolite, either in its ionized or acid form (Connett et al. 1990; Brooks, 1986; Connett et al. 1984). The use of lactate as a precursor for glycogen synthesis in skeletal muscle (glyconeogenesis) has been the subject of considerable debate (Bar and Blanchaer 1965;Connett 1979; Hermansen and Vaage 1977; Hill 1924; Meyerhof (a) 1920; Meyerhof (b) 1920). However, it has now been determined that glyconeogenesis is a significant process in mammalian skeletal muscle (Bonen et al. 1990; Talmadge et al. 1989), and the relative contribution of lactate as a precursor for glycogen synthesis in muscle compared to the major glycogenic precursor, glucose, has recently been elucidated (Bonen et al. 1990). Historically, skeletal muscle is often considered to be primarily a producer of lactate, however, in some circumstances lactate can be taken up by a skeletal muscle and used as an energy substrate very efficiently (Richter et al. 1988). It has been recognized for some time that lactate is an important substrate for the normally functioning mammalian heart (Katz 1977). Recently, there has been speculation that lactate efflux from non-exercising muscle and subsequent uptake in working muscle may be a means of redistributing carbohydrate stores (Brooks 1986; McDermott et al 1987; Tinker et al. 1986). Yet, since lactate is ubiquitous, the concentrations of this metabolite in any body compartment is related to the membrane's permeability to either the acid and/or ionized form. Therefore, a potential level of regulation for lactate disposal resides at the membrane level via a specific monocarboxylate transporter that has been identified in several tissue types, including skeletal muscle

(Balkovetz et al. 1988; Brand and Stansbury 1981; Edlund and Halestrap 1988; Fafournoux et al. 1985; Deuticke; 1978; McDermott and Bonen 1989; Watt et al. 1988; Koch et al. 1981).

The focus of this paper is to review the current literature and present some new information on lactate metabolism and transport in skeletal muscle. Specifically, we will address the following issues: a) the importance of lactate as a glycogen precursor in skeletal muscle compared to glucose as a glycogen precursor; b) the use of lactate as an oxidative substrate in muscle and whether this is regulated by the muscle's endocrine milieu; c) the existence of a specific transporter for lactate in muscle and whether the transport of lactate could be rate limiting for metabolism; and d) the regulation of the lactate transporter.

1) METABOLIC END PRODUCTS OF LACTATE METABOLISM

1.1) MUSCLE GLYCOGEN RESTORATION BY LACTATE AND GLUCOSE.

Glucose as a substrate for glycogen synthesis (glycogenesis) has been known for some time (Bonen et al. 1989). However, glycogen can also be synthesized from lactate (glyconeogenesis). This reconversion of lactate into glycogen was first postulated by Meyerhof (Meyerhof (a) 1920; Meyerhof, et al. 1925)) and Hill (Hill 1924)) for frog skeletal muscle, and this has been reconfirmed by Connet (Connett 1979). Glyconeogenesis has now also beeen observed in mammalian skeletal muscle (Bonen et al. 1990; McLane and Holloszy 1979). McLane and Holloszy (McLane and Holloszy 1979) showed that non-contracting, perfused rat hindlimb muscles synthesized glycogen from lactate, and Johnson and Bagby (Johnson and Bagby 1988) demonstrated that glyconeogenesis occurred in the post-exercise period in rats. More recently, Talmadge et al. postulated that lactate can be used as a glycogen precursor in active as well as resting muscle (Talmadge et al. 1989).

Estimations of the relative contribution of glucose and lactate to glycogen synthesis vary widely, ranging from 50 to 95% in human muscle (Hermansen et al. 1977) and from 10 to 66% in rat muscles (Bonen et al. 1990; McLane et al. 1979). These approximations are gross estimates only and require a considerable number of arguable assumptions in humans (Hermansen et al. 1977), and in animals (McLane et al. 1979). There are, however, intrinsic differences in the rates of glycogenesis

and glyconeogenesis in different types of skeletal muscles (Fig 1). The greatest rates of glycogenesis occur in muscles rich in FOG and SO fibers, and the lowest rates in FG muscle fibers. Conversely, the rates of glyconeogenesis are most rapid in FG and FOG muscle fibers and slowest in SO muscle fibers (Fig 1). The reason for these differences in glyconeogenesis appear to be related to the greater activity of fructose 1,6 bis -phosphatase (McLane et al. 1979). Whether the activity of this enzyme is critical to glyconeogenesis is not known, since the pathway for muscle glyconeogenesis (in contrast to glycogenesis) is not known. The current hypothesis is that glyconeogenesis in mammalian muscle probably occurs by the energetically unfavourable reversal of the pyruvate kinase reaction to permit the formation of phospoenolpyuvate directly from pyruvate (McLane et al. 1979; Shiota et al. 1984), rather than via the 'malic enzyme' route proposed by Connet (Connett 1979) for frog sartorius muscle.

Insulin and muscle contractility are known to promote glucose transport and increase the activity of glycogen synthase I (for review see Bonen et al. 1989) Whether there are physiological stimuli for glyconeogenesis is not known. Studies in our laboratory have shown that glyconeogenesis is not altered by corticosterone, glucagon or insulin. (Fig 2). However, acidic pH (~6.5) of the extracelluar milieu (pHe) increases glyconeogenesis, (ie. increased rates by 40-50%)(Fig. 3) (Bonen et al. 1990). This may be related to the increased rate of lactate transport which also is stimulated by an inwardly directed proton gradient (see below). The physiological significance of this may be that glyconeogenesis is increased in the post-exercise period when the lactate levels are high and the pHe is reduced.

Despite the potential for glyconeogenesis, recent work in this laboratory has shown that at best only about 30% of muscle glycogen can be obtained from lactate (10mM) compared to the equivalent quantities of glucose (5mM glucose, or 10mM expressed as 3 carbon lactate equivalents), and then only in FT muscles. In soleus muscle negligible quantities of lactate are synthesized to glycogen (<5%) compared to that obtained from glucose (Fig 4).

In summary, a) glucose is the primary substrate for skeletal muscle glycogen, however, lactate can also provide a substantial quantity in FG and FOG muscle, b) glycogenesis and glyconeogenesis are increased by a reduction in pH to levels that are similar to those found in intensively exercised muscles, and c) there appears to be no regulation of glyconeogenesis by hormones that stimulate or inhibit glycogenesis in muscle or hormones that stimulate gluconeogenesis in liver.

1.2) LACTATE OXIDATION IN SKELETAL MUSCLE

It has long been known that lactate can be taken up by both inactive and active muscle and oxidized to CO_2 and water. Lactate removal by skeletal muscle has predominantly been considered as a means of recovery from a lactic acidosis rather than as a useful substrate during exercise in skeletal muscle. In contrast, the importance of lactate as an efficient energy source during contraction in cardiac muscle has been accepted for some time (Katz 1977).

In skeletal muscle the fate of glucose and lactate appear to be connected since the presence of lactate diverts glucose away from the glycolytic pathway towards glycogen formation. We have direct evidence to suggest that lactate may well be a very useful substrate in skeletal muscle. Recent studies in our laboratory have shown that when a muscle is bathed in a buffer containing lactate (5mM), the muscle consumes less glucose for oxidation and also redirects glucose to glycogen synthesis. Thus, lactate has a sparing effect on glucose oxidation in skeletal muscle (Fig 5). The sparing effects of lactate on glucose metabolism implies that lactate is efficiently oxidized as an energy substrate.

We (McDermott et al. 1987), and others (Brooks 1986; Tinker, et al. 1986) have proposed the idea that lactate may be produced in both active and inactive muscle and used elsewhere as a substrate. Specific evidence in our laboratory shows that glycogen is lost during exercise in non-exercising muscle and that this is metabolized primarily to lactate in such muscles (McDermott et al. 1987; McDermott et al. 1990). However, far from being a new idea, the first suggestion of such a mechanism dates back to 1930 when Owles (Owles 1930) postulated that lactate may be produced in some muscles and subsequently oxidized in other muscles, or even in adjacent fibers of the same muscle. The rejuvenated 'lactate shuttle' (Brooks, 1986; McDermott, et al. 1990) hypothesis makes considerable teleological sense, however, the importance of this mechanism in providing substrate to active muscle during exercise has yet to be quantified. One possible benefit may be the sparing of glucose utilization by muscle by directing this substrate to glycogen when lactate is available (see above).

One dichotomy which results from the idea that lactate is produced by non-exercising muscle is that muscle has been observed to act as a lactate 'sink' in some situations ((Gollnick et al. 1986; McGrail et al. 1978; Richter et al. 1988). This may

be resolved if one considers the driving forces behind lactate movement. We hypothesize that the prevailing lactate concentrations determine whether lactate is produced or utilized by non-exercising muscle. When arterial lactate concentrations increase dramatically then the non-active muscle may act as a 'sink' because the lactate concentration gradient is directed inward, but when the arterial lactate concentration is stable (1-2mM) and the intramuscular lactate concentration increases, due to the adrenergic stimulus of exercise, lactate may efflux from the non-exercising muscle. Therefore the direction of lactate movement into or out of non-exercising muscle may be related to the lactate concentration gradient.

An interesting study by Richter et al. (Richter et al. 1988) lends some support to this hypothesis, in this study substrate usage in exercising humans was measured by arterial- venous catheterization and blood flow measurements across knee extensor muscles, with subsequent analytical determination of substrate concentrations. During work with large muscle masses it was found that arterial lactate concentrations increased to ~6mM, at this point it was found that net lactate release from the leg reverted to net lactate uptake. Also, when exercise was performed with one leg and two arms the uptake of lactate was greater in the working leg than in the resting leg.Previous work in this laboratory has shown a high relationship between oxygen consumption and lactate removal from the blood (McGrail et al. 1978). These studies support the idea that lactate can be used as a substrate by working muscle, as well as by resting muscle. These data indicate the complexity of metabolism *in vivo*, and the discrepancies which can result from the prevailing systemic and intracellular conditions.

The rate of lactate oxidation by muscle is related to the transmembrane concentration gradient in a linear manner (Fig 6). Another factor which may influence the rate of lacate utilization in skeletal muscle is the endocrine milieu. In view of the potent effect of epinephrine on muscle glycogen metabolism ((Dietz et al. 1980; Nesher et al. 1980; McDermott, et al. 1987; Stainsby et al. 1985; Stainsby et al. 1987), we also assessed its effect on lactate oxidation. Exposure of soleus muscle strips to epinephrine (10^{-6}M) resulted in an increase in the amount of $^{14}CO_2$ produced from ^{14}C- L-lactate in the incubation buffer. Incubation with the beta receptor antagonist propranolol negated the increased oxidation of lactate in the presence of epinephrine (Fig. 7). These data indicate that the epinephrine-stimulation

of lactate oxidation may be linked to the beta-adrenergic cascade- cAMP system which also stimulates skeletal muscle glycogenolysis (Dietz et al. 1980) and exerts an inotropic effect on muscle contraction (Nesher et al. 1980)

Epinephrine might mediate these effects on lactate oxidation in a relatively non-specific way by enhancing the metabolic rate of the cell, as opposed to having a direct effect on the lactate transporter itself. The enhanced metabolic rate is usually associated with the enhanced activity of key rate limiting enzymes in glycolysis (Trivedi and Danforth 1966). However, it is also possible that when the metabolic rate is enhanced, but glycolytic rate is not disproportionately increased, that the greater rate of lactate utilization may be due to elevated pyruvate removal for oxidative metabolism in the Krebs cycle. Enhanced pyruvate removal thus maintains the inwardly directed lactate concentration gradient, which would dissipate if lactate were allowed to accummulate. The potential importance of the beta adrenergic system for lactate oxidation in vivo has yet to be determined.

2) LACTATE TRANSPORT IN SKELETAL MUSCLE

In the previously described studies we have shown the importance of lactate as a) an oxidative substrate and b) a potential glyconeogenic precursor in skeletal muscle. In view of the central role of L-lactate in skeletal muscle metabolism (Gladden 1989; Gollnick et al. 1986; Connett, et al. 1990; Katz and Sahlin 1988) there would clearly be an advantage in having a specific transport system mediating rapid L-lactate traversal of the muscle membrane. The role of carrier-mediated transport processes in the movement of L-lactate have been described in detail for several tissue types (Balkovetz et al. 1988; Fafournoux et al. 1985; Trosper and Philipson 1987; Deuticke et al. 1978; Spencer and Lehninger 1976). In contrast, there is evidence suggesting the presence of an L-lactate carrier in the sarcolemmal membrane of skeletal muscle cells(Koch et al. 1981; Juel and Wibrand 1989). The key evidence of showing a saturable, carrier-mediated process for L-lactate transport, while accounting for cellular metabolism of the transported solute and measuring accurate initial rates of unidirectional transport, has not yet been provided.

2.1) Lactate transport kinetics and effects of inhibitors.

Previous investigations have suggested that lactate transport is mediated by two

components in skeletal muscle cells: a carrier mediated system and a non-saturable, diffusive process (Watt, et al., 1988). Recent studies in isolated muscle (Koch et al. 1981; McDermott et al. 1989; Juel et al. 1989) and in the perf ·d rat hindquarter (Watt et al. 1988) suggest the presence of a putative carrier for L-lactate in skleletal muscle. Carrier-mediated transport of L-lactate has been implicated in skeletal muscle lactate transport primarily by the effects of several monocarboxylate inhibitor compounds on L-lactate uptake in intact cell preparations (Juel et al. 1989; Juel 1988; Koch et al. 1981).

State of the art methods developed by membrane biochemists are now being utilized to study transport phenomena in skeletal muscle(Sternlicht et al. 1988; Kinget al. 1989). These methods involve the use of highly purified plasma membranes which re-seal to form vesicular structures. Because of the sealed nature of these sarcolemmal vesicles the transport of radiolabelled substrates into the intra-vesicular space across the membrane can be carried out using a vacuum filtration method (Grimditch et al. 1985; Trosper et al. 1987). There are several advantages to these studies; a) very rapid kinetic measurements can be carried out (<5 second uptake measurements), which are crucial for initial rate measurements, b) there is no interference from metabolism of the transported solute, and c) both the intra-vesicular and extra-vesicular milieu can be closely controlled by the investigator. Studies in our laboratory on isolated membrane vesicles corroborate the idea that a saturable, stereospecific, carrier for L-lactate exists in skeletal muscle plasma membranes (Fig 8). Lineweaver-Burke transformation of the concentration dependent L-lactate transport into the vesicles yields a Km value of ~4mM (Fig. 8). In addition, a non-saturable diffusive component of the transmembrane lactate movement is present in the isolated membrane vesicles and also in isolated muscle strip preparations. This diffusive component of lactate flux is illustrated by a) the incomplete saturation of lactate transport with increasing lactate concentrations (Fig.8,10), and also the incomplete inhibition of lactate flux by monocarboxylate and protein inhibitors (Tables 1,2)

Further proof of the existence of a membrane carrier for L-lactate is provided by the effects of various inhibitor compounds on L-lactate uptake. The inhibition of L-lactate uptake by the presence of pyruvate and alpha- cyano-4- hydroxycinnamate suggests that the carrier may be a general monocarboxylate carrier (Table 1).The sulphhydryl group modifier, N-ethylmaleimide, had a marked effect on reducing L-lactate uptake providing persuasive evidence that a protein is involved in the lactate flux. The possibility arises that lactate could be transported by the inorganic anion

exchanger. However, this hypothesis is refuted by the observation that the inorganic anion exchanger, SITS, had no effect on lactate uptake (Table 2). Complete substitution of Na^+ in the extracelluar and intracellular medium, using methylglucamine as the substituting ion, had no effect on lactate transport indicating that lactate is not co-transported with sodium ions (Table 2).

The measurement of unidirectional flux for kinetic analysis is most easily and accurately accomplished in an isolated membrane vesicle preparation (Clausen 1986). However, while this elegant method allows the study of transport systems in the natural membrane matrix in the absence of cell metabolism, it also divorces transport processes from the metabolic processes that they serve, and thus eliminates a potential level of regulation, ie. the coupling of mass solute transport with cell metabolism. In addition, because the preparation of purified isolated vesicles is a lengthy procedure (~24 hrs) and does not leave the intracellular signalling mechanisms intact, it does not easily allow insight as to how an acute alteration in the metabolic or hormonal milieu of the cell might alter the vectorial flux of solutes. Therefore, a combined approach (Katz 1977) would be useful in investigating a transport system of interest ; ie. to use both an isolated vesicle preparation and an intact cell preparation, in order to begin to study not only the basic characteristics of a particular transport system (sarcolemmal preparation) but also how acute changes in the cellular metabolic / hormonal status might regulate transport processes (muscle strip preparation).

In order to validate this approach to the study of cellular transport we carefully compared the transport kinetics of unidirectional L-lactate transport in isolated skeletal muscle sarcolemmal vesicles, and L-lactate uptake in an intact cell, muscle strip preparation. By assuming that ~12% of cell wet weight is protein and ~2% of the total protein is sarcolemmal membrane protein (Frank et al. 1984), we calculated that under similar uptake conditions (pH 7.4, 1mM L-lactate) the transport rates were quite similar, ie. 0.05 nmol · mg $protein^{-1}$· s^{-1} in isolated intact muscle strips and 0.06 nmol · mg $protein^{-1}$· s^{-1} in the SL vesicles. Furthermore, there is also considerable consistency in the data if the two preparations are compared. For example, Fig. 10 shows that when the transport data are expressed as a percentage of the maximal transport rates for each preparation, the concentration dependent uptake curves show similar trends with the initial linear portion of the curves for each prepartion having similar slopes. The concentrations at which the transport saturation occurs are similar for both preparations. In addition, the uptake data as a function of changing external pH also show similar trends (Fig 11). These

observations indicate that the uptake measurements made in the intact muscle strips, if performed under initial rate conditions, give an acceptable index of membrane transport. Therefore, the combined approach of verifying intact cell uptake measurements with membrane vesicle studies to determine the similarity of the observed transport behaviour has potential benefits. If the comparison between the preparations show similar trends and respond to manipulations in the same direction, then physiological manipulations of the intact cell may be performed, which are not always possible in the vesicle preparation, and a useful index of membrane transport can be still be obtained. This approach may prove useful in the investigation of how acute changes in hormone binding alter cell membrane L-lactate transport, or indicate how an alteration in cell metabolism might alter the membrane transport of lactate, or provide insight into how the separate processes of membrane transport and intracellular metabolism are co-ordinated.

In the aforementioned studies the transport of lactate was studied from the extracellular compartment into the intracellular compartment, however the transport of lactate in the opposite direction (ie. from the intracellular to the extracellular compartment) is also of considerable importance, especially when glycolytic flux is high. Therefore, it is interesting to consider if lactate transport behaviour is similar in both directions. Connet et al. (Connett et al. 1984) provided some insight into this question in their studies on isolated vascularly intact dog gracilis muscle. In these studies a period of electrical stimulation was used to manipulate intracellular lactate concentrations in the range; 1-25mM, the subsequent rate of lactate efflux was then measured in the venous outflow from the muscle. From these data it can be clearly seen that as the intracellular concentration of lactate is increased (via the stimulation protocol) above ~12mM there is a plateau in the rate of efflux of lactate (Fig 9), these data are consistent with the saturable carrier hypothesis, and therefore complement the studies which have examined transport behaviour in the opposite direction. However, accurate determination of the symmetry of the transport system in terms of the rates of transport in both directions have yet to be investigated.

2.2) Is lactate transported in its ionized or acid form?

The possibility that lactate transport may be related to the protonated (HLa) rather than the ionic (La^-) form deserves some consideration even though lactic acid is almost completely in the dissociated form at normal physiological pH. In fact, the possibility that the protonated form is the permeant form would explain the observation that acidic external pH enhances lactate transport. In order to evaluate whether lactate entry is related to the external concentration of the non-dissociated

lactic acid (HLa) rather than the lactate ion (La^-), the rates of lactate uptake for the SL vesicle and muscle strip preparations were plotted against calculated values of non-dissociated lactic acid, based on a pK_a of 3.86 at room temperature (Gladden 1989), for two different manipulations :
a) constant pH (7.4) and varied concentrations of total lactate (0.5;1;10;25 mM);
b) constant L-La (1mM) and varied external pH (8.0; 7.4;6.5; 6.0).
If the non-dissociated form is the only permeant form then both manipulations should yield the same uptake for a given concentration of non-dissociated lactic acid (HLa). The discrepancy in the uptake curves for the two manipulations (Fig12) shows that lactate transport was much greater when changes in the acid form (HLa) corresponded to changes in the total lactate concentration, and therefore the ionic form. The dissimilar uptake data for similar concentrations of external undissociated La, indicates that lactate uptake is not simply related to the external concentration of undissociated lactic acid, and therefore, the uptake of lactate is more closely related to changes in the ionic form of L-lactate.

2.3) Lactate production and maximal transport The intracellular acidification associated with muscle fatigue is generally thought to be related to a lactic acidosis (Fitts and Holloszy 1976; Juel 1988; Katz et al. 1988; Mainwood and Renaud 1985; Metzger and Fitts 1987). Despite the general consensus that lactic acid production is responsible for the cellular acidification it is important to note that the terminal reaction in the anaerobic production of lactic acid does not produce net H^+, and therefore does not lead to acidification of the cell interior. Busa and Nuccitelli (Busa and Nuccitelli 1984) carefully point this out, as the complete balanced equations for glycolysis accounting for mass and charge yield no surplus H^+ when lactic acid is produced (see equation 1 below);

Net reaction for glycolysis:

$$\text{Glucose} + 2\text{Mg ADP}^{2-} + 2\text{Pi}^{2} \text{----------} > 2\ \text{lactate}^{-} + 2\ \text{Mg ATP}^{2-} + 2\text{H}_2\text{O} \quad (1)$$

However, it is the subsequent hydrolysis of ATP generated fom glycolysis that leads to the production of H^+ (see equation 2 below);

Net rection for ATP hydrolysis:

$$Mg\ ATP^{2-} + H_2O \text{ ----------> } Mg\ ADP^{2-} + Pi^{2-} + H^+ \qquad (2)$$

However, because lactic acid production is indirectly associated with muscle fatigue, an interesting issue concerns the capacity for lactate transport compared to lactate production in skeletal muscle. The maximal rate of lactate production in skeletal muscle has been reported to be ~0.25 umol· g^{-1}· s^{-1} (Meyer and Terjung 1979). If we convert the vesicle derived transport data on lactate transport to the same units, based on the previously cited assumptions, the maximal rate of lactate transport is ~0.03 umol · g^{-1}·s^{-1}. Assuming that the transport rates are symmetrical (maximal inward transport ≃ maximal outward transport) then the maximal rate of lactate production is ~8 times the maximal lactate transporting capacity of intact skeletal muscle. It is therefore clear from this comparison that at times when muscle glycolytic flux is maximal the membrane transport of lactate would be limiting for lactate removal. This supports the numerous studies in the literature which have reported the onset of an intracellular lactic acidosis in skeletal muscle when the glycolytic flux is maximal (Gollnick, et al., 1986).

2.4) Molecular aspects of the lactate transporter

Considerable homogeneity seems to exist between the properties of the lactate transporters in several tissue types. An L-lactate carrier with pH dependency and sensitivity to similar monocarboxylate and protein inhibitors has been postulated for Ehrlich ascites cells (Spencer et al. 1976), hepatocytes (Fafournoux et al. 1985; Edlund et al. 1988) , cardiac cells (Trosper et al. 1987) , erythrocytes (Deuticke et al. 1978), placental brush border membranes (Balkovetz et al. 1988) , and intact skeletal muscle (Watt et al. 1988) (Table 3 compares the characteristics of lactate transport in these tissues).Several attempts have been made to identify the lactate transporting protein in several tissues. A protein of molecular size 40-50 kD was found to be labelled by a radioactive analog of DIDS (4,4'- diisothiocyanato- 2,2' - dihydrostilbenedisulfonate) in rabbit erythrocyte ghosts , DIDS inhibited lactate

transport in the same preparation (Jennings and Adams-Lackey 1982) . In a subsequent study, Donovan and Jennings (Donovan and Jennings 1985) reported a decrease in the labelling of this protein by [^{3}H] H_2 DIDS when other lactate transport inhibitors were included in the incubation. However, the specificity of these inhibitors has been questioned (Poole and Halestrap 1988; Poole et al. 1990). A lactate binding protein has been purportedly isolated from rat hepatocyte plasma membranes with an apparent M_r of 40 Kd, determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS page)(Welch et al. 1984). Whether this protein is the lactate carrier protein has yet to be confirmed. Poole and Halestrap (Poole and Halestrap 1989) have since been able to reconstitute solubilized rat and rabbit erythrocyte membrane proteins into liposomes, these proteoliposomes were shown to have an accelerated rate of monocarboxylate transport, indicating that the lactate transport protein can be reconstituted and retain it's transport capacity. A subsequent report from the same group has recently documented substantial differences in the affinity of the lactate carrier from cardiac myocytes and erythrocytes for various monocarboxylates (myocytes 10 fold higher than erythrocytes) (Poole 1990), possibly implying the presence of distinct carriers in these tissues. One feasible explanation for these observations is that there is a family of related tissue specific lactate carrier proteins which have different affinities for the substrate. Further molecular characterisation of the lactate transport carrier protein will help in elucidating the extent of the homology between different tissues.

3) LACTATE TRANSPORT :EFFECTS OF ACUTE AND CHRONIC CONTRACTILE ACTIVITY

3.1) Contractile regulation of lactate uptake.

It is well known that a bout of sustained contractile activity enhances glucose uptake in skeletal muscle for a prolonged period after cessation of the stimulus. This post-contraction enhancement of glucose transport is sustained above basal levels for several days after exercise (Bonen et al. 1988). As the transport of glucose is mediated by a membrane bound transporter protein this permissive effect of contraction on glucose uptake is thought to involve a recruitment of more

transporters to the muscle membrane and/or a change in the intrinsic activity of the transporter (Bonen et al. 1988). Lactate uptake also seems to be mediated by a membrane protein and we therefore hypothesised that skeletal muscle lactate transport might be regulated by contraction in an analogous manner to glucose transport. After 30 min. of *in situ* electrical stimulation (The hindquarter muscles of one leg innervated by the sciatic nerve were electrically stimulated to contract with 300 ms trains at a supramaximal 10-V intensity and a 1 Hz frequency. Trains consisted of 3 separate impulses in 300 ms, each 0.3 ms in duration. The stimulation period was 30min. in duration.), glucose and lactate uptake measurements were made on stimulated and control rat soleus muscles (the control limb was subjected to all manipulations, suchas the sciatic nerve isolation, except for the electrical stimulation.). It was found that depite a significant increase in glucose transport after the stimulation protocol in the electrically stimulated muscle compared to the control muscles, there was no difference in lactate uptake between stimulated and contralateral non-stimulated soleus muscles (Fig. 13). These preliminary data indicate that the lactate transporter is not regulated by an acute bout of contraction in a similar manner to the glucose transporter.

3.2) Effects of chronic contractile activity on lactate transport.

Lactate accummulation in skeletal muscle at any work rate is reduced after a period of endurance training (Favier et al. 1986). One explanation for this phenomena is the increased oxidative capacity of the muscle and a reduction in lactate production (Favier et al. 1986). Conversely, Donovan and Brooks have argued that the production of lactate is unaffected by training but the clearance rate (the ratio of lactate removal to lactate concentration) is altered (Donovan and Brooks 1983). A more recent study has furthered the case for the 'enhanced clearance' hypothesis, reporting that endurance trained rats have an enhanced capacity to dissipate an exogenously infused lactate load compared to sedentary rats (Donovan and Pagliassotti 1989; Donovan and Pagliassotti 1990). The mechanisms which mediate the enhanced efficiency for lactate removal in trained animals have not been elucidated. The clearance rate could potentially be altered by 1) enhanced clearance in other organs such as the heart, liver and kidney (Brooks, 1986), 2) increased uptake in non-contracting muscle for oxidation,(Richter, et al., 1988) and 3) removal

by contracting oxidative fibers (Richter et al. 1988). In reality probably all of the aforementioned factors play a role in the enhanced lactate clearance seen with training. The muscle membrane transport of lactate can be seen to be a fundamental aspect of such clearance, both in the need for La efflux from muscle which is producing lactate, and also for lactate influx into less active muscle for oxidation and/or glyconeogenesis.

Transmembrane lactate flux may well be an important 'unconsidered' aspect of the training response because lactate efflux in muscle is known to be greatly exceeded by the rate of lactate production when glycolysis is accelerated (see previous comparison of the rates of lactate flux and transport). Therefore, an alteration in the capacity of the membrane to increase the rate of lactate transport would result in a reduced intracellular concentration for a given rate of lactate production. Thus, in view of the foregoing discussion, it was hypothesized that the lactate transporting capacity of skeletal muscle would be increased as a result of endurance training.

In order to test this hypothesis purified sarcolemmal vesicles were isolated from endurance trained and sedentary control animals and lactate transport measurements were made to assess the membrane capacity for lactate transport. When the lactate transport capacity of the membranes was assessed at a lactate concentration (1mM) below the Km value (~4mM), an enhanced capacity for lactate transport was observed in the trained animals (~30% stimulation of transport) (Fig. 14A). Conversely, when the experiments were carried out at high external lactate concentrations corresponding to Vmax of the transport system (50mM) there was no difference in the lactate transport capacity of the membranes isolated from sedentary or endurance trained rats (Fig. 14 B). These observations imply that there may be a change in the affinity of the existing transporters with training (ie. altered Km), without a simultaneous increase in the number of transporters in the membrane (ie. no change in Vmax) (Fig 15). This would effectively mean that at normal physiological concentrations of lactate (1-10mM) the muscle membrane capacity for transporting lactate is enhanced.

4) Speculation concerning pH as a second messenger

In both the SL vesicle and muscle strip preparations we have consistently observed that an inwardly directed proton gradient markedly stimulates the vectorial flux of L-lactate (Fig. 11). The dependence of the lactate uptake on a proton gradient is not without precedence as studies on other tissues have also also shown a marked increase in lactate flux when confronted with inwardly directed lactate and proton gradients (Balkovetz et al. 1988). This mechanism of proton coupled symport for various organic solutes is being recognized as a recurring motif of regulation in several mammalian transport systems. Recently the role of pH as a potential intracellular messenger has received some attention (Busa et al. 1984). It is interesting to consider this possibility in relation to the co-ordination of skeletal muscle glycolysis (lactate production) and the mass transport of lactate.

The question is; how does the muscle cell maintain intracellular lactate concentration within tolerable limits in the face of markedly varying rates of lactate production and transport ?. Clearly, if the production (glycolysis) and transport mechanisms for lactate operate independently and in an uncoordinated manner, it is difficult to see how the intracellular lactate concentration can be homeostatically regulated to maintain reasonable intracellular constancy. Obviously some form of 'cross talk' between the two processes would be necessary to co-ordinate their behaviour. Intracellular pH has been shown to have an important role in regulating glycolytic flux, primarily by it's allosteric effects on key rate limiting enzymes (Trivedi et al. 1966) , and a pH gradient is also a potent regulator of lactate transport . Therefore, a speculative hypothesis can be proposed in which both processes may be regulated by the pH status of the intracellular and extracellular milieu to control the production (glycolysis) and efflux/influx mechanisms simultaneously and in a co-ordinated manner (Fig. 16). If we pursue this hypothesis and consider the following scenario; the muscle cell is contracting vigorously and glycolytic flux is high, the intracellular concentrations of lactate and H^+ are increasing exponentially to levels which would endanger cell integrity. Two mechanisms would be simultaneously mobilized by the 'pH signal' to inhibit a further build up of these potentially hazardous metabolic by-products : a) the decrease in pHi due to the increased H^+ would inhibit key glycolytic enzymes to effectively sharply reduce the further production of lactate and H^+, and b) the outwardly directed pH gradients would simultaneously stimulate the lactate transport system to efflux lactate and H^+ into the extracellular compartment which is at a higher pH. Thus, a return towards constancy of the intracellular milieu is achieved.

Summary

We have reviewed lactate transport and metabolism in skeletal muscle with an emphasis on recent information. In this review a number of significant points are made. These are summarized below:

(i) Glyconeogenesis is a significant process in white FT skeletal muscle, supplying at best 30% of the precursor for glycogen synthesis compared to glycogen derived from glucose.

(ii) Lactate can be efficiently oxidized in muscle diverting glucose to the 'anabolic' glycogenic pathway, as opposed to the 'catabolic' glycolytic pathway.

(iii) Lactate oxidation in skeletal muscle is stimulated by epinephrine.

(iv) Membrane transport of L-lactate is mediated by a specific monocarboxylate transporter.

(v) When glycolytic flux is maximal the membrane transport of lactate may be rate limiting for lactate removal and lead to intracellular accummulation.

(vi) Lactate transport into muscle is very sensitive to a pH gradient.

(vii) We hypothesize that pH may act as a cellular signalling mechanism to co-ordinate glycolytic flux and lactate transport simultaneously.

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Legends

Fig. 1. A. Glyconeogenesis and glycogenesis in perfused hindlimb muscles (mean ± SE, n=3-5 animals). Perfusions were performed with cell free media for 20 min. (glyconeogenesis) or 60 min. (glycogenesis). Rat hindlimb perfusions were performed in a manner similar to those described by our lab elsewhere (McDermott et al 1989). RG, red gastrocnemius; WG white gastrocnemius, SOL soleus.
Glycogenesis : no insulin SOL> RG> WG ($p<0.05$, t test); +insulin SOL>WG, RG>WG.
Glyconeogenesis: RG> SOL, WG>SOL ($P<0.05$, t test).

B: Rates of glycogenesis and glyconeogenesis in relation to muscle fiber composition of the rat hindlimb. Glyconeogenesis and glycogenesis data are from Fig A and muscle fiber type data are from McDermott et al 1987.

Fig. 2. Effects of corticosterone on glyconeogenesis in soleus and extensor digitorum longus muscles incubated in vitro. Values are means ± SE. Data are based on 28 control muscles and 4 muscles at various hormone concentrations.

Fig. 3. Effects of pH on glyconeogenesis in EDL and SOL muscles incubated in vitro (means ±SE) . Data are based on 10-16 muscles per point. Effects of pH are significant for each muscle ($P<.05$, ANOVA).

Fig 4. Relative contributions (%) of lactate (La) and glucose (gluc) to formation of glycogen in soleus and EDL muscles incubated in vitro. Basal glycogenesis (5mM glucose, no insulin) and glyconeogenesis at two lactate concentrations (10 and 20 mM) are shown in relation to rate of glyconeogenesis [100%; 5mM glucose, 10nM insulin (ins)]. Data are calculated from mean values, N= 5-8 muscles per point.

Fig. 5 Sparing effect of L-lactate (5mM) on glucose metabolism in mouse soleus and EDL muscles incubated in vitro. A: glucose oxidation in the presence and absence of insulin (10nM) with and without 5mM L-lactate in the incubation buffer. B: Glycogenesis from glucose in the presence and absence of insulin (10nM) with and without 5mM L-lactate in the incubation buffer. Values are means ± SE, N= 5 muscles per point.

Fig. 6 - Oxidation of [U-^{14}C] lactate as a function of time in isolated rat soleus muscles. Results are expressed as umol substrate oxidized / g wet wt. B- Effect of medium lactate concentration on lactate oxidation. Rates are based on $^{14}CO_2$ production from [U-^{14} C] lactate. Data is adapted from Connett and Pearce (1980).

Fig. 7 The oxidation of L-lactate in incubated rat soleus muscle strips in 3 conditions; a) control, b) with 10^{-6}M epinephrine in the incubation buffer, c) with 10^{-6}M epinephrine and 10^{-5}M propranolol in the incubation buffer. Values are means ± SE, N=5 muscles per point.

Fig. 8 Dependence of L-lactate transport on external L-lactate concentration in sarcolemmal vesicle experiments. Values are means of triplicates for at least 3 separate vesicle preparations *Inset* Lineweaver Burke plot of data. Slope, which is least squares fit of the data, gives apparent Km for L-lactate of ~4mM.

Fig. 9. The efflux of lactate from skeletal muscle as a function of the intracellular lactate concentration. Intramuscular lactate was manipulated by electrical stimulation. These data were adapted from the study of Connet et al .

Fig. 10. Dependence of L-lactate transport on external L-lactate concentration in sarcolemmal vesicle experiments and muscle strip preparations. Data are expressed as a % of the maximal rate of transport in order to facilitate comparison between the two preparations. Values are means of triplicates for at least 3 separate vesicle preparations. For the muscle strips values are means ± SE, N=6-13 muscle strips per point.

Fig. 11. Initial rate of L-lactate transport in sarcolemmal vesicle and muscle strip preparations, as a function of external pH. Internal pH is 7.4. Values are means ± SE of triplicate determinations for 4 experiments with different vesicle preparations.Values for muscle strips are means ± SE, N= 5 muscles per point.

Fig. 12. Relation between external undissociated form of L-lactic acid and the rate of transport in sarcolemmal vesicles. Variations of undissociated form were obtained in two ways: constant pH (7.4) and increasing concentration of total lactate (0.5, 1,10,25 mM); constant concentration of lactate (1mM) with changes in the external pH (8.0, 7.4,6.5,6.0).Values are means ± SE for 4 experiments.

Fig. 13 The effects of electrical stimulation on the uptake of D-glucose and L-lactate in skeletal muscle strips. Hindlimb muscles from one leg were electrically stimulated via the sciatic nerve for 30 min. (). After the stimulation period the stimulated and contralateral non-stimulated soleus muscles were stripped and incubated in vitro for lactate and glucose uptake measurements. A: Uptake of DOG in control and stimulated muscles. B: uptake of L-lactate in control and stimulated muscles at three different external lactate concentrations.Values are means ± SE, N= 12 muscles per point.

Fig. 14. The effects of endurance training (5 weeks) on lactate transport in isolated sarcolemmal vesicles. A: Transport measurements taken at external concentration of 1mM L-lactate at pH 7.4 and 6.5. B: Transport measurements taken at external concentration of 50 mM L-lactate.Values are means of triplicates for at least 3 separate vesicle preparations.

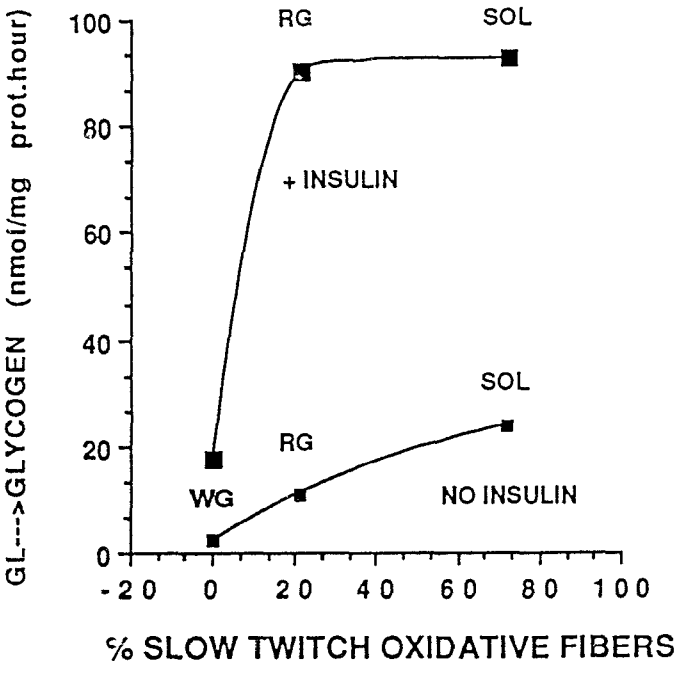
Fig. 15. A hypothetical explanation of how endurance training might alter the kinetics of lactate transport in skeletal muscle, based on the experimental data in Fig. 14.

Fig. 16. A schema depicting the effects of intracellular pH (pHi) on the energy generating pathways and transmembrane lacate flux in skeletal muscle (See text for explanation).

LA---->GLYCOGEN (nmol/mg prot.20min)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
SOLEUS
RG
WG
MUSCLE

GI---->GLYCOGEN (nmol/mg prot.h)
0
20
40
60
80
100
120
140
SOL
RG
WG
MUSCLE
+ insulin
no insulin

Figure. 1a


GL--->GLYCOGEN (nmol/mg prot.hour)
100
80
60
40
20
0
RG
SOL
+ INSULIN
SOL
RG
WG
NO INSULIN
-20
0
20
40
60
80
100
% SLOW TWITCH OXIDATIVE FIBERS

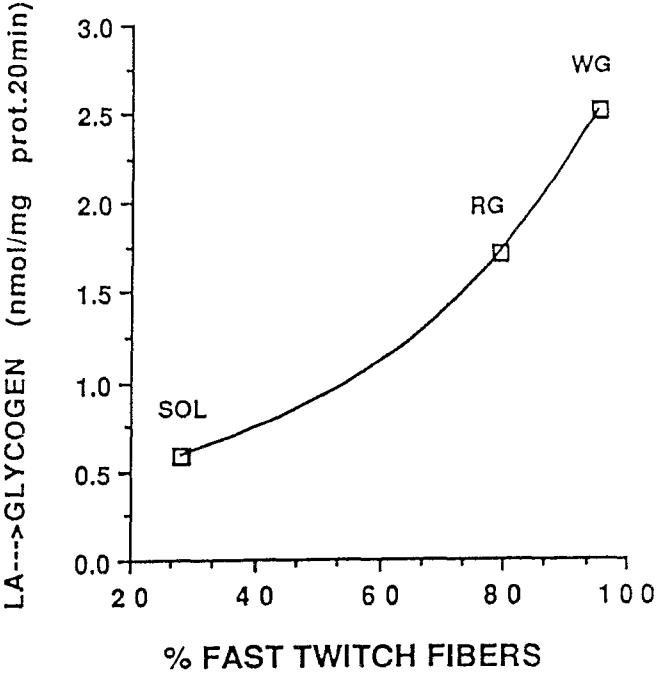
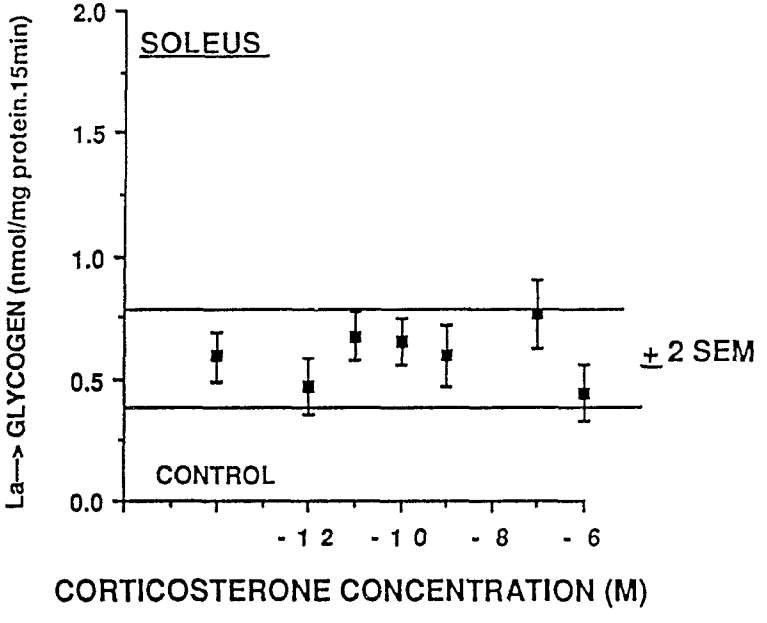

LA--->GLYCOGEN (nmol/mg prot.20min)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
WG
RG
SOL
20
40
60
80
100
% FAST TWITCH FIBERS

Figure. 1b


SOLEUS
2.0
1.5
1.0
0.5
0.0
La---> GLYCOGEN (nmol/mg protein.15min)
± 2 SEM
CONTROL
-12
-10
-8
-6
CORTICOSTERONE CONCENTRATION (M)

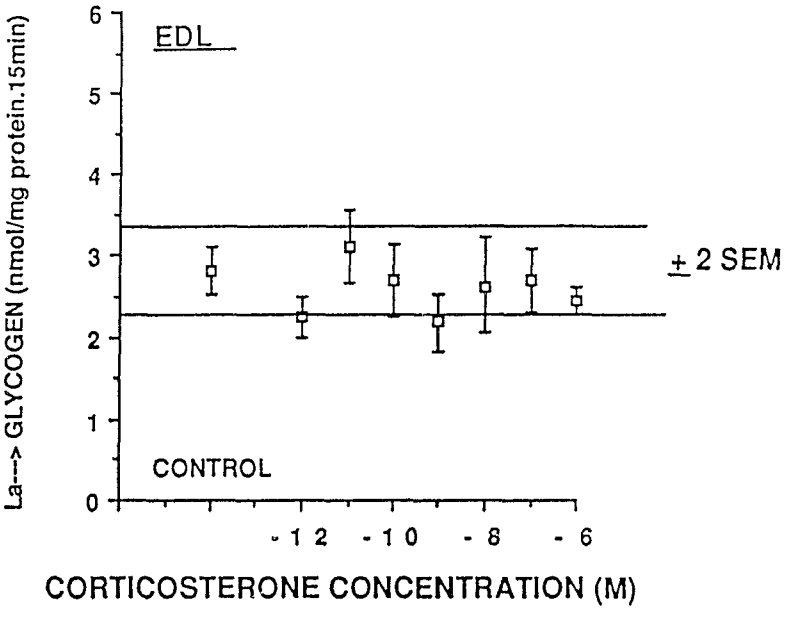

EDL
6
5
4
3
2
1
0
La---> GLYCOGEN (nmol/mg protein.15min)
± 2 SEM
CONTROL
-12
-10
-8
-6
CORTICOSTERONE CONCENTRATION (M)

Figure.2

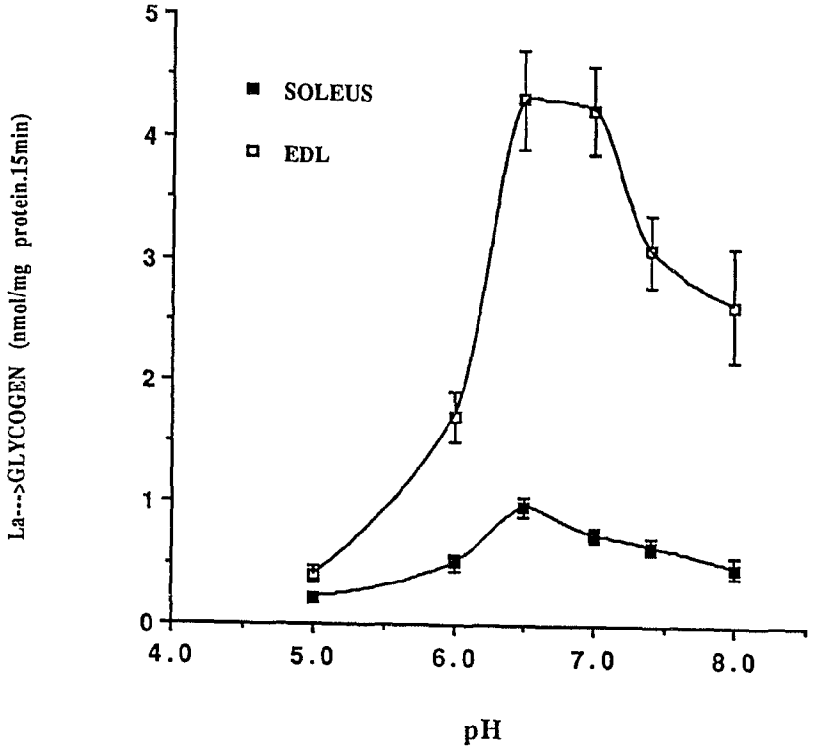

La--->GLYCOGEN (nmol/mg protein.15min)
SOLEUS
EDL
5
4
3
2
1
0
4.0
5.0
6.0
7.0
8.0
pH

Figure.3

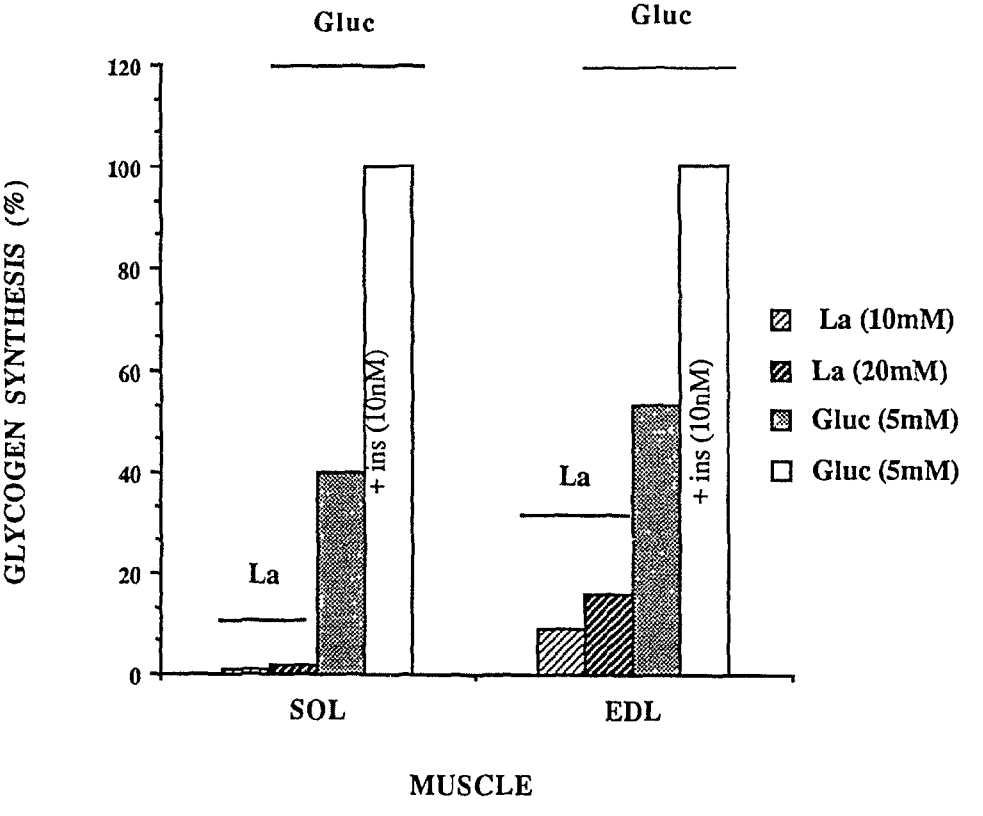

Gluc
Gluc
120
100
80
60
40
20
0
GLYCOGEN SYNTHESIS (%)
La
+ ins (10mM)
La
+ ins (10nM)
La (10mM)
La (20mM)
Gluc (5mM)
Gluc (5mM)
SOL
EDL
MUSCLE

Figure. 4

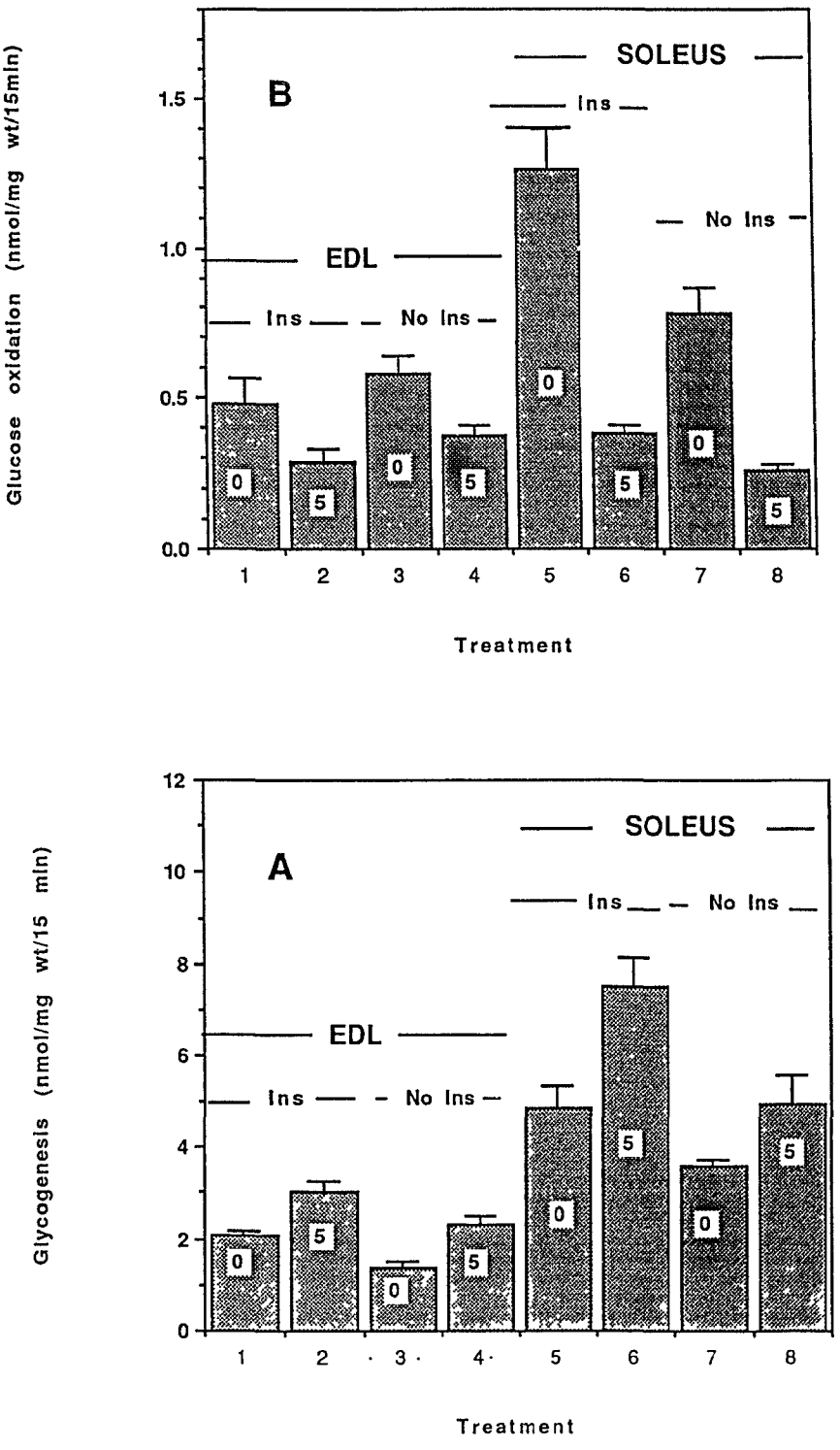
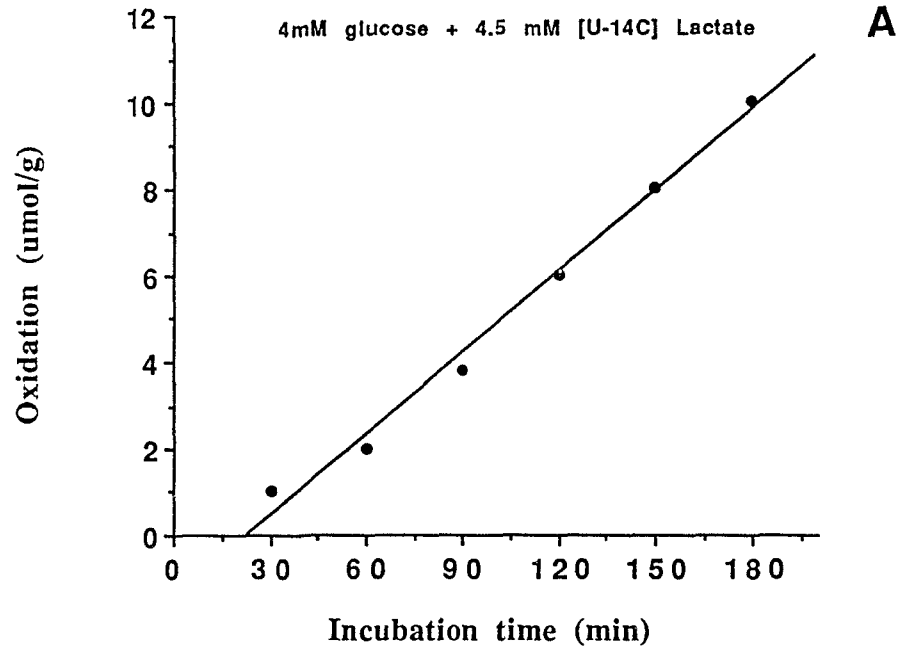

B
SOLEUS
Ins
No Ins
EDL
Ins
No Ins
Glucose oxidation (nmol/mg wt/15min)
1.5
1.0
0.5
0.0
0
5
0
5
0
5
0
5
1
2
3
4
5
6
7
8
Treatment
A
SOLEUS
Ins
No Ins
EDL
Ins
No Ins
Glycogenesis (nmol/mg wt/15 min)
12
10
8
6
4
2
0
0
5
0
5
0
5
0
5
1
2
3
4
5
6
7
8
Treatment

Figure.5


A
4mM glucose + 4.5 mM [U-14C] Lactate
Oxidation (umol/g)
0
2
4
6
8
10
12
0
30
60
90
120
150
180
Incubation time (min)

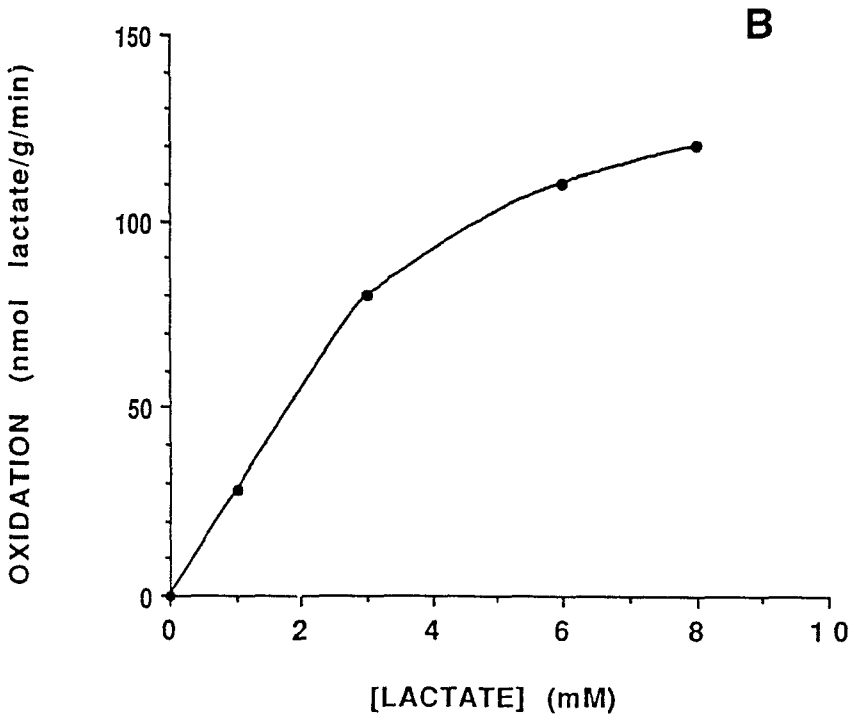

B
OXIDATION (nmol lactate/g/min)
0
50
100
150
0
2
4
6
8
10
[LACTATE] (mM)

Figure.6

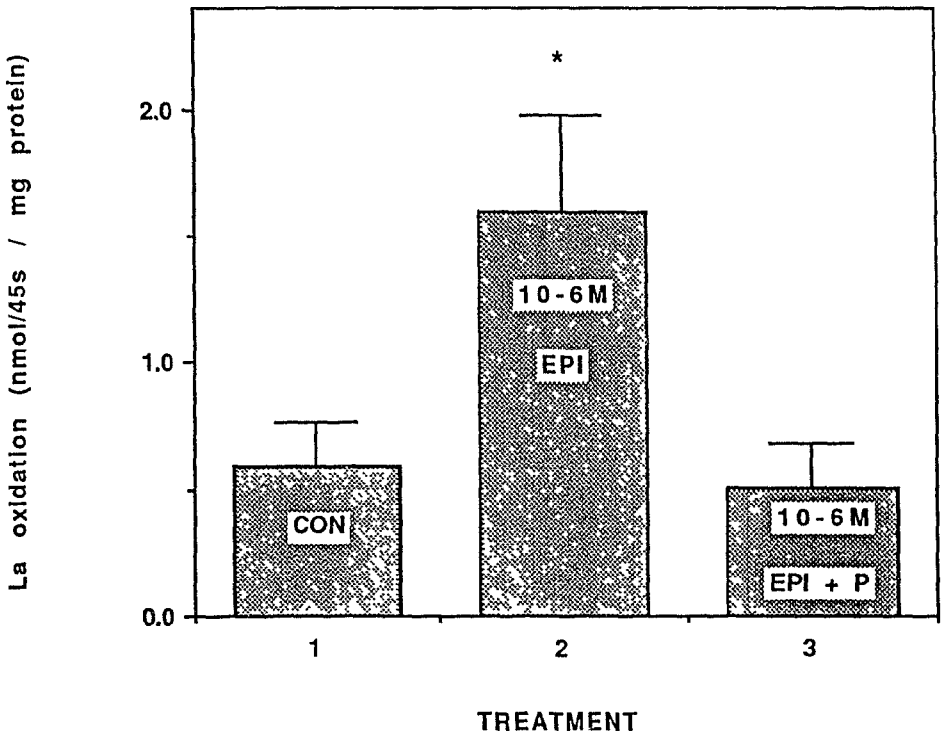

La oxidation (nmol/45s / mg protein)
2.0
1.0
0.0
*
10-6M
EPI
CON
10-6M
EPI + P
1
2
3
TREATMENT

Figure.7

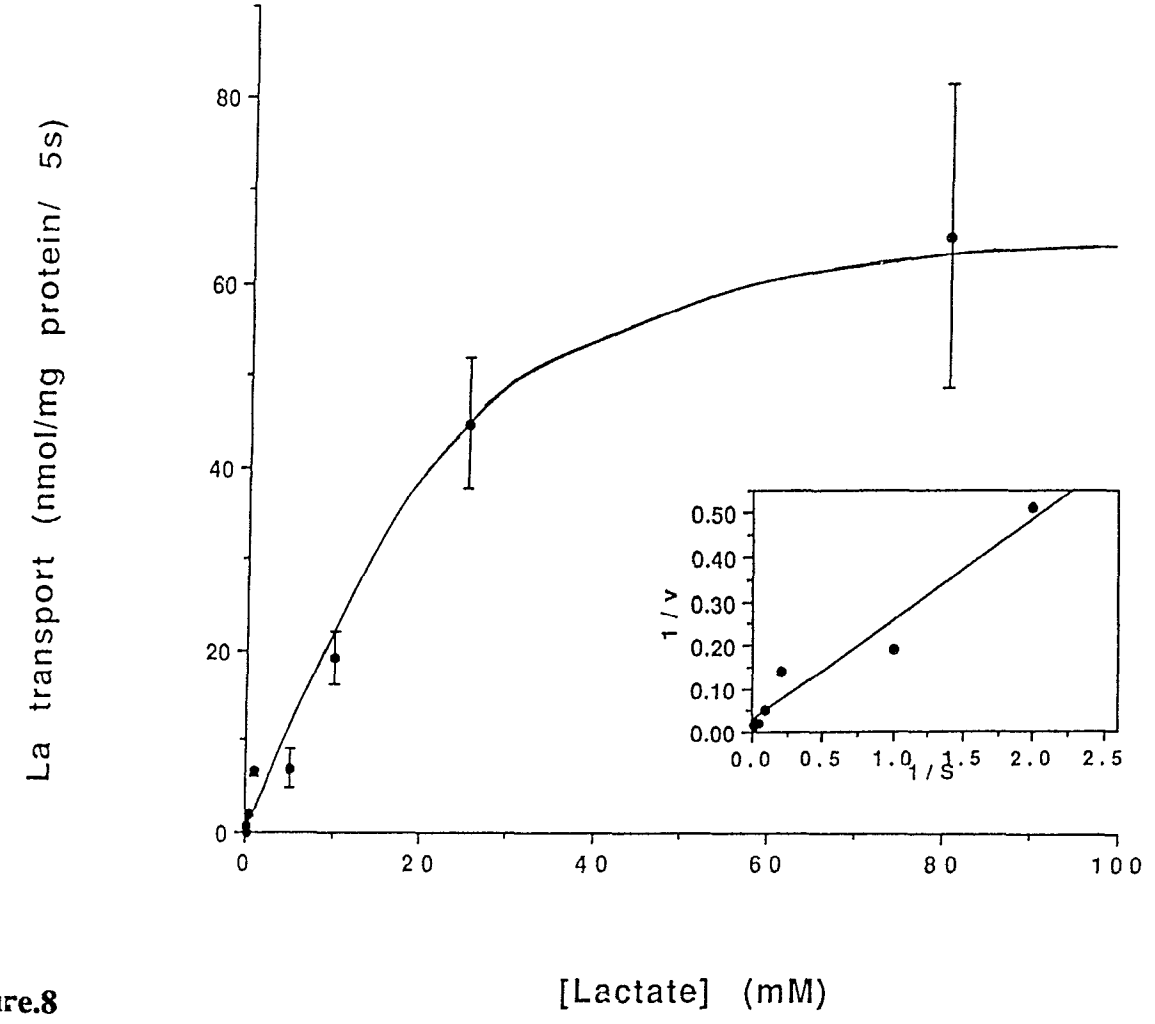

La transport (nmol/mg protein/ 5s)
80
60
40
20
0
0
20
40
60
80
100
[Lactate] (mM)
1 / v
0.50
0.40
0.30
0.20
0.10
0.00
0.0
0.5
1.0
1.5
2.0
2.5
1 / S

Figure.8

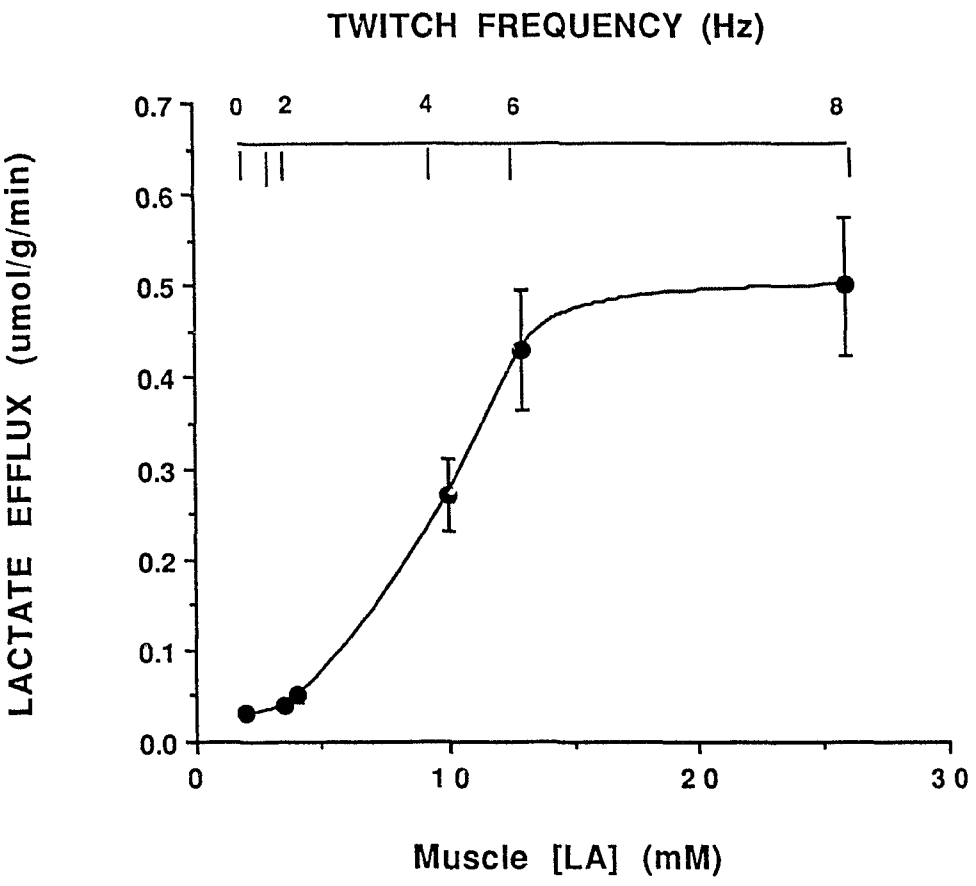

TWITCH FREQUENCY (Hz)
0
2
4
6
8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
LACTATE EFFLUX (umol/g/min)
0
10
20
30
Muscle [LA] (mM)

Figure.9

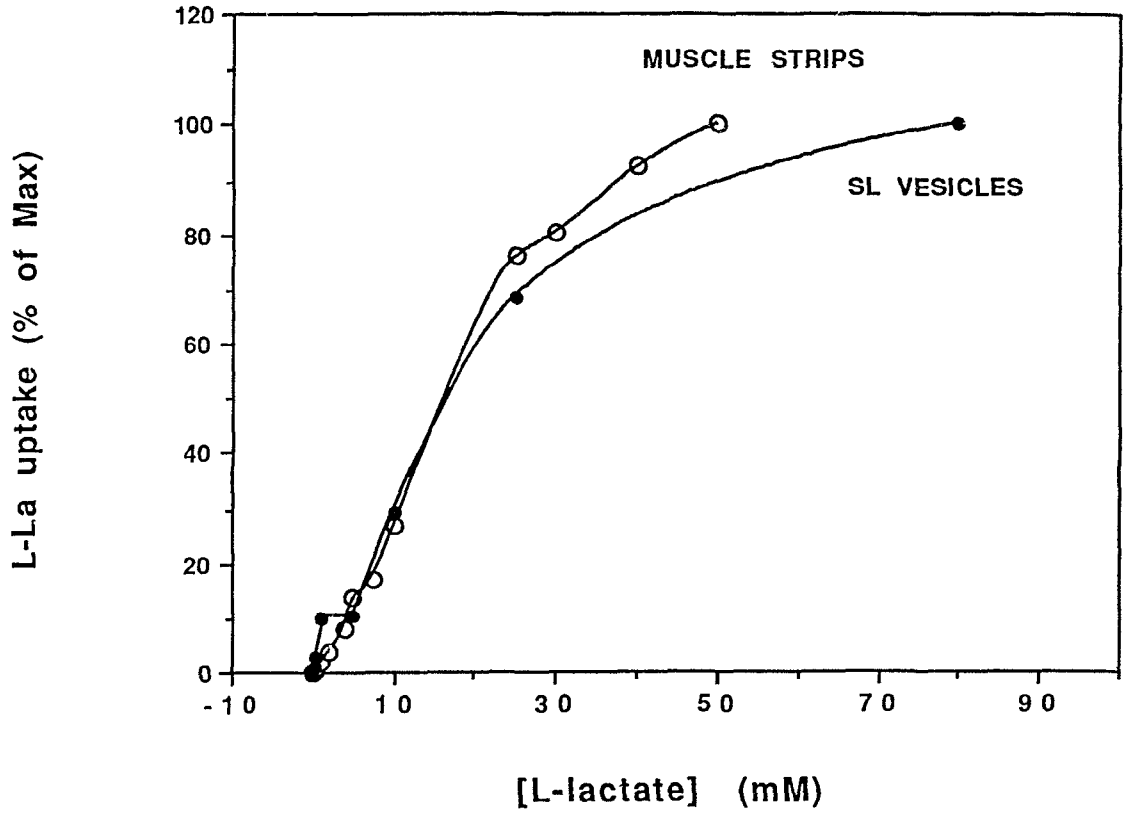

MUSCLE STRIPS
SL VESICLES
L-La uptake (% of Max)
[L-lactate] (mM)
120
100
80
60
40
20
0
-10
10
30
50
70
90

Figure.10

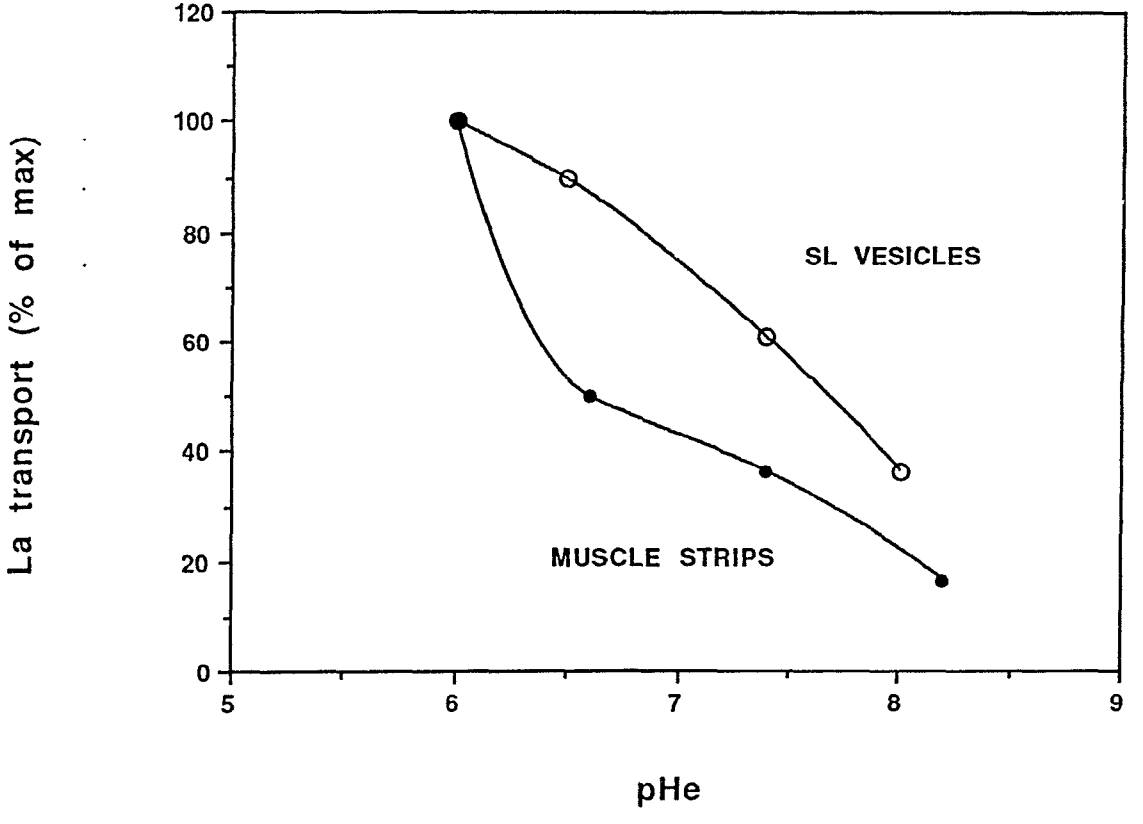

La transport (% of max)
120
100
80
60
40
20
0
5
6
7
8
9
pHe
SL VESICLES
MUSCLE STRIPS

Figure.11

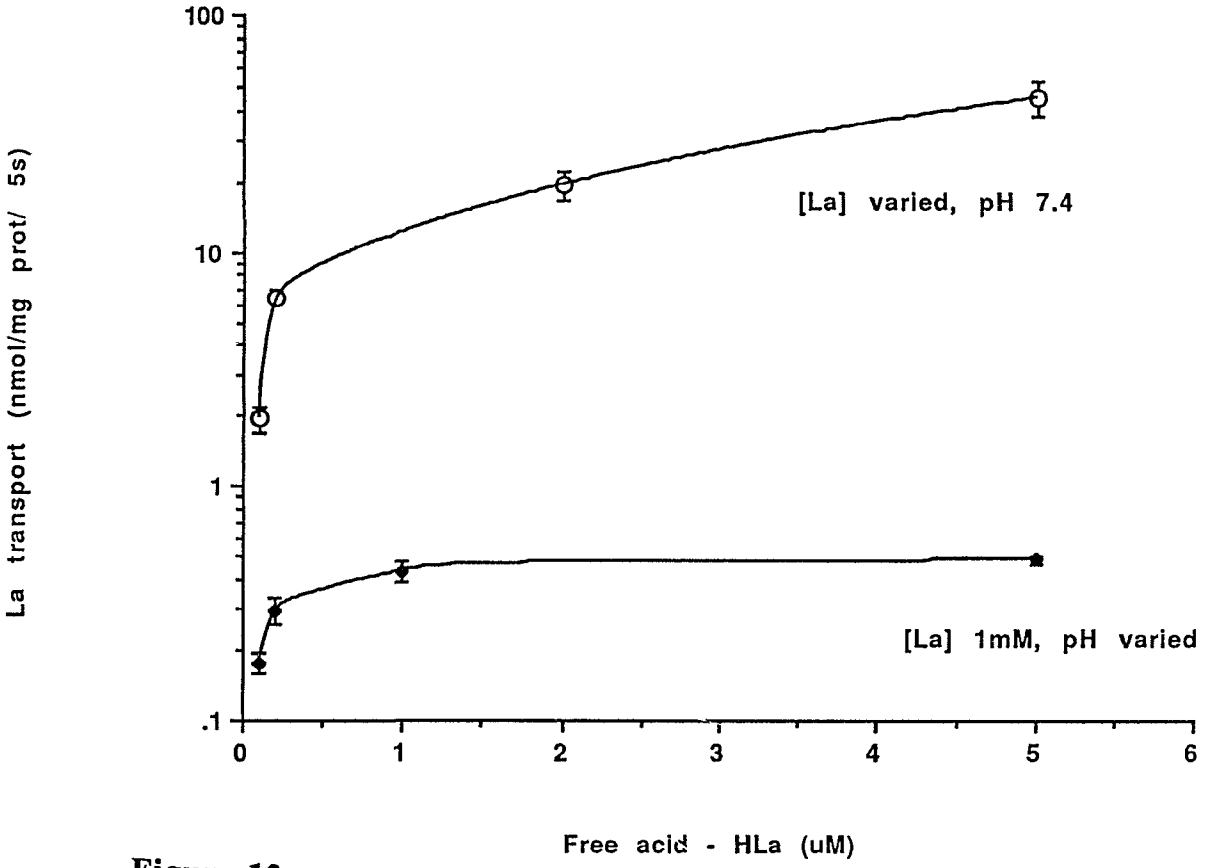
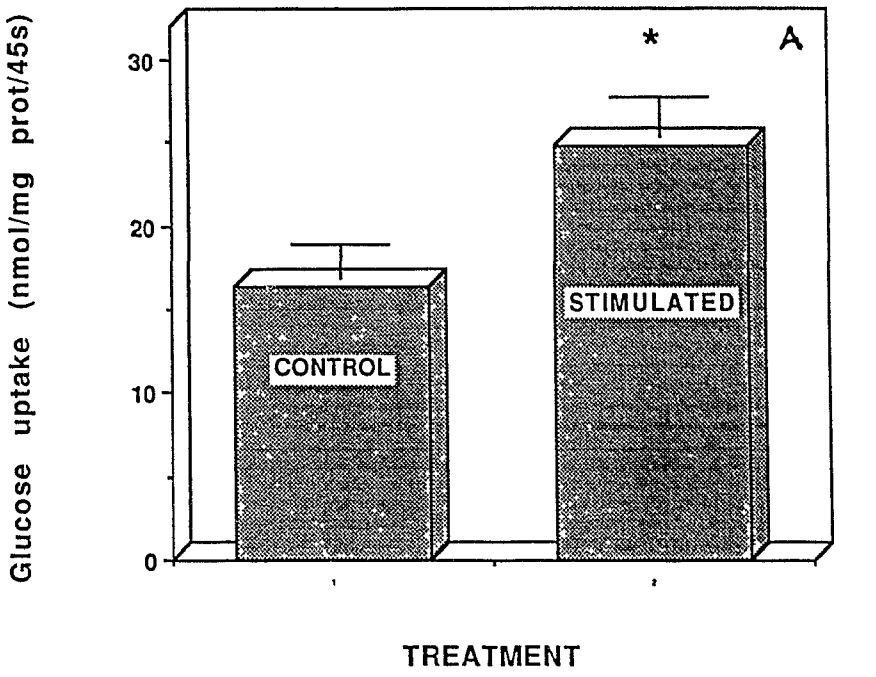

100
10
1
.1
0
1
2
3
4
5
6
La transport (nmol/mg prot/ 5s)
Free acid - HLa (uM)
[La] varied, pH 7.4
[La] 1mM, pH varied

Figure.12


Glucose uptake (nmol/mg prot/45s)
30
20
10
0
*
A
CONTROL
STIMULATED
TREATMENT

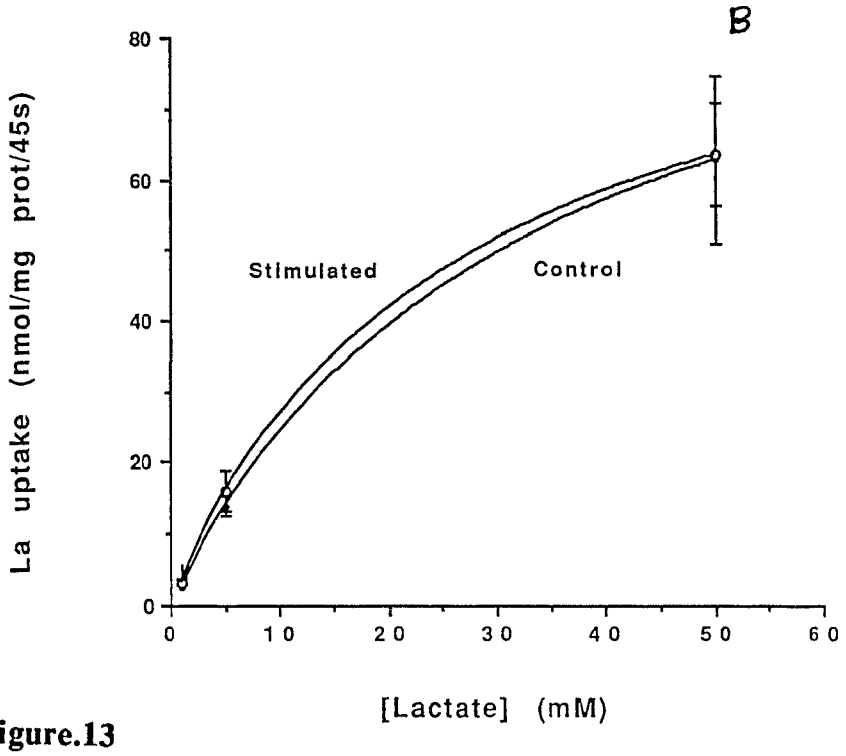

B
La uptake (nmol/mg prot/45s)
80
60
40
20
0
Stimulated
Control
0
10
20
30
40
50
60
[Lactate] (mM)

Figure.13

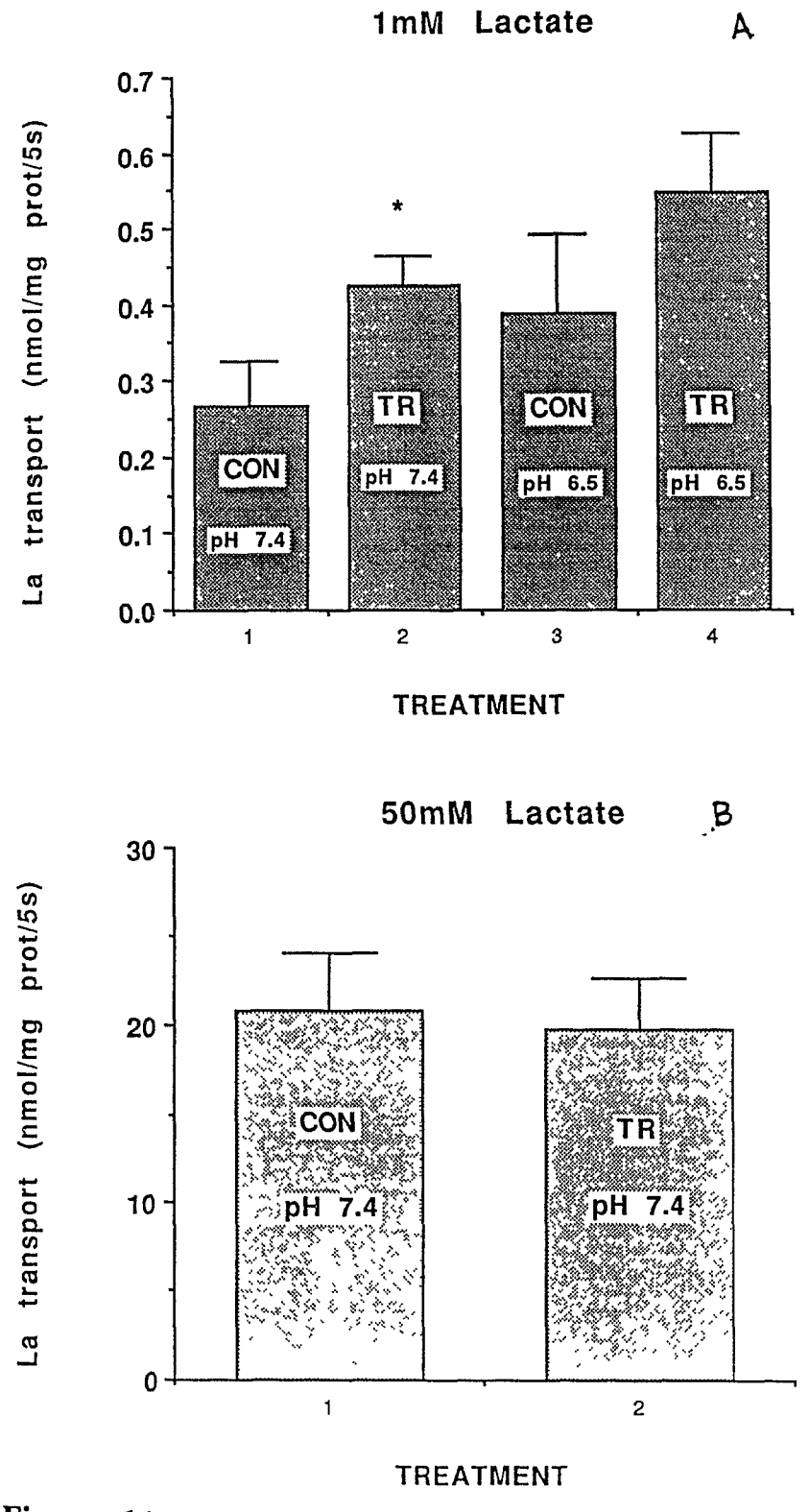

1mM Lactate
A
La transport (nmol/mg prot/5s)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
CON
pH 7.4
TR
pH 7.4
CON
pH 6.5
TR
pH 6.5
1
2
3
4
TREATMENT
50mM Lactate
B
La transport (nmol/mg prot/5s)
30
20
10
0
CON
pH 7.4
TR
pH 7.4
1
2
TREATMENT

Figure. 14

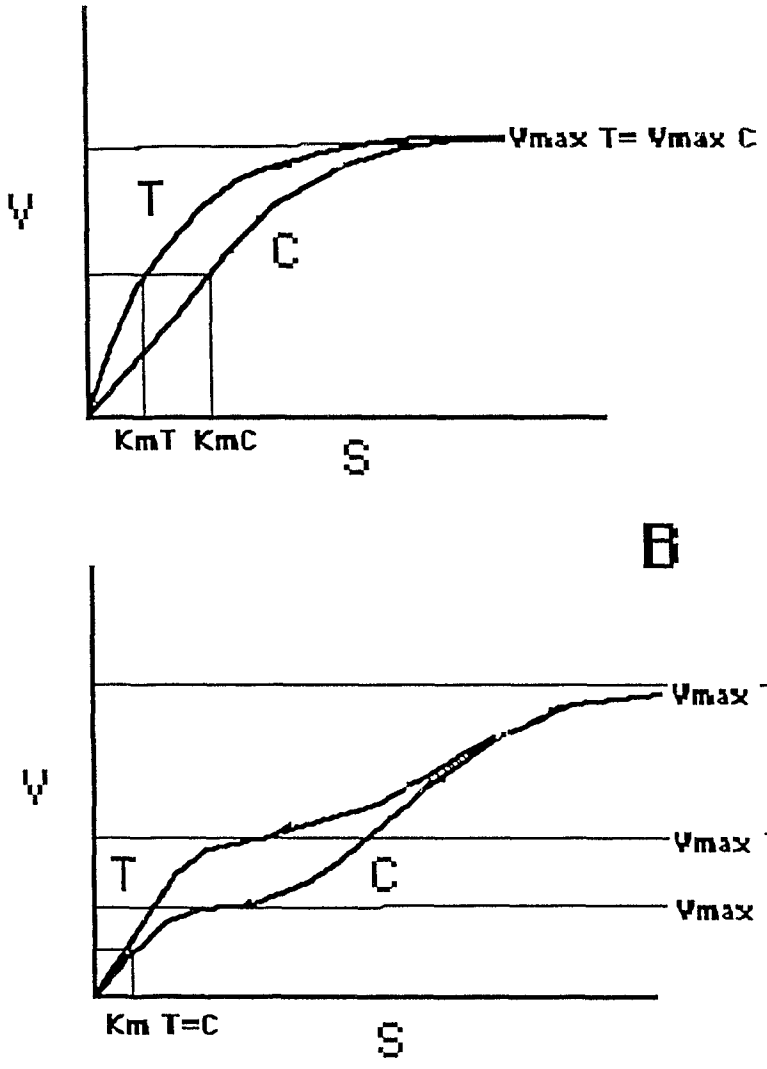


Figure.15

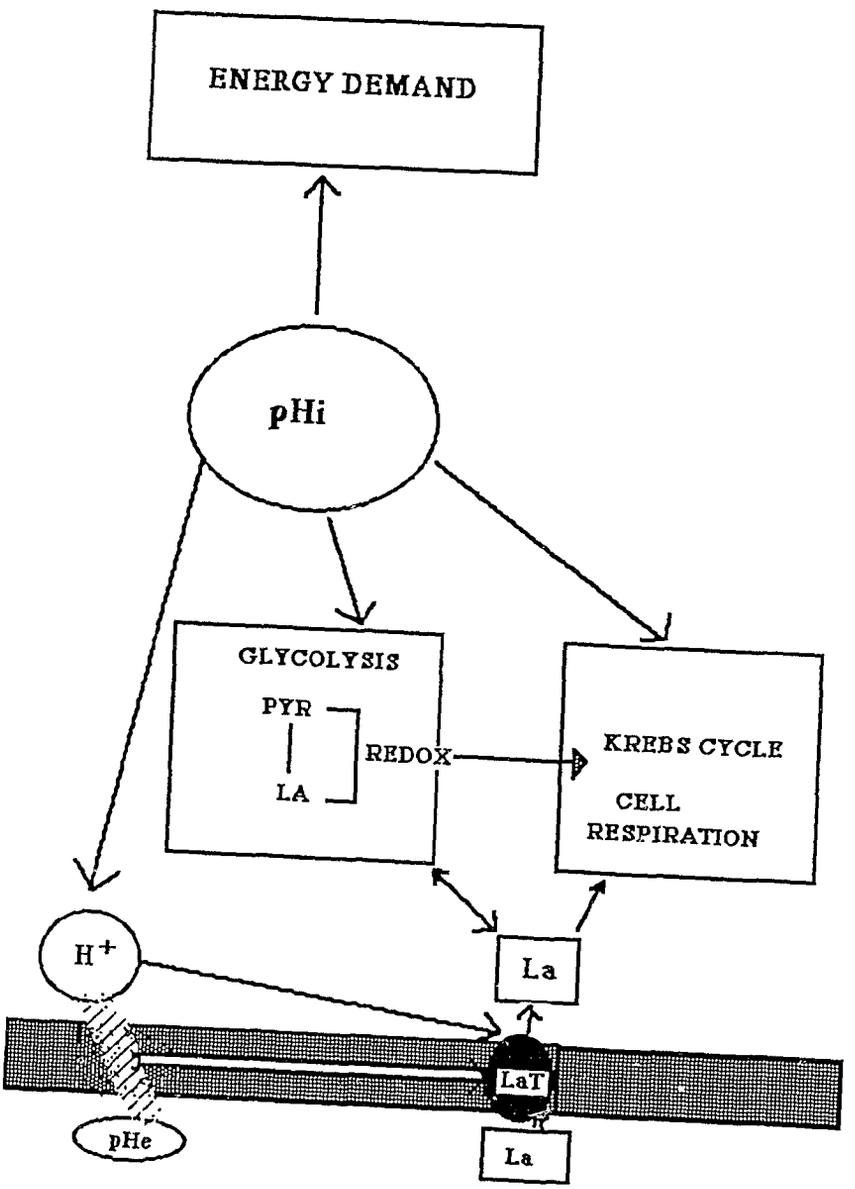

ENERGY DEMAND
pHi
GLYCOLYSIS
PYR
LA
REDOX
KREBS CYCLE
CELL
RESPIRATION
H+
La
LaT
pHe
La

Figure.16

Table . Effects of monocarboxylate inhibitors on inital rate of L- lactate uptake.

Inhibitor	Ratio La: Inhib	Uptake nmol/mg/5s	% inhibition	Significance
Control	-	0.191 ± 0.022	-	-
Pyruvate (10mM)	1: 66	0.037 ± 0.009	81	p<.05
a-cyano-h (5mM)	1: 33	0.032 ± 0.013	83	p<.05
D- Lactate (10mM)	1:66	0.146 ± 0.014	-	N.S.

Table . Effects of protein modifiers on the initial rate of L-lactate uptake

Inhibitor	Uptake nmol/mg/5s	% inhibition of initial uptake	Significance
Control	0.191 ± 0.022	-	-
N-ethylmaleimide (20mM)	0.027 ± 0.007	86	p<.05
SITS	0.195 ± 0.047	-	N.S
Na^+ substitution	0.152 ± 0.029	-	N.S.

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