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Robert James Talmadge
Louisiana State University and Agricultural & Mechanical College

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Metabolic pathways associated with glycogen storage in chronically active and normal skeletal muscle

Talmadge, Robert James, Ph.D.
The Louisiana State University and Agricultural and Mechanical Col., 1991
METABOLIC PATHWAYS ASSOCIATED WITH
GLYCOGEN STORAGE IN CHRONICALLY ACTIVE
AND NORMAL SKELETAL MUSCLE

A Dissertation

Submitted to the Graduate Faculty of the
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Doctor of Philosophy

in

The Department of Zoology and Physiology

by

Robert James Talmadge
B.S., The University of Connecticut, 1983
M.S., Louisiana State University, 1987
August, 1991
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I dedicate this work to my mother Mary Jane Talmadge and in the memory of my father Arthur Reed Talmadge Sr., who supported my every effort. His guidance continues to lead me and always will.

I thank my advisor, Dr. Harold Silverman, for his guidance, support, and advice throughout my years at L.S.U. I thank my committee members, Drs. Sue Bartlett, John Caprio, Thomas Dietz, Joseph Siebenaller, and John Trant for their advice. I would also like to thank Drs. Scott Powers and Bruce Tedeschi for serving on my committee prior to their leaving L.S.U. Finally, I thank S. Mills, Y.T. Hsu, L. Younger, Dr. J. Scheide, and R. Bouchard for contributing to the completion of this dissertation.
<table>
<thead>
<tr>
<th>TABLE OF CONTENTS</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acknowledgements</td>
<td>ii</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>iii</td>
</tr>
<tr>
<td>List of Tables</td>
<td>iv</td>
</tr>
<tr>
<td>List of Figures</td>
<td>v</td>
</tr>
<tr>
<td>General Abstract</td>
<td>viii</td>
</tr>
<tr>
<td>Chapter One: Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Chapter Two: Glycogen synthesis from lactate by chronically active muscles</td>
<td>11</td>
</tr>
<tr>
<td>Chapter Three: Glyconeogenic and glycogenic enzymes in chronically active and normal muscle</td>
<td>20</td>
</tr>
<tr>
<td>Chapter Four: Variation of glycogen along the length of single muscle fibers from normal and chronically active muscles</td>
<td>55</td>
</tr>
<tr>
<td>Chapter Five: Glucose uptake and glycogen synthesis from glucose by normal and chronically active muscle</td>
<td>75</td>
</tr>
<tr>
<td>Chapter Six: Glyconeogenesis by skeletal muscle: Possible involvement of PEPCK</td>
<td>105</td>
</tr>
<tr>
<td>Chapter Seven: Summary</td>
<td>129</td>
</tr>
<tr>
<td>References</td>
<td>133</td>
</tr>
<tr>
<td>Appendix I: Glyconeogenesis from alanine by diaphragm and other skeletal muscles</td>
<td>144</td>
</tr>
<tr>
<td>Appendix II: Copyright Permission Letter</td>
<td>174</td>
</tr>
<tr>
<td>Curriculum Vitae</td>
<td>176</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

### Chapter II
1. $^{14}$C-lactate incorporation into glycogen by 'isolated' gastrocnemius muscles
2. Effects of 95% O$_2$-5% CO$_2$ on $^{14}$C-lactate incorporation

### Chapter III
1. Phosphorylase activity in normal and pseudomyotonic muscles
2. Fiber type specific phosphorylase activity

### Chapter IV
1. Individual fiber statistics for glycogen content

### Chapter V
1. Blood glucose levels in normal and dy$^{23}$ mice

### Chapter VI
1. Glycogen contents in *in vitro* incubated EDL and SOL muscles with 3-MPA

### Appendix I
1. Effect of insulin on glyconeogenesis from alanine and glycogenesis from glucose
2. Effect of alanine concentration on alanine utilization
3. Comparison of glyconeogenesis from alanine and lactate
# LIST OF FIGURES

## Chapter II

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glycogen content of normal and (dy^2J) muscles</td>
<td>14</td>
</tr>
<tr>
<td>2</td>
<td>Effect of denervation on lactate contents of normal and (dy^2J) muscles</td>
<td>14</td>
</tr>
<tr>
<td>3</td>
<td>Autoradiographs of normal and (dy^2J) gastrocnemius muscles following (^{14}C)-lactate administration</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>Quantitation of (^{14}C)-lactate autoradiography</td>
<td>16</td>
</tr>
<tr>
<td>5</td>
<td>Fiber types in (dy^2J) gastrocnemius muscle</td>
<td>16</td>
</tr>
</tbody>
</table>

## Chapter III

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Putative pyruvate kinase bypass enzymatic pathway</td>
<td>23</td>
</tr>
<tr>
<td>2</td>
<td>Glycogen synthase activity in normal and (dy^2J) muscles</td>
<td>31</td>
</tr>
<tr>
<td>3</td>
<td>Histochemistry of glyconeogenic and other enzymes in normal gastrocnemius muscle</td>
<td>32</td>
</tr>
<tr>
<td>4</td>
<td>Fiber type characteristics of normal superficial gastrocnemius muscles</td>
<td>34</td>
</tr>
<tr>
<td>5</td>
<td>Histochemistry of glyconeogenic and other enzymes in (dy^2J) gastrocnemius muscle</td>
<td>36</td>
</tr>
<tr>
<td>6</td>
<td>Glycogen synthase activities in specific fiber types of (dy^2J) gastrocnemius muscle</td>
<td>38</td>
</tr>
<tr>
<td>7</td>
<td>Activities of the pyruvate kinase bypass enzymes</td>
<td>40</td>
</tr>
<tr>
<td>8</td>
<td>Malic enzyme activities in specific fiber types of (dy^2J) gastrocnemius muscle</td>
<td>42</td>
</tr>
<tr>
<td>9</td>
<td>Fructose 1,6-bisphosphatase activity in normal and (dy^2J) muscles</td>
<td>44</td>
</tr>
<tr>
<td>10</td>
<td>Histochemistry of phosphorylase activity in normal and (dy^2J) gastrocnemius muscle</td>
<td>47</td>
</tr>
<tr>
<td>Chapter IV</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>---------------------------------------------------------------------------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>1 Linear relationship between camera exposure time and light intensity</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>2 Serial sections of normal superficial gastrocnemius muscle stained for glycogen content</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>3 Serial sections of dy^{2J} superficial gastrocnemius muscle stained for glycogen content</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>4 Glycogen content along the lengths of specific fiber types in normal and dy^{2J} superficial gastrocnemius muscle</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>5 Glycogen content along the lengths of muscle fibers from normal and dy^{2J} superficial gastrocnemius muscle</td>
<td>69</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter V</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 2-Deoxyglucose uptake in vivo</td>
<td>85</td>
</tr>
<tr>
<td>2 3-O-methylglucose uptake in vivo</td>
<td>86</td>
</tr>
<tr>
<td>3 Glycogen synthesis from ^14C-glucose in vivo</td>
<td>87</td>
</tr>
<tr>
<td>4 Linear relationship between glycogen content and glycogen synthesis from ^14C-glucose in vivo</td>
<td>88</td>
</tr>
<tr>
<td>5 Autoradiographs of normal and dy^{2J} superficial gastrocnemius muscles following ^14C-glucose administration</td>
<td>91</td>
</tr>
<tr>
<td>6 Insulin stimulated 2-deoxyglucose uptake in vitro</td>
<td>93</td>
</tr>
<tr>
<td>7 Insulin stimulated glycogenesis by EDL muscle in vitro</td>
<td>94</td>
</tr>
<tr>
<td>8 Insulin stimulated glycogenesis by SOL muscle in vitro</td>
<td>95</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter VI</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Effect of pH on glyconeogenesis in vitro</td>
<td>112</td>
</tr>
<tr>
<td>2 Effect of lactate concentration on glyconeogenesis in vitro</td>
<td>113</td>
</tr>
<tr>
<td>3 Effect of gas phase on glyconeogenesis in vitro</td>
<td>114</td>
</tr>
</tbody>
</table>
LIST OF FIGURES (Continued)

Chapter VI (continued)

4 Effect of contractile activity on glyconeogenesis in vitro 115
5 Dose dependent effect of 3-MPA on glyconeogenesis in vitro 117
6 Maximal effect of 3-MPA and quinolinic acid on glyconeogenesis in vitro 118
7 Glycogen contents following in vitro incubation 119

Appendix I

1 Alanine aminotransferase activity 152
2 Incorporation of $^{14}$C-alanine into glycogen in vivo 153
3 2-Deoxyglucose uptake by diaphragm muscle in vitro 154
4 Glycogenesis by diaphragm muscle in vitro 155
5 Effect of alanine concentration on glyconeogenesis from alanine by diaphragm muscle in vitro 158
6 Effect of pH on glyconeogenesis from alanine by diaphragm muscle in vitro 159
7 Glyconeogenesis from alanine by normal and dy$^{2J}$ muscle 160
8 Pathway of glycogen synthesis from alanine 164
To determine the pathways used for glycogen synthesis in chronically active and normal muscle, muscles from C57Bl/6J dy2J/dy2J (dy2J) mice were evaluated biochemically and histochemically.

The gastrocnemius muscle (GAST) of the dy2J mouse, a chronically active muscle, contained twice as much glycogen and lactate as control GAST. The dy2J triceps muscle (TRI), which is not chronically active, showed no elevation in glycogen content. An intraperitoneal injection of 14C-lactate resulted in increased incorporation of 14C into glycogen by dy2J compared to control GAST. Both normal and dy2J GAST incorporated 14C into glycogen in an in situ preparation, indicating direct glycogen synthesis from lactate. Autoradiography revealed that high glycogen containing muscle fibers in the dy2J GAST have the highest capacity for glyconeogenesis.

Glycogen synthase (GS), malic enzyme (ME) and phosphoenolpyruvate carboxykinase (PEPCK) are all elevated in dy2J GAST, but not in dy2J TRI compared to normal controls. High glycogen fibers in the dy2J had higher activities of GS and ME than any other fibers.

The variation in glycogen content along the length of single muscle fibers increased with increasing glycogen content. However, the variation was low enough in all fiber types to allow for a single histochemical section to be a good predictor of glycogen in that fiber.

Glucose uptake and glycogen synthesis from glucose (glycogenesis) were elevated in chronically active muscles, in vivo and in vitro. The diaphragm muscle had the highest rates of glucose uptake and
glycogenesis. Insulin stimulation of glucose uptake and glycogenesis were enhanced in chronically active muscles from dy^2^J mice.

Specific inhibitors of PEPCK inhibited glyconeogenesis in skeletal muscle, demonstrating the involvement of PEPCK. Previous contractile activity had no effect on glyconeogenic rates. Glyconeogenic rates were linearly dependent on substrate concentration and had a pH optimum of 6.6.

Normal and chronically active muscles utilize both lactate and glucose for glycogen synthesis. Chronically active muscles store increased amounts of glycogen. The increased glycogen may provide the chronically active muscle with some additional resistance to fatigue.
CHAPTER I. INTRODUCTION
The C57B16J dy^{2J}/dy^{2J} (henceforth dy^{2J}) mouse suffers from pseudomyotonia, a chronic and repetitive neural stimulation of the hindlimb muscles (Rasminsky 1978). The nerves innervating the hindlimb show an abnormal lack of myelination at the spinal roots (Jaros and Jenkison 1983). The abnormal neural activity arises both spontaneously and as a result of ephaptic "cross-talk" of impulses between adjacent axons at the spinal roots (Rasminsky 1978). Therefore, the hindlimb musculature of this animal can be used as a model for chronic contractile activity in the study of muscle plasticity and the effects of contractile activity on metabolism.

The superficial region of the gastrocnemius muscle (GAST) in the dy^{2J} mouse was shown to increase in oxidative capacity (Hargroder et al. 1986) with age. The superficial GAST normally consists of a uniform population (~98%) of muscle fibers that contain low densities of mitochondria (Silverman and Atwood 1982), have low oxidative capacities (Silverman and Atwood 1980), and stain histochemically as fast-twitch fibers (Dribin and Simpson 1977). Thus, these fibers are classified as fast-twitch glycolytic muscle fibers (FG) according to the fiber type classification scheme of Peter et al. (1972). In the superficial region of the dy^{2J} GAST, the fibers remain fast-twitch (Younger and Silverman 1984). However, some fibers in this region show increases in mitochondrial density (Silverman and Atwood 1982) and oxidative capacity (Silverman and Atwood 1980). Thus, some fibers have become fast-twitch oxidative glycolytic (FOG). Many of the fibers which have not increased in oxidative capacity appear to store abnormally high amounts of glycogen (Younger and Silverman 1984). It is hypothesized
that the high glycogen fibers may not receive as much neural stimulation as other fibers in the dy^2J GAST. The fibers that receive the most stimulation increase in oxidative capacity, as it has been shown that exercise (Barnard et al. 1970, Holloszy and Booth 1976) and chronic stimulation (either direct or indirect) (Pette et al. 1973, Pette and Tyler, 1983) of fast-twitch muscle results in an increased oxidative capacity of the stimulated muscle. The fibers that do not receive as much stimulation in the dy^2J GAST may make use of available substrates and convert these into glycogen, the stored form of carbohydrate in muscle. One probable substrate is lactic acid. It was shown that lactic acid can accumulate in an oxidative muscle despite adequate oxygen supply (Connett et al. 1984, Connett et al. 1986). Thus, lactate is expected to accumulate in chronically active muscles undergoing fiber type transition and could be used for glycogen synthesis. However, the possibility of a direct pathway for the synthesis of glycogen from lactate in muscle is only now becoming widely accepted. Chapter II of the dissertation provides evidence that this occurs in the chronically active GAST of dy^2J mice.

The present dogma is that during high intensity exercise or contractile activity, skeletal muscles utilize glycogen as a major fuel for ATP production. If the exercise reaches a high level of intensity, the muscle can become glycogen depleted. It has also been found that during exercise the liver responds to humoral cues to metabolize its glycogen to form glucose. The glucose formed and released by the liver during exercise is utilized by the muscle for either aerobic or anaerobic ATP production. The anaerobic and aerobic (under certain
production of ATP by glycolysis results in the formation of lactic acid, which is released from the muscle into the blood supply, allowing lactate levels in the sarcoplasm to accumulate more slowly and allowing glycolysis to proceed. At the end of exhaustive, high intensity exercise both the liver and muscles are depleted of glycogen and blood lactate is elevated.

A substantial percentage of the lactate produced by the active muscles is transported to the liver via the blood supply where lactate is converted into glucose via the gluconeogenic pathway. The newly formed glucose can then be used for glycogen synthesis by either the liver or the skeletal muscle. It was shown that the glycogen supply in the liver is replenished after that in skeletal muscle (Fell et al. 1980). Thus, immediately following exercise, the following cycle of events occurs: (a) the formation of lactate at the muscle from endogenous glycogen and blood glucose, (b) the transport of lactate to the liver via the circulation, (c) the formation by the liver of glucose from lactate, (d) the release of the newly formed glucose into the blood, and (e) the utilization of blood glucose by the muscle for glycogen synthesis. This cycle of events was initially described by Himwich et al. (1928) and further studied by Cori (1931), and is termed the Cori cycle.

One possible alternative to the Cori cycle is that glycogen could be synthesized from lactate by skeletal muscle directly. The possibility of glyconeogenesis occurring in skeletal muscle has received little attention compared to gluconeogenesis by the liver or
glycogen synthesis from glucose by skeletal muscle, which are two primary components of the Cori cycle. The first investigation of direct glyconeogenesis by skeletal muscle, using amphibian muscle, demonstrated a relationship between lactate uptake and glycogen replenishment following *in vitro* contractions (Meyerhof et al. 1925). However, subsequent studies were unable to duplicate these findings (Eggleton and Evans 1930, Sacks and Sacks 1935). The studies by Eggleton and Evans (1930) demonstrated that despite lactate concentrations of 0.12% (13 mM), frog muscle would not synthesize glycogen from lactate. However, skeletal muscles of eviscerated dogs were able to resynthesize glycogen from lactate. Furthermore, two-thirds of blood lactate was removed by the dog liver following exercise; thus, one-third of the blood lactate could have been utilized by skeletal muscle for glycogen synthesis. These data suggest both the Cori cycle and a direct glyconeogenic pathway in skeletal muscle for glycogen resynthesis (Eggleton and Evans 1930).

Sacks and Sacks (1935) demonstrated that during early recovery lactic acid was lost from the muscle. Also, during this time, muscle carbohydrate was decreased. However, if any of the lactic acid produced during exercise was used for glycogen synthesis, muscle carbohydrate should increase during the initial recovery time. What was not taken into account by Sacks and Sacks (1935) was the fiber type specificity for glycogen synthesis from lactate. Perhaps, some fibers are synthesizing glycogen from lactate, while others utilize carbohydrate for ATP production. Sacks and Sacks (1935) also hypothesized that all of the lactic acid produced by the muscle entered
the blood where it could then enter into the Cori cycle.

Later studies with radiolabeled isotopes of pyruvate and lactate demonstrated glyconeogenesis could occur in skeletal muscle (Cavert and Boyd 1956, Gourley and Suh 1967, Hiatt et al. 1958, Moorthy and Gould 1969, and Warnock et al. 1965). However, the exact pathway for conversion of pyruvate to phosphoenolpyruvate was unknown. Initially, pyruvate kinase (PK) reversal was proposed as the mechanism because there was little randomization of the $^{14}$C-labeled carbons from the lactate into the glucosyl moiety of the glycogen molecule (Hiatt et al. 1958). Glycogen synthesis from lactate in the liver shows extensive randomization of label, due to the involvement of mitochondrial intermediates (Landau et al. 1955, Lorber et al. 1950). Thus, it was proposed that mitochondrial intermediates were not involved in skeletal muscle glyconeogenesis because randomization of the $^{14}$C-label would occur at the fumarase step of the Krebs cycle. One possible mechanism for glycogen synthesis from lactate explaining $^{14}$C-lactate incorporation without label randomization was the reversal of the PK reaction.

A second proposed mechanism utilizes cytosolic malic enzyme, malate dehydrogenase, and phosphoenolpyruvate carboxykinase (PEPCK) as a series of bypass enzymes to circumvent the PK reaction (Bendall and Taylor 1970, and Connett 1979). This was proposed due to the thermodynamic irreversibility of the PK reaction. All of these enzymes have been shown to be present in the cytosol of skeletal muscle fibers (Nolte et al. 1972, Pette 1968).

Studies utilizing a specific inhibitor for PEPCK, 3-mercaptopicolinic acid (3-MPA), yielded conflicting conclusions. The
addition of 3-MPA to an in vitro amphibian muscle preparation significantly decreased the amount of $^{14}$C incorporated into glycogen from $^{14}$C-lactate (Connett 1979), suggesting PEPCK is involved in skeletal muscle glyconeogenesis. However, 3-MPA was shown to be ineffective in inhibiting glyconeogenesis by skeletal muscle in perfused rat (Shiota et al. 1984) and rabbit hindlimbs (Pagliasotti and Donovan 1990). Therefore, in mammalian muscle, PK reversal is the only alternative that takes into account both the lack of randomization of lactate carbon in glycogen synthesized from lactate and the lack of any effect of 3-MPA on glyconeogenic rates. It was also demonstrated that under appropriate conditions the reversal of PK is thermodynamically feasible (Dyson et al. 1975). Thus, the pathway for conversion of pyruvate to phosphoenolpyruvate remains unclear. Chapters III and VI of this dissertation address this issue and indicate the involvement of PEPCK in skeletal muscle glyconeogenesis.

A recent study examined the maximal capacities for skeletal muscle to synthesize glycogen from lactate in vitro and some possible regulatory factors (Bonen et al. 1990). Bonen et al. (1990) determined that glyconeogenesis has a pH and a lactate concentration dependence and that the maximal rate approaches 30% of that for glycogen synthesis from glucose in the extensor digitorum longus muscle of mice. This is a substantial level of glyconeogenesis because during high intensity exercise the concentrations of lactate in the blood are much higher than blood glucose (Hermansen and Vaage 1977). It was also demonstrated that insulin and corticosterone, both of which affect glycogen synthesis from glucose, have no affect on glycogen synthesis
from lactate (Bonen et al. 1990).

The capacities for different muscle fiber types to synthesize glycogen from lactate has also been evaluated (Bonen et al. 1990, Johnson and Bagby 1988, McLane and Holloszy 1979). In all of these studies, fast-twitch glycolytic fibers showed the highest rates of glyconeogenesis. All of these studies have focused on whole muscles which contain primarily a single fiber type. No study has evaluated the capacity for specific fiber types in a muscle of mixed fiber type to synthesize glycogen from lactate.

While it now appears that some skeletal muscles utilize lactate for glycogen synthesis under some circumstances, the importance of this pathway to mammalian muscle energetics has been questioned (Gaeser and Brooks 1984). It is clear, however, that fish (Batty and Wardle 1979) and reptiles (Gleeson 1985, Gleeson and Dalessio 1990) utilize glyconeogenesis to replenish muscle glycogen after exercise. In humans, it was estimated that only 10% of muscle glycogen is synthesized from glucose during recovery from a short-term highly intense bout of exercise, whereas the remainder comes primarily from lactate (Hermansen and Vaage 1977). It was also estimated that only 10% of the lactate produced in the muscle passes into the circulation, less than 15% is oxidized to CO$_2$ and the remaining 75% is used for glycogen synthesis (Hermansen and Vaage 1977). Despite controversy over the importance of glyconeogenesis in skeletal muscle, this pathway may play an important role in the resynthesis of muscle glycogen following intense activity in humans and other mammals.

This dissertation consists of five separate chapters designed to
investigate the glyconeogenic and glycogenic pathways in normal and chronically active muscles. Chapter II reports the utilization of lactate for glycogen synthesis by normal and chronically active muscles in an in situ preparation with an emphasis on the fiber type involvement. This paper was published in the Journal of Applied Physiology with J. I. Scheide and H. Silverman as co-authors. In Chapter III, the enzymes involved in glyconeogenesis and glycogen synthesis and breakdown were determined in whole muscle and single muscle fibers using histochemical techniques. Chapter III was accepted for publication in the Journal of Applied Physiology, with H. Silverman as co-author and will appear in the July 1991 issue. Chapter IV is a study on whether or not muscle fibers, in particular muscle fibers that appear to store high amounts of glycogen, show variations in glycogen content along their length. Chapter IV was submitted for publication in the Anatomical Record, with Y.T. Hsu and H. Silverman as co-authors. In Chapter V, the capacities of insulin stimulated glucose uptake and glycogenesis are evaluated in both normal and chronically active muscles. Chapter V was submitted to the American Journal of Physiology, with H. Silverman as co-author. An in vitro analysis of glycogen synthesis from lactate was performed in Chapter VI. Chapter VI established the involvement of PEPCK in glyconeogenesis. Chapter VI also determined the pH optimum, and the response to contractile activity of glyconeogenesis and completes the body of the dissertation. It was submitted to the American Journal of Physiology with H. Silverman as co-author. A summary chapter (Chapter VII) is followed by an appendix which describes closely related work. The appendix is an
investigation of the utilization of alanine, a three carbon amino acid, as a glyconeogenic substrate in the diaphragm, a chronically active muscle under normal conditions, and in hindlimb muscles from normal and dy<sup>2J</sup> mice in vitro. A major portion of this work was performed by S.A. Mills for whom I served as a direct supervisor. This work was submitted to Respiration Physiology with S.A. Mills and H. Silverman as co-authors.
CHAPTER II. GLYCOGEN SYNTHESIS FROM LACTATE IN
NORMAL AND CHRONICALLY ACTIVE MUSCLE

Journal of Applied Physiology
Glycogen synthesis from lactate in a chronically active muscle

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TALMADGE, ROBERT J., JOHN I. SCHEIDE, AND HAROLD SILVERMAN. Glycogen synthesis from lactate in a chronically active muscle. J. Appl. Physiol. 66(5): 2231-2238, 1989.—In response to neural overactivity (pseudomyotonia), gastrocnemius muscle fibers from C57B1/6J dy2/dy2 mice have different metabolic profiles compared with normal mice. A population of fibers in the fast-twitch superficial region of the dy2 gastrocnemius stores unusually high amounts of glycogen, leading to an increased glycogen storage in the whole muscle. The dy2 muscle also contains twice as much lactate as normal muscle. Activation of the peripheral nervous system leads to preferential incorporation into glycogen in the dy2 muscle compared with normal muscle. To determine whether skeletal muscles were incorporating lactate into glycogen without body organ input, gastrocnemius muscles were bathed in 10 mM [14C]lactate with intact neural and arterial supply but with impeded venous return. The contralateral gastrocnemius serves as a control for body organ input. By using this in situ procedure, we demonstrate that under conditions of high lactate both normal and dy2 muscle can directly synthesize glycogen from lactate. In this case, normal whole muscle incorporates [14C] lactate into glycogen at a higher rate than dy2 whole muscle. Autoradiography, however, suggests that the high-glycogen-containing muscle fibers in the dy2 muscle incorporate lactate into glycogen at nearly four times the rate of normal or surrounding muscle fibers.

Glycogen synthesis; dy2 mice; pseudomyotonia; dystrophic mice; muscular dystrophy

MANY STUDIES have been done identifying the effects of chronic muscle stimulation using the technique of artificial, indirect muscle stimulation (23). The dy2 recessive autosomal mutant of the C57B1/6J mouse strain suffers from a chronic, intermittent, neural stimulation (pseudomyotonia) of the hindlimb musculature. The pseudomyotonic muscle is chronically stimulated by the motor nerve at a variable frequency, up to 100 Hz or in some cases by continuous activity lasting up to half an hour (24). The hindlimb muscles of this mouse can therefore serve as another model of chronic muscle activity.

Skeletal muscles of mice affected by pseudomyotonia display an abnormal periodic acid-Schiff (PAS) staining pattern (34). This pattern is the result of an increase in glycogen storage in some of the fast-twitch, glycolytic (FG) fibers of the superficial region of the gastrocnemius muscle. Similarly, it has been found that chronic, low-frequency stimulation of the extensor digitorum longus and tibialis anterior (both fast-twitch muscles) leads to a similar PAS staining pattern (12, 18). Thus, chronic muscle activity leads to an increased glycogen content in some muscle fibers of the active muscle.

We hypothesize that the high activity of the pseudomyotonic fast-twitch muscle fibers in the superficial region would cause an accumulation of lactate in the whole muscle. Secondly, that the locally produced lactate would be used by the less active or unaffected motor units for glycogen synthesis.

Recent studies have demonstrated that glycogen synthesis from lactate by skeletal muscle occurs in a variety of animals (1, 2, 9, 26). It has been suggested that perhaps this pathway is operative in many vertebrates, but it is only important in the lower vertebrates (8). However, McLane and Holloszy (19) have demonstrated that perfused mammalian skeletal muscle does indeed have the capacity to synthesize glycogen from lactate at physiologically significant rates. Similarly, Johnson and Bagby (14) have demonstrated that glycogen synthesis from lactate may occur after exercise in the rat.

In this study we demonstrate that lactate and glycogen levels are increased in the dy2 muscle and that both normal and pseudomyotonic muscles of mice are capable of utilizing lactate as a substrate for gluconeogenesis. We have measured the uptake of 14C into a glycogen fraction after an intraperitoneal injection of 14C-labeled lactate and shown that the high glycogen muscle fibers incorporate lactate into glycogen with the use of autoradiographic techniques. Functionally isolated muscles with intact nerve and arterial supply were bathed in [14C]-lactate to confirm the ability of muscle fibers to incorporate lactate into glycogen independently of internal body organs.

METHODS

Animals. A colony of C57B1/6J dystrophic (dy2/dy2) mice, henceforth (dy2), has been maintained at the Life Sciences Animal Care Facility at Louisiana State University for the last 7 yr. Mice were judged to be pseudomyotonic (dy2) by observation of the hindlimbs and the progressive loss of hindlimb coordination during exercise. Normal mice are either +/dy2 or +/+ as the dystrophic allele is an autosomal recessive (20). None of the normal animals showed any tendency toward impaired hindlimb function. Mice were between 3 and 6 months of age at the time of experimentation.

Animals to be denervated were first anesthetized with pentobarbital at 60 mg/kg body weight. The right leg
was always used for denervation and the leg used as an internal control. Unoperated animals were used as additional controls. The sciatic nerve and its branch supplying the gastrocnemius were cut at the sciatic notch and about 1 cm of nerve removed. The whole procedure routinely took <15 min and the animals were allowed to recover for 1 day. Gastrocnemius muscles were removed and used for the determination of lactic acid as will be described later.

**Administration of [14C]lactate.** Whole animal labeling experiments were performed by intraperitoneal injection as follows. Normal and dy mice were injected with 1 ml of uniformly labeled [14C]lactate (New England Nuclear Research Products) in 10 mM lactic acid. The vehicle consisted of a modified Liley's saline containing 137 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 24 mM NaHCO₃, and 1 mM NaH₂PO₄ (4). The specific activity of the [U-14C]lactate injection was 11.1 MBq/mmol lactate (Bq = 1 disintegration/s). The pH of the final working saline was 6.5.

The mice were killed by cervical dislocation 3 h after injection and the tissues placed in either 30% KOH for biochemical isolation of glycogen or mounted on cryostat chucks in preparation for autoradiography.

For the isolated hindlimb experiments, anesthetized animals had the skin and the biceps complex of the lower leg carefully dissected away to expose the gastrocnemius muscle, leaving the arterial supply, neural supply, and tendon of origin intact. The Achilles tendon (gastrocnemius insertion tendon) was then cut to free the gastrocnemius leaving the muscle with an adequate arterial supply. The exterior of the muscle was kept damp using Liley's saline during dissection. The gastrocnemius muscle was freed from underlying connective tissue and although still attached to the animal at its origin was then positioned carefully into a 0.6-ml container. Venous return is severed during the dissection. This procedure results in an in situ preparation. The dy gastrocnemius muscle maintains pseudomyotonic activity after this procedure.

The container was filled with Liley's saline containing 10 mM lactate and [U-14C]lactate with a specific activity of 3.7 MBq/mmol. The pH of the solution was 6.6. The muscle was maintained in the bath for 1 h, during which time additional volumes of labeled saline were added to maintain a constant volume. This was necessary because of siphoning of the bath solution out of the container, caused by tissue contact with the lip of the container. In some cases the bath was gassed with 95% O₂-5% CO₂ throughout the experiment. At the completion of the bath time period the gastrocnemius muscle was dissected out and washed in unlabeled saline containing 10 mM lactate for 5 min to remove any extracellular [14C] from the muscle. In addition, the contralateral gastrocnemius muscle, both triceps brachi, and a portion of liver tissue were removed for analysis. All dissection occurred while the animal remained anesthetized. All tissues were prepared for biochemical analysis of glycogen by immersion in 30% KOH as described above. Alternatively, the two gastrocnemius muscles were prepared for autoradiography. The animal was killed by cervical dislocation immediately after dissection.

**Histological and autoradiography.** Cryostat sections were stained for glycogen content using the PAS technique of Luna (17). If slides were to be analyzed by autoradiography for [14C] uptake, then they were subjected to PAS staining and postfixed in 100% ethanol for 5 min. The slides were dried and dipped in melted Kodak NTB3 emulsion under safelight conditions. After removing excess emulsion from the back of the slides, they were placed section side up on a metal tray for 15 min. They were then transferred to exposure boxes section side up. These boxes were wrapped in aluminum foil to prevent light leakage and stored in a 4.5°C cold room. Slides were developed at varying exposure times ranging from 1 to 4 wk. Slides were developed as follows: 2 min in a one-half dilution of Kodak Dektol developer followed by a brief wash in distilled water and fixed in 30% sodium thiosulfate for 8 min. The sections were then dehydrated in a graded ethanol series, cleared in 100% xylene, mounted in Permount, and air-dried at room temperature. Representative slides showing the lowest nonspecific exposure background and the best tissue preservation were chosen for analysis. Silver grain deposition was quantified using a microscope equipped with the Bioquant statistical package (Biosquant, Nashville, TN) and an IBM personal computer. Demonstration of myofibrillary ATPase and succinic dehydrogenase activity was accomplished as in Younger and Silverman (34).

**Biochemical isolation and quantification of muscle glycogen.** Tissues to be analyzed for glycogen content went through an initial glycogen isolation protocol using the procedures of Hassid and Abraham (11). The procedure was sufficient to separate unincorporated [14C]lactate in the tissue from the isolated glycogen fraction (data not shown). Thus there was no contamination of the final pellet by [14C]lactate and no interference in liquid scintillation spectrophotometry determination of [14C] in the glycogen fraction. Glycogen content was determined using Anthrone methodology (6, 11).

**Lactate determination.** Lactate determination was by means of a coupled enzyme reaction, measuring reduced NAD produced by lactate dehydrogenase. The assay was conducted in a 0.5 M glucose-0.1% hydrazine buffer containing 5 mg NAD, 25,000 U lactate dehydrogenase/μl, 25 μl deproteinized sample in perchloric acid, and H₂O to give a final volume of 3 ml. NADH production was monitored spectrophotometrically at 360 nm. Lactate measurements are expressed as micrograms lactate/g wet wt.

**Liquid scintillation spectrophotometry procedures.** Total [14C] in a tissue fraction was determined by pseudo wet ashing the tissue in 1 ml concentrated nitric acid at 70°C for 30 min. A 10%- aliquot of the nitric acid digest was used for determination of [14C] in the fraction. For determination of [14C] incorporation into glycogen a 100-μl aliquot of the glycogen-isolated fraction was used. The aliquot was placed into 10 ml of liquid scintillation counting mixture. Counting continued until a 2 SD spread of 2.0% was reached or to a maximum time of 50 min. For intraperitoneal injection experiments, counting
was for 10 min or until 2 SD spread described above was reached.

**Analytic procedures.** Statistical analysis of the data included pairwise Student's t tests and analysis of variance to determine significant differences. When significant differences became apparent with the use of analysis of variance, this test was followed by Duncan's multiple-range comparisons. All values reported in the results are reported as means ± SE.

**RESULTS**

**Glycogen and lactate content in normal and pseudomyotonic muscles.** The glycogen content of normal mouse gastrocnemius muscle was 1.81 ± 0.36 mg glycogen/g wet wt, whereas the triceps brachii (triceps) muscle contained 1.58 ± 0.20 mg/g wet wt (Fig. 1). In contrast, glycogen content in the skeletal muscles of dystrophic mice were 3.77 ± 0.34 mg/g wet wt for gastrocnemius and 2.19 ± 0.29 mg/g wet wt for triceps. A twofold increase in glycogen is seen in dy^2^ gastrocnemius muscle over normal muscle (P < 0.05), although the dy^2^ triceps, a muscle which does not receive pseudomyotonic stimulation, shows no increase in glycogen compared with normal muscle (Fig. 1). These results are consistent with the suggestion that pseudomyotonic muscle has increased glycogen content (34).

Total lactate content in pseudomyotonic gastrocnemius muscles was nearly twice that found in the normal gastrocnemius muscle as measured by enzymatic assay (519.4 ± 73.4 μg lactate/g wet wt vs. 312.6 ± 81.4 μg/g wet wt, P < 0.05, Fig. 2). The dy^2^ lactate concentration is estimated to be 8 mM if 0.76 ml tissue water/g wet wt is assumed for the analyzed muscles (30). As early as 24 h after experimental denervation of the pseudomyotonic gastrocnemius, lactate content was reduced to control levels (399.4 ± 89.6 μg/g g wet wt for normal and 398.2 ± 53.5 μg/g wet wt for dy^2^). However, the dy^2^ contralateral muscle, which retained its neural supply and therefore its pseudomyotonic activity, continues to show high lactate content (599.1 ± 99.3 μg/g wet wt vs. normal 435.8 ± 125 μg/g wet wt).

**[14C]lactate incorporation.** After a 1-ml intraperitoneal injection of 10 mM [U-^14C]lactate at a specific activity of 11.1 MBq/mmol, and a 3-h metabolic incubation period, significant labeling of the glycogen fraction isolated from the gastrocnemius muscle was observed. A threefold increase in the incorporation rate is observed in the pseudomyotonic muscle vs. the normal muscle such that dy^2^ gastrocnemius incorporates 175.33 ± 31.67 Bq into the glycogen fraction from the [14C]lactate and normal muscle incorporates 54.67 ± 17.33 Bq (n = 8 for both dy^2^ and normal, P < 0.05).

Analysis of autoradiographs (Fig. 3, A and B) of normal and pseudomyotonic gastrocnemius muscle reveals that it is primarily a select population of fibers that show deposition of 14C intracellularly from a [14C]lactate intraperitoneal injection. These select fibers are all intensely stained by PAS methodology, indicating high glycogen content. In normal muscle these fibers are absent and correspondingly one does not see any fibers with a high degree of 14C deposition. In contrast, normal fibers show uniform labeling. Such experiments indicate only 14C incorporation but cannot separate gluconeogenic activity of the muscle fibers themselves from the metabolic contribution of body organs such as the kidney and liver.

In situ experiments were done to isolate the muscle contribution from body organ contribution. In these experiments, muscles with intact neural and arterial blood supply and bathed in 10 mM [14C]lactate (specific activity = 3.7 MBq/mmol lactate) for 1 h were able to incorporate 14C into the glycogen fraction (Table 1). Both normal and dy^2^ gastrocnemius muscles were capable of incorporating [14C]lactate into glycogen. The normal muscle did so at a significantly (P < 0.05) greater rate than the dy^2^. The contralateral gastrocnemius, triceps muscles, heart, and liver were also assayed for 14C in their glycogen fractions. In all cases, no 14C counts above background were detected in the isolated glycogen of these tissues (Table 1). The background level counts in
FIG. 3. Autoradiographs of superficial gastrocnemius muscles after exposure to \(^{14}C\) lactate. Muscles are stained with periodic acid-Schiff for demonstration of glycogen. A and B: normal and dystrophic muscles exposed to label, by intraperitoneal injection, respectively. Note uniform labeling of normal fibers in A. In B variation in glycogen content is seen in \(dy^{23}\) muscle, and arbitrary grouping of fibers is documented: dark or high-glycogen fibers (dk), intermediate fibers (i), and light fibers (l). Note large numbers of grains seen over dark fibers and relatively few grains appearing over light fibers. C and D: normal and \(dy^{23}\) muscles that have been exposed via the in situ bath procedure. Note relative lack of grains in C, with only outer rim of fibers showing any accumulation of grains. D shows a similar pattern to that seen in B, but again grain quantity appears to decrease gradually with distance from periphery of muscle to center. Dark fibers still show largest accumulation of grains, but those on surface show more grains than dark fibers in intermediate fibers, 100 \(\mu\)m.

The tissues were always within the standard error term for a no label control that contained only counting mixture and 100 \(\mu\)l of distilled water.

To determine whether \(O_2\) or \(CO_2\) was limiting to incorporation, some bath experiments were performed with 95% \(O_2\)-5% \(CO_2\) bubbled through the bath solution during the experiment. There were no differences in incorporation observed when gas was applied vs. without gas in either normal or \(dy^{23}\) muscle preparations (Table 2).

Autoradiography of the bathed pseudomyotonic muscles reveals again that the population of fibers that contain high amounts of glycogen show an increased deposition of label. Normal muscle does not contain this population of fibers and did not show any fibers depositing more label than any others. Presumably because of the short time period allowed for the labeled lactate to diffuse into the tissue from the bath, only the outer rim of fibers (43 to 4 fibers deep) displayed any major label deposition (Fig. 3, C and D).

Quantitative analysis of deposited grains in the bathed muscle experiment was used to assess \(^{14}C\) incorporation by individual muscle fibers. Fibers in pseudomyotonic muscle were arbitrarily assigned to one of three groups based on PAS staining intensity (dark, light, and intermediate) and normal fibers made up a fourth group. Five superficial fibers from each of the four groups were
TABLE 1. \(^{14}C\) incorporation into glycogen fraction of tissues taken from mice after "isolated" gastrocnemius exposure to \(^{14}C\) lactate in vivo

<table>
<thead>
<tr>
<th>Tissue</th>
<th>(d_\text{14}^C) Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastrocnemius</td>
<td>203.0±23.0 (8) 373.0±57.0 (8)</td>
</tr>
<tr>
<td>Contralateral</td>
<td>0 (8)</td>
</tr>
<tr>
<td>Triceps</td>
<td>0 (8)</td>
</tr>
<tr>
<td>Liver</td>
<td>0 (7)</td>
</tr>
<tr>
<td>Heart</td>
<td>0 (6)</td>
</tr>
</tbody>
</table>

Values are means ± SE in nmol lactate per g muscle wet wt incorporated per h for no. of samples indicated in parentheses. All values recorded as zero indicate that measured \(^{14}C\) in tissue was not above background. * Significantly different from normal, \(P < 0.05\).

TABLE 2. Effects of 95% O\(_2\)-5% CO\(_2\) on \(^{14}C\) incorporation into glycogen fraction of tissues from mice after "isolated" gastrocnemius exposure to \(^{14}C\) lactate in vivo

<table>
<thead>
<tr>
<th>Animal</th>
<th>With Gas</th>
<th>Without Gas</th>
</tr>
</thead>
<tbody>
<tr>
<td>(d_\text{14}^C)</td>
<td>205.0±40.0 (4) 212.0±28.0 (4)</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>354.0±67.0 (4)</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE in nmol lactate per g muscle wet wt incorporated per h for no. of samples indicated in parentheses. No significant differences were found.

FIG. 4. Graphic analysis of exposed silver grains found over arbitrarily established fiber groups represented in Fig. 3, C and D. Fiber types are dark, intermediate, and light fibers from the \(d_\text{14}^C\) muscle and normal muscle fibers. Values represent mean of 5 fibers for each fiber group after in vivo bath exposure to labeled lactate. Error bars, SE. Dark \(d_\text{14}^C\) fibers are significantly different from all other groups \((P < 0.05)\).

Randomly chosen from a single representative autoradiograph and analyzed. The number of exposed silver grains per fiber cross-sectional area are shown in Fig. 4. As demonstrated in Fig. 4, the PAS-intense (dark, high glycogen content) fibers contained the most grains/\(\mu m^2\) at 0.295 ± 0.029. This value is almost 8 times greater than that found in pseudomyotonic light fibers (0.038 ± 0.016 grains/\(\mu m^2\), \(P < 0.001\)), 2 times greater than intermediate fibers (0.145 ± 0.030 grains/\(\mu m^2\), \(P < 0.05\)), and almost 3.5 times greater than fibers from normal muscle (0.085 ± 0.050 grains/\(\mu m^2\), \(P < 0.01\)).

ATPase histochecmistry reveals that the superficial region of the gastrocnemius muscle in \(d_\text{14}^C\) mice contains virtually all fast-twitch fibers (Fig. 5). This region in the normal mouse is composed entirely of FG fibers (7). In the \(d_\text{14}^C\) mouse, most of these fibers demonstrate an increased oxidative capacity above normal (27). However
the high-glycogen fibers have not increased in oxidative capacity. These findings are similar to previous studies (34). These high-glycogen fibers are the group that shows enhanced label incorporation after exposure to labeled lactate as seen in Fig. 3.

**DISCUSSION**

Younger and Silverman (34) demonstrated that some fibers found in the gastrocnemius muscle of dy2 mouse stored unusually high amounts of glycogen whereas others appeared depleted and still others appeared normal. Thus, it appears that the increase in total glycogen in the whole muscle is the result of the presence of a select population of fibers that have increased their glycogen stores. These fibers are located in the superficial region of the gastrocnemius (34), a region which does not suffer from the degenerative changes associated with the dy2 condition early in life (7, 27) (see Fig. 5). This region is occupied primarily by fast-twitch fibers (Fig. 5) (98% fast-twitch glycolytic in the normal muscle) (7), whereas it is primarily the oxidative fibers in the deep region of the muscle that degenerate early in life (3, 27). Therefore, the area of interest is considered to be nondegenerative.

Maier and Pette (18) and Hudlicka et al. (12) have demonstrated a similar glycogen-staining pattern in normal fast-twitch muscle subjected to chronic, indirect stimulation. Therefore, the staining pattern seen in the superficial gastrocnemius of the dy2 mouse appears related to the overactivity imposed upon this muscle. Further evidence for this point is the rapid loss of high-glycogen fibers after either denervation (29) or phenytoin therapy (34), both of which remove the pseudomypotonic overactivity.

We demonstrate that pseudomyotonic muscles also contain significantly higher amounts of lactate than do normal controls. The high lactate in the dy2 gastrocnemius is presumably produced by the increased activity of the fast-twitch fibers that are originally glycolytic in metabolic profile (10, 27). Some of these fibers are clearly active as documented by electromyographic recording from this region (27). We hypothesize a causal relationship between a high lactate environment and glycogen synthesis from lactate by some fibers situated in this environment. Support for this argument is as follows. Denervation reduces glycogen content (29) and lactate concentration as demonstrated in this study. Further, experiments on normal muscle suggest that glycogenogenesis from lactate by skeletal muscle is a viable alternative for lactate removal from active muscles (13, 21).

Johnson and Bagby (14) and Stevenson et al. (32) have demonstrated that rat fast-twitch muscle will utilize lactate to replenish glycogen supplies after exercise, only when high blood lactate levels are maintained for an extended period of time. This is similar to the situation of the dy2 muscle, as it is continually bathed in lactate presumably produced by the active motor units in the muscle.

This study confirms the capacity for normal and pseudomyotonic muscles to synthesize glycogen directly from lactate. The process occurs at physiological concentrations of lactate. In the dy2 muscle, the lactate concentration is calculated to be ~8 mM using a muscle water estimate of 76% (30). Further, under certain exercise conditions, blood lactate concentrations can easily exceed this level in humans (6); thus all experiments were performed at high but physiologically relevant lactate concentrations.

Injection of [14C]lactate into mice, suffering from pseudomyotonia, resulted in labeling of the isolated glycogen fraction from skeletal muscles. The rate of incorporation was significantly greater in the pseudomyotonic muscle vs. the normal muscle. This is consistent with in vitro experiments that demonstrate that the incorporation of 14C from lactate into glycogen by rodent skeletal muscle is dependent on the lactate concentration presented to the muscle (19). Because the lactate concentration of the pseudomyotonic muscle is significantly greater than the normal muscle it incorporates more lactate into glycogen than normal muscle.

Because the label was introduced via an intraperitoneal injection, the contribution of body organs (liver, kidney) to labeling of muscle glycogen must be considered. It is well documented that the liver (15, 16, 33) and the kidneys (22) utilize lactate for glycogenesis. Newly synthesized glucose can then be transported to the muscle by the vascular system and subsequently used for glycogen synthesis by the muscle, thus contributing to the label observed in the muscle glycogen.

Therefore, skeletal muscles were isolated away from the body organs while still maintaining an in situ preparation. In these experiments both normal and dy2 gastrocnemius muscles were subjected to high lactate conditions with [14C]lactate added to follow its incorporation into glycogen. The sciatic nerve and arterial vasculature remain intact allowing the muscle to remain viable and pseudomyotonia to persist in the dy2 muscle. In this preparation the contralateral limb controls for body organ input. If labeled lactate were to enter the blood supply during the incubation period, incorporation of labeled glucose produced by the body organs into glycogen would be observed in the contralateral limb.

With the use of autoradiography, only the high-glycogen fibers are observed to incorporate any appreciable amount of lactate. It is probable that these fibers are actively synthesizing glycogen although the surrounding fibers are the actively contracting pseudomyotonic fibers that produce the high lactate environment. In every case, whether dy2 or normal, the contralateral muscles showed no incorporation of 14C into glycogen. Forelimb triceps and liver of animals with bathed hindlimbs also showed no incorporation of 14C into glycogen. Thus the hindlimb was sufficiently isolated away from the internal body organs, allowing demonstration of direct muscle incorporation. The incorporation rate of 14C from lactate into glycogen by normal gastrocnemius muscle exposed to high lactate was 374 nM·g−1·h−1 at 10 mM lactate. McIvane and Holloszy (19) observed an incorporation rate of 11,300 nM·g−1·h−1 at 12 mM lactate during perfusion in rat hindlimb muscle.

Several differences in protocol are likely responsible for the lower incorporation rates observed in our studies. In our bath experiment, the lactate passed to the muscle...
fibers through the connective tissues instead of the vascular system as in McLane and Holloszy’s (19) experiments. Autoradiography demonstrates that only those fibers located at the periphery of the muscle were subject to the lactate bath conditions (because of diffusion and dilution effects). In this regard our data are underestimates of total [14C]lactate incorporation, because only peripheral fibers had access to the [14C]lactate and the whole muscle, including central fibers, was used in the biochemical analysis.

Other differences that may affect glycogenogenesis from lactate include the pH and CO₂ concentrations in the bathing or perfusing solutions. Bendall and Taylor (2) have suggested that high pH and high CO₂ concentrations are stimulating glycogen synthesis from lactate. McLane and Holloszy (19) maintained a pH of 7.3 and gassed the perfusate with 95% O₂-5% CO₂. In our experiments, the pH of the bathing solution was 6.6 after the addition of the 10 mM lactic acid. Bubbling the bath with 95% O₂-5% CO₂ had no effect on incorporation.

The actively contracting pseudomyotonic whole muscle incorporated [14C]lactate into its glycogen fraction at a rate (208 nM·g⁻¹·h⁻¹) significantly less than the normal muscle under identical conditions. This is presumably the result of the presence of the many actively contracting (pseudomyotonic) fibers located in the superficial region of the pseudomyotonic muscle (28, 29). These contracting fibers, which continue to contract under anesthesia (27), would be producing or oxidizing lactate, but not using it for glycogen synthesis. Because of anesthesia all fibers in normal muscle are inactive; therefore all fibers can be in a glycogenic mode as opposed to the δ² muscle in which only the inactive fibers can be in a glycogenic mode. The autoradiography data are consistent with this thought (see Fig. 3A). In the δ² muscle, the darkly staining (high glycogen) fibers contain almost eight times the amount of label as the light fibers and twice as much as the intermediate fibers. Normal muscle fibers on the other hand are similar to the intermediate fibers in the δ² muscle. Thus the PAS dark fibers are synthesizing glycogen at high rates directly from lactate, intermediate and normal fibers at lower rates, and light fibers may actually be in net glycogenolytic phase. The whole muscle glycogen synthesis from lactate rate is decreased to a level below that observed when normal muscle is exposed to high lactate conditions. There is a greater than normal incorporation of lactate into glycogen under these conditions by those fibers responsible for the increased glycogen content in the δ² muscle.

Sahlin (25) has recently offered two hypotheses for the fate of lactic acid produced by active skeletal muscle. One hypothesis states that some muscle fibers produce lactate whereas other surrounding fibers utilize lactate as an oxidizable energy source. The second one states that lactate acid equilibrates with pyruvate and thus oxidation of pyruvate will also contribute to lactate removal. Stanley and Brooks (31) offer a third possibility that states that some fibers both produce and oxidize lactate. We suggest that although some fibers in active muscle may produce and/or oxidize lactate and that lactate will equilibrate with pyruvate, lactate can also be used by some motor units of an active muscle as a substrate for glycogen synthesis. Lastly, we conclude that one explanation for the presence of the high glycogen-containing fibers within chronically active skeletal muscle would be an increased usage of the lactate to glycogen pathway by less active motor units because of the presence of a high lactate environment. The increased glycogen stores within these fibers may then provide the muscle with an increased resistance to fatigue as discussed by Hudlicka et al. (12).

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REFERENCES


Chapter III: GLYCOGENIC AND GLYCOGENIC ENZYMES IN CHRONICALLY ACTIVE AND NORMAL SKELETAL MUSCLE
INTRODUCTION

Vertebrate skeletal muscle has the capacity to synthesize glycogen from lactate (Bonen et al. 1990, Connett 1979, McLane and Holloszy 1979, Pagliasotti and Donovan 1990, Talmadge et al. 1989). Chronically active muscles, including the pseudomyotonic gastrocnemius from the dy²J (dystrophic) mouse, apparently have an increased capacity to utilize lactate for glycogen synthesis (Talmadge et al. 1989). Pseudomyotonic muscles, as well as other chronically active fast-twitch muscles (Hudlicka et al. 1977, Maier and Pette 1987), contain muscle fibers with elevated glycogen levels (Younger and Silverman 1984). These fibers in the pseudomyotonic muscle have an increased ability to take up lactate in in situ experiments. While all fibers in the muscle show some capacity to incorporate lactate into glycogen, the specialized high glycogen fibers do so at a much higher rate than other fibers (Talmadge et al. 1989). The specialized fibers apparently do not undergo as much contractile activity as the majority of fibers in the muscle since the specialized fibers do not have an increased oxidative capacity, as do the majority of fibers (Younger and Silverman 1984). Thus, it is hypothesized that these less active fibers take advantage of the extracellular lactate released from chronically active fibers and utilize it for glycogen synthesis (Talmadge et al. 1989).

In this report, we analyze the activities of glyconeogenic enzymes in whole muscle extracts of both normal and chronically active muscles. The enzymatic adaptations leading to the high lactate incorporation in the muscle, and particularly the specialized
glyconeogenic fibers, have not been investigated. These adaptations may be important components of muscle fiber response to exercise, aerobic training, and abnormal neural activity. Chronic muscular activity leads to changes in the enzymes associated with the contractile apparatus, sarcoplasmic calcium regulation and oxidative energy production (Hargroder et al. 1986, Pette 1984). Exercise training also leads to adaptations associated with contractile function (Baldwin et al. 1986), and with the enzymes associated with the production of ATP (Holloszy and Coyle 1984). Adaptations in the enzymes associated with glyconeogenesis in response to chronic contractile activity or aerobic training have not been well studied.

The enzymatic process by which skeletal muscle converts lactate into glycogen is unknown, but it is known that the process is not a mass action reversal of the glycolytic pathway (Krebs and Woodford 1965). There are two steps in the glycolytic pathway considered to be irreversible: the reaction catalyzed by phosphofructokinase (PFK, EC 2.7.1.11), and the reaction catalyzed by pyruvate kinase (PK, EC 2.7.1.40, see Fig. 1). The bypass reaction utilized to circumvent PFK requires fructose 1,6 bisphosphatase (FBPase, EC 3.1.3.11). Two possibilities exist for the conversion of pyruvate to phosphoenolpyruvate in skeletal muscle. The first involves the formation of oxaloacetate and the subsequent formation of phosphoenolpyruvate (PEP) by phosphoenolpyruvate carboxykinase (PEPCK, EC 4.1.1.31). In the liver, oxaloacetate is formed from pyruvate by the enzyme pyruvate carboxylase (PC, 6.4.1.1), but PC is not present in
CHAPTER III.

Figure 1. Proposed bypass reactions for pyruvate conversion to phosphoenolpyruvate, involved in the glyconeogenic pathway in skeletal muscle. Pathway 1 involves the simple reversal of pyruvate kinase. Pathway 2 involves 3 cytosolic enzymes (malic enzyme, malate dehydrogenase, and phosphoenolpyruvate carboxykinase) and 2 intermediates (malate and oxaloacetate). The larger arrow at PK represents the kinetically favored direction. Abbreviations: EN, enolase; PK, pyruvate kinase; LDH, lactate dehydrogenase; ME, malic enzyme; MDH, malate dehydrogenase; and PEPCK, phosphoenolpyruvate carboxykinase.
skeletal muscle (Keech and Utter 1963). Therefore, oxaloacetate may be formed by a two step cytosolic pathway in muscle, involving the formation of malate from pyruvate by malic enzyme (ME) (NADP⁺-linked malate enzyme, EC 1.1.1.40) and subsequently the formation of oxaloacetate from malate by malate dehydrogenase (MDH, EC 1.1.1.37) (Connett 1979). Oxaloacetate is converted to phosphoenolpyruvate by PEPCK. All three of these enzymes are present in the cytosol; therefore, mitochondria may not be involved. The second possibility involves the reversal of pyruvate kinase and under certain conditions the kinetics of the PK reaction favor the formation of phosphoenolpyruvate (Dyson et al. 1975).

In this study, we examined whole muscle enzymatic activities of skeletal muscle glyconeogenic enzymes: glycogen synthase (GS, EC 2.4.1.11); the putative pyruvate kinase bypass enzymes, ME, MDH, and PEPCK; the PFK bypass enzyme, FBPase; and a key glycogen catabolic enzyme, glycogen phosphorylase (PHOS, EC 2.4.1.1). Secondly, we utilized histochemical techniques to assess muscle fiber type specificity for GS, PHOS, ME, and MDH.
MATERIALS AND METHODS

Normal C57B16J and C57B16J dy^2J/dy^2J mice were housed in the Life Sciences Vivarium at Louisiana State University. This colony was maintained at the university for approximately 11 years. The mice used in this study were between 3 and 6 months of age and represent mature, adult mice.

Gastrocnemius (hindlimb) and triceps (forelimb) muscles were typically removed from the animal following cervical dislocation, weighed and homogenized in an appropriate buffer dependent upon the particular enzyme to be assayed. Homogenization was carried out on ice using a ground glass tissue homogenizer and ice cold homogenization buffers. The gastrocnemius muscle of the dy^2J mouse receives a pseudomyotonic, chronically and repetitively active, neural supply (Rasminsky 1978). This muscle eventually degenerates; however, at the ages used in this study, only the deep, primarily oxidative portion of the muscle undergoes significant degeneration (Butler and Cosmos 1977, Hargroder et al. 1986). Therefore, this muscle is used as a model for chronic muscular activity (Talmadge et al. 1989). The triceps muscles of the dy^2J mice in our colony do not show any pseudomyotonia or any changes associated with the dy^2J condition (Hargroder et al. 1986, Talmadge et al. 1989). Thus, the triceps muscle is an internal control. Although similar to the gastrocnemius by having a deep oxidative and superficial glycolytic region, the percentages of specific fiber types in the triceps muscle are different from the gastrocnemius. Thus, direct comparisons should be viewed with caution.
For GS measurements, freshly dissected whole muscles were homogenized on ice in 9 volumes of a buffer consisting of 50 mM Tris-HCl, 5 mM EDTA, and 250 mM sucrose (pH 7.8). The homogenate was then centrifuged at 16,000g and the supernatant used for enzyme activity measurements. The filter paper assay of Thomas, Schlender and Larner (1968) was used to measure glycogen synthase activity. Briefly, 30 µl of the homogenate was added to 60 µl of the assay mixture which consisted of 50 mM Tris-HCl (pH 7.8), 5.0 mM EDTA, 6.7 mM UDP-14C-glucose (0.05 µCi/µmole), and 10 mg/ml rabbit liver glycogen and incubated at 30°C for 10 min. The assay was run in the presence and absence of 6.7 mM glucose-6-phosphate. Seventy-five µl of the assay mixture was removed and spotted onto filter paper squares (Schleicher and Schuell grade 903). The filter paper squares were then washed twice in 66% ethanol (25 min and 10 min) and once in acetone (5 min). Finally, the filter papers were air dried and placed into liquid scintillation vials containing 10 ml of counting cocktail and 14C counted by typical liquid scintillation procedures in a Beckman LS8000 liquid scintillation counter (Talmadge et al. 1989).

For PEPCK analysis, the muscle tissue was homogenized in 9 volumes of buffer consisting of 100 mM Tris-HCl, 1 mM EDTA, 5 mM MgCl2, and 20 mM 2-mercaptoethanol (pH 7.5). The homogenate was centrifuged at 600g for 10 min at 4°C and the supernatant used for PEPCK activity analysis. The procedure used was a modification of method A of Duff and Snell (1982). Briefly, 50 µl of sample was mixed with 450 µl dH2O and 500 µl double strength buffer to yield a final concentration of 100 mM Imidazole-HCl (pH 6.6), 3 mM phosphoenolpyruvate, 2 mM inosine
diphosphate, 2 mM MnCl₂, 2 mM dithiothreitol, 2.5 mM NADH, 50 mM NaH¹⁴CO₃ (40 mCi/mol), 2.5 µg/ml antimycin A, and 12 units/ml MDH. The mixture was incubated at 25°C for 15 min and stopped by addition of 1 ml of 2 M HCl. One half ml of the preparation was evaporated to dryness at 85°C to remove unincorporated ¹⁴C. The dried precipitate was dissolved in 0.5 ml dH₂O and this suspension was added to 10 ml scintillation cocktail and ¹⁴C was counted as previously described.

Tissues to be analyzed for ME were homogenized in 9 volumes of homogenization buffer consisting of 50 mM Tris-HCl (pH 7.4). For MDH the tissues were homogenized in 9 volumes of 0.1 M NaPO₄ buffer. Homogenates for analysis of both enzymes were centrifuged at 600g for 15 min and the supernatants used for enzymatic analysis. Standard procedures for analysis of NADP and NAD dependent dehydrogenases were performed at 25°C (Hsu and Lardy 1969, Smith 1983). Fructose 1,6 bisphosphatase was measured according to the procedure of Krebs and Woodford (1965) at 25°C on tissue supernatants homogenized in 9 volumes of 20 mM Tris-HCl (pH 7.4), and 1 mM EDTA and centrifuged at 2,500g for 15 min.

For measurement of PHOS, the procedures of Schmidt and Wegener (1990) were followed at 25°C. The tissues were prepared according to Conlee et al. (1979) except that the Dowex treatment of the extracts was omitted. The protein content of the final homogenate was determined using the Biorad, Coomasie brilliant blue dye reagent assay (Bradford 1976).
Histochemical Analysis

Gastrocnemius muscles were frozen in liquid nitrogen or Freon cooled by liquid nitrogen immediately following removal from the animal. Twelve μm thick sections were taken on a Cryocut cryostat, air dried for 30 min, and subsequently stained for myosin adenosine triphosphatase (ATPase) activity according to the method of Staron et al. (1983). In the mouse, muscle fibers typically classified as type I are slow-twitch, oxidative (SO); type IIA are fast-twitch, glycolytic (FG); and type IIB are fast-twitch, oxidative, glycolytic (FOG). This is different from other mammals in which type IIA and IIB fibers are reversed in oxidative capacity (Reichman and Pette 1982). Serial 12 μm thick (20 μm for glycogen synthase) sections were then stained for glycogen, by the PAS technique (Maier and Pette 1987), as well as MDH, ME, succinate dehydrogenase (SDH, Troyer 1980), GS (Takeuchi and Glenner 1961) and PHOS (Eränkö and Palkama 1961).

Tissue sections were viewed on a Zeiss light microscope with camera lucida attachment. Camera lucida drawings were made to match serial sections of single fibers stained by different histochemical techniques, which allowed for the assessment of multiple enzymatic activities in single muscle fibers. Alternatively, tissue sections were viewed on a Nikon Microphot FXA with camera attachment for light microphotography.

Fibers were subjectively assigned to one of four classes based on PAS staining intensity (high, medium, low, and depleted). Enzymatic activities (GS, ME, MDH, and PHOS) were similarly evaluated. For GS, ME, and PHOS fibers were assigned to one of four classes: no measured
activity (-), low activity (+), intermediate activity (++), and high activity (+++). For MDH and SDH fibers were assessed to be either high or low in enzymatic activity.

Statistical analysis was performed on treatment means using Student's t-tests to determine significant differences. Statistical significance was set at p < 0.05.
RESULTS

Pseudomyotonic (dy^2J) gastrocnemius muscle shows greater than normal GS activity in whole skeletal muscle homogenates (figure 2). This increase in GS activity is seen in both the glucose-6-phosphate stimulated and non-stimulated activities (Fig. 2A,B). Thus, the total GS activity (glucose-6-phosphate stimulated) is nearly 23% greater in the dy^2J gastrocnemius as compared to the normal gastrocnemius (p < 0.05). There is approximately 44% more I form (active form, non glucose-6-phosphate stimulated) GS activity in the dy^2J gastrocnemius compared to control (p < 0.05). The increase in the I form of GS results from an increase in the percentage of GS in the active form from 39% in the control gastrocnemius to 45% in the dy^2J gastrocnemius (Fig. 2C). The triceps muscles do not show any difference (normal vs. dystrophic) in either glucose-6-phosphate stimulated or non-stimulated activities, nor are these values different from those found in the normal gastrocnemius muscle.

Histochemistry demonstrates that the FG fibers (according to ATPase histochemistry and SDH activity, Fig. 3A,B) store low amounts of glycogen (Fig. 3C and 4A). The FG fibers also show a relatively uniform and low (+) staining for GS activity in the normal superficial gastrocnemius muscle (Fig. 3D, fiber 1, and Fig. 4B). Fig. 3E and 3F show homogeneous low staining for MDH activity and high ME activity. Fig 4C demonstrates that 91% of all superficial gastrocnemius fibers from normal mice have high ME activity. Thus, 90% of all fibers in the normal superficial gastrocnemius are FG, low in glycogen, low in GS
CHAPTER III.

Figure 2. Glycogen synthase (GS) activity in normal and dystrophic mouse muscles performed on whole muscle homogenates. A) Total GS measured in the presence of 6.7 mM glucose-6-phosphate (G-6-P). B) Active GS (I form) activity measured in the absence of G-6-P. C) Percentage of glycogen synthase in the active (I) form. Assays are as described in Materials and Methods. Dy^{21} values which are significantly (p < 0.05) different from normal are represented by * . Reported values are means ± standard error of the mean (SEM), n = 16 for each group.
CHAPTER III.

Figure 3. Serial sections of normal, superficial gastrocnemius muscle. Stained for A) pH 4.3 pre-incubation myosin ATPase, B) succinate dehydrogenase (SDH), C) glycogen (PAS), D) glycogen synthase (GS), E) malate dehydrogenase (MDH), and F) malic enzyme (ME). Fiber 1 represents a typical fast-twitch glycolytic (FG) fiber found in this region. It is characterized by low SDH and MDH activities, low amounts of glycogen and GS activity and high ME activity. Fiber 2 represents a fast-twitch oxidative glycolytic (FOG) fiber which is found in the minority in this region. It shows high SDH activity, low glycogen, and slightly higher GS activity, while MDH and ME activities are similar to the surrounding fibers. The bar in A equals 35 μm. All other micrographs are at the same final magnification.
CHAPTER III.

Figure 4. Fiber type characteristics in the normal superficial gastrocnemius muscle. Percentages of the total number of fibers analyzed for: A) Glycogen content, either high, medium, low, or depleted; B) GS activity, either no (-), low (+), intermediate (++), or high (+++) activity; and C) malic enzyme (ME) activity, either no, low, intermediate, or high activity. All quantifications were based on staining intensity as described in materials and methods. The data are pooled from 3 normal animals and represent 800 total fibers. The same 800 fibers were used for all three analyses.
activity and high in ME activity. Ten percent have variations in either oxidative capacity, glycogen, GS activity, ME activity or combinations of these.

The superficial region of the pseudomyotonic (dy^{2J}) gastrocnemius muscle contains exclusively fast-twitch fibers according to ATPase histochemistry (Fig. 5A). Some of the fast-twitch fibers have low SDH capacity (Fig. 5B, fiber 1) and are FG fibers, while other fast-twitch fibers stain high for SDH activity (Fig. 5B, fibers 2 and 3) and are FOG fibers. In this muscle, the glycogen staining intensity appears to be related to the GS activity (Fig. 5C). The majority (80%) of fibers depleted of their glycogen show low levels or no GS activity based on the relative scale (Fig. 6). Those fibers containing low levels of glycogen show a wide range of GS activities. Approximately 55% of all low glycogen fibers have low or no GS activity. Medium and high level glycogen fibers typically have higher levels of GS activity. About 60% of the medium glycogen fibers show intermediate or high levels of activity. About 65% of the high glycogen fibers have intermediate or high levels of GS activity. Clearly, the high glycogen fibers have the greatest percentage (25%), which were scored as having the highest level of GS activity.

The activity of phosphoenolpyruvate carboxykinase was higher in the pseudomyotonic gastrocnemius muscle as compared to the normal control (p < 0.05, Fig. 7A). The forelimb triceps of the dy^{2J} mouse, a muscle which does not receive any pseudomyotonic input, shows similar levels of PEPCK activity as the normal mouse triceps and this activity is also similar to that found in normal gastrocnemius muscle.
CHAPTER III.

Figure 5. Serial sections of dy²⁻, superficial gastrocnemius muscle stained as in Fig. 3. Fiber 1 is a high glycogen fiber which shows high GS activity. All other characteristics are similar to normal FG fibers (ie low SDH and MDH, and high ME activities). Fiber 2 is an intermediate glycogen fiber which shows typical FOG characteristics (ie high SDH and MDH activities, intermediate GS activity and low ME activity). Fiber 3 is a low glycogen fiber which also shows FOG characteristics. The bar in A equals 35 μm. All other micrographs are at the same final magnification.
CHAPTER III.

Figure 6. Quantification of GS staining intensity in different fiber types (based on glycogen content) in dy²J muscle. GS activity is subjectively quantified based on staining intensity as described in Materials and Methods. The percentage of fibers of a given fiber type staining at the different intensities is plotted on the y-axis. The data are pooled from 3 dy²J animals and represent percentages of the total fiber number for a given fiber type. A total of 823 fibers were examined 133 high, 385 medium, 215 low glycogen, and 90 glycogen depleted fibers. A) Percentages of glycogen depleted fibers staining at identified intensities. B) Percentages of low glycogen containing fibers staining at identified intensities. C) Percentages of medium glycogen containing fibers staining at identified intensities. D) Percentages of high glycogen containing fibers staining at identified intensities.
CHAPTER III.

Figure 7. Activities of the putative pyruvate kinase bypass reaction enzymes in whole muscle homogenates. A) PEPCK, B) ME, C) MDH. Assays are as described in Materials and Methods. Dy² values which are significantly (p < 0.05) different from normal are represented by *. Reported values are means ± standard error of the mean (SEM), n = 12 for PEPCK (all groups), 10 for ME, and 8 for MDH.
Both ME and MDH activities are significantly higher in homogenates of dy2J gastrocnemius than in normal gastrocnemius (p < 0.05 for both enzymes), whereas the triceps muscles from normal and dy2J animals have similar activities for both enzymes (Fig. 7B,C). The high oxidative fibers in the superficial dy2J gastrocnemius (Fig. 5B) also stain intensely for MDH activity (Fig. 5E); thus, high oxidative fibers (based on SDH staining intensity) show high levels of MDH as well. ME appears to stain most intensely fibers that are low in oxidative capacity (Fig. 3F and 5F). Thus, in the dy2J muscle, it is the high glycogen, high GS, FG fibers which stain most intensely for ME (Fig. 8). Approximately 93% of the glycogen depleted fibers in the dy2J superficial gastrocnemius muscle have low or no ME activity (Fig. 8A), and 76% of low glycogen fibers have low or no ME activity (Fig. 8B). Medium level glycogen containing fibers show 30% with intermediate to high ME activity (Fig. 8C), and 66% of high glycogen fibers are intermediate to high in ME activity.

Fructose 1,6 bisphosphatase has a different response to pseudomyotonia than the other enzymes presented. The pseudomyotonic gastrocnemius muscle has reduced FBPase activity as compared to normal (p < 0.05, Fig. 9). Similar to the other enzymes studied, there is no difference in enzymatic capacity between the two triceps muscles (Fig. 9).

The total activity of PHOS was significantly reduced in dy2J gastrocnemius muscles, as was the activity of PHOS present in the active form (Table 1). However, there was no measurable difference in the percentage of PHOS present in the active form. The percentage of
CHAPTER III.

Figure 8. Quantification of ME staining intensity in different fiber types (based on glycogen content) in dy^2J muscle. ME activity is quantified based on relative staining intensity as described in Materials and Methods. The percentage of fibers of a given fiber type staining at the different intensities is plotted on the y-axis. The data are pooled from 3 dy^2J animals and represent percentages of the total fiber number for a given fiber type. A total of 845 fibers were examined 195 high, 330 medium, 265 low glycogen, and 55 glycogen depleted fibers. A) Percentages of glycogen depleted fibers staining at identified intensities. B) Percentages of low glycogen containing fibers staining at identified intensities. C) Percentages of medium glycogen containing fibers staining at identified intensities. D) Percentages of high glycogen containing fibers staining at identified intensities.
CHAPTER III.

Figure 9. Activity of FBPase in whole muscle homogenates. Assays are as described in Materials and Methods. Dy²J values which are significantly (p < 0.05) different from normal are represented by *. Reported values are means ± standard error of the mean (SEM), n = 14 normals and 17 dy²J muscles.
CHAPTER III.

TABLE 1. Phosphorylase activity in normal and pseudomyotonic muscles.

<table>
<thead>
<tr>
<th>Muscle</th>
<th>Animal</th>
<th>n</th>
<th>No AMP added</th>
<th>AMP (3 mM)</th>
<th>% of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastrocnemius</td>
<td>Normal</td>
<td>6</td>
<td>0.097 ± 0.015</td>
<td>0.309 ± 0.021</td>
<td>31.6 ± 4.8</td>
</tr>
<tr>
<td></td>
<td>dy^{2J}</td>
<td>6</td>
<td>0.045 ± 0.004*</td>
<td>0.158 ± 0.018*</td>
<td>28.9 ± 1.4</td>
</tr>
<tr>
<td>Triceps</td>
<td>Normal</td>
<td>6</td>
<td>0.100 ± 0.016</td>
<td>0.291 ± 0.022</td>
<td>33.7 ± 3.8</td>
</tr>
<tr>
<td></td>
<td>dy^{2J}</td>
<td>6</td>
<td>0.091 ± 0.006</td>
<td>0.318 ± 0.017</td>
<td>29.2 ± 3.1</td>
</tr>
</tbody>
</table>

Values are means ± SEM (Standard error of the mean), n is the number of muscles used of each type. All animals were anesthetized with pentobarbital at the time of tissue collection by freeze clamping. It should be noted that pseudomyotonic activity persists in the dy^{2J} gastrocnemius during anesthesia. Assays performed in the presence of 3 mM AMP yield total activity. One unit (U) is defined as the amount of enzyme necessary to produce 1 μMol of hexose-phosphate per minute. * denotes statistically significant dy^{2J} value from corresponding normal value at the p < 0.01 level by Student's T Test.
PHOS in the active form was approximately 30% for both normal and \( \text{dy}^{2J} \) gastrocnemius muscles. The triceps muscles showed no differences in total PHOS activity, PHOS activity in the active form, nor in the percentage of PHOS in the active form.

Histochemical staining for PHOS activity (Fig. 10) demonstrates that in normal superficial gastrocnemius muscle PHOS activity is high (Fig. 10B). Table 2 shows that nearly all fibers in this region have high or intermediate levels of PHOS activity, with 94% showing high activity.

The \( \text{dy}^{2J} \) superficial gastrocnemius shows a much different staining pattern (Fig. 10D). Fibers showing higher levels of glycogen appear to have higher levels of PHOS activity. Nearly 90% of all high glycogen fibers have high to intermediate levels of PHOS activity (Table 2). Seventy percent of fibers containing medium levels of glycogen have intermediate PHOS activity. Low glycogen containing fibers and glycogen depleted fibers tend to have low levels of PHOS activity. Over 63% of both low glycogen and glycogen depleted fibers have low or no PHOS activity.
CHAPTER III.

Figure 10. Serial sections of normal (A, and B) and dy²J (C, and D) superficial gastrocnemius muscle stained for glycogen (A, and C) and phosphorylase activity (B, and D). Three normal fibers are labeled with an ‘n’ to aid in fiber matching. Four dy²J fibers are identified according to glycogen content. Fiber 1 containing high glycogen. Fiber 2 represents a medium glycogen fiber. Fiber 3 represents a low glycogen fiber and fiber 4 a glycogen depleted fiber. Note the corresponding decrease in PHOS activity with decreasing glycogen content.
CHAPTER III.

TABLE 2. Phosphorylase activity (relative staining intensity) in specific muscle fiber types* from dy²J and normal mice.

<table>
<thead>
<tr>
<th>Phosphorylase Relative Intensity</th>
<th>dy²J Glycogen Content</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High</td>
<td>Medium</td>
</tr>
<tr>
<td>-</td>
<td>1.6</td>
<td>3.0</td>
</tr>
<tr>
<td>+</td>
<td>9.1</td>
<td>17.7</td>
</tr>
<tr>
<td>++</td>
<td>42.4</td>
<td>70.6</td>
</tr>
<tr>
<td>+++</td>
<td>46.9</td>
<td>8.7</td>
</tr>
</tbody>
</table>

* Fiber types were based on glycogen content (PAS staining intensity).

** Numbers represent the percentage of fibers within a fiber type class displaying a relative measure of phosphorylase staining intensity.

The data are pooled from 3 dy²J and 3 normal animals. A total of 1095 dy²J fibers were examined, 243 high, 436 medium, 305 low glycogen fibers, and 111 glycogen depleted fibers. A total of 1041 normal fibers were examined and treated as a single group due to the homogeneous staining for glycogen content.
DISCUSSION

We have demonstrated that GS activity as well as activities of some key glyconeogenic enzymes, PEPCK and ME, are elevated in a chronically active muscle that stores increased amounts of glycogen. The fiber type distribution of GS and ME in the chronically active muscle demonstrates that the high glycogen fibers have adapted to increase glycogen synthesis and to increase the usage of glyconeogenesis as a means for supporting the increased glycogen stored in this muscle.

The total activity of GS is increased by approximately 23% in the chronically active dy²J gastrocnemius over what is observed in the normal gastrocnemius. There is also a 44% increase in the I form (active form) of GS. Thus, not only is there an increase in the total amount of GS present, represented by the increase in total activity, but there is also a greater percentage of the increased enzyme pool in the active form. The increased GS activity helps to explain the increased levels of glycogen found in this muscle (Talmadge et al. 1989). However, others have found that increased levels of endogenous glycogen correspond with decreased levels of GS activity (Conlee 1978, Henriksen et al. 1989) and decreased rates of glycogen synthesis from glucose as the substrate (Hutber and Bonen 1989). In the dy²J muscle, GS activity remains high despite the increased glycogen content. The maintenance of high GS activity allows muscle glycogen to reach levels well above normal.

We demonstrate that it is primarily the fibers with high and intermediate levels of glycogen that show high levels of GS activity.
Normal muscle typically contains fibers that are low in glycogen and a minority (less than 3%) of fibers that are intermediate in glycogen content. The sub-population of fibers (high and intermediate glycogen containing fibers) that is primarily responsible for the increased glycogen content in the dy²J muscle shows high GS activity. This is in contrast to the inverse relationship typically observed between glycogen content and GS activity (Conlee et al. 1978, Henriksen et al. 1989). Perhaps at some point during chronic muscular activity this inverse relationship is negated allowing the muscle fiber to increase its glycogen supply to greater than normal levels. The increased glycogen may provide this fiber with some additional resistance to fatigue (Hudlicka et al. 1977, Talmadge et al. 1989).

We observed that all three bypass enzymes necessary for the formation of PEP from pyruvate in the pathway of glycogen synthesis from lactate were elevated in the dy²J gastrocnemius. Our data provide evidence that the enzymatic pathway by which pyruvate is converted to PEP is through the three step process involving ME, MDH, and PEPCK as opposed to being a reversal of the pyruvate kinase step. The elevation of MDH activity in this muscle probably plays little role in the adaptation for pyruvate conversion to PEP, since this is an oxidative enzyme. The MDH increase is more likely due to the overall increase in oxidative capacity observed in virtually all cases of chronic muscular activity. This suggestion is supported by the histochemical data showing that the fibers having high levels of oxidative capacity in the dy²J gastrocnemius typically do not show high levels of glycogen. The elevation in ME and PEPCK probably plays a more direct role in the
adaptations of this pathway to supply increased PEP for glyconeogenesis, since malic enzyme histochemical preparations demonstrate a preferential staining of FG fibers in normal and dy^{23} muscle for ME activity. The FG fibers in the dy^{23} muscle show a higher glycogen content, increased GS activity, high ME activity and enhanced glyconeogenesis. Pagliasotti and Donovan (1990) found that glyconeogenesis occurs at higher rates in muscles composed primarily (>99%) of FG fibers in the rabbit. We also found that glyconeogenesis occurs at higher rates in muscle fibers that are low in oxidative capacity (FG fibers) within a muscle of mixed fiber type in the mouse (Talmadge et al. 1989). Since the high glycogen FG fibers show a high level of ME activity, and ME is a PK bypass enzyme, the PK bypass enzymes appear to be important in glyconeogenesis. In rat glycolytic muscle, ME and PEPCK are found predominantly in the cytosolic compartment with a small percentage in the mitochondria (Nolte et al. 1972). A more oxidative muscle had low cytosolic and high mitochondrial levels despite similar total activity levels. The high cytosolic ME and PEPCK in FG muscle may explain the higher capacity of FG fibers for glyconeogenesis and is in agreement with the extramitochondrial, pyruvate kinase bypass pathway proposed by Connett (1979).

PEPCK is thought to be one of the rate limiting enzymes in glyconeogenesis; thus, an increase in the levels of PEPCK would signify an increase in the usage of this pathway. Other laboratories (Connett 1979, Pagliasotti and Donovan 1990, Shiota et al. 1984) utilized mercaptopicolinic acid (MPA), an inhibitor of PEPCK, in an effort to
elucidate the pathway by which pyruvate is converted into PEP.
Mercaptopicolinic acid significantly reduced the synthesis of glycogen from lactate in amphibian muscle, demonstrating the involvement of PEPCK (Connett 1979). However, MPA showed no effect on glyconeogenesis in mammalian muscle, suggesting that PEPCK is not involved in glyconeogenesis in skeletal muscle (Pagliasotti and Donovan 1990, Shiota et al. 1984). In the present experiment, we observed an increase in PEPCK activity in a muscle which contains fibers that show enhanced glyconeogenesis, which is circumstantial evidence that PEPCK is involved in mammalian skeletal muscle glyconeogenesis. Another possibility is that in normal mammalian muscle PK reversal is adequate for glyconeogenesis, but in the chronically active muscle glyconeogenic rates may be increased in some fibers, such that PK reversal would become limiting. An adaptive increase in the activities of the bypass enzymes may then become necessary to support the increased glyconeogenic rates.

Our study supports the hypothesis that the increased glycogen observed in the dy^{2J} gastrocnemius is synthesized, at least in part, from locally produced lactate. If six carbon intermediates were acting as the initial substrate, there would be no need for increased levels of the bypass enzymes. Other three carbon substrates, such as alanine, could enter the pathway at the same point as lactate. But, if glucose were the primary source, it would enter the pathway at glucose-6-phosphate and not be used by the bypass reactions. It is curious that the measured activity of fructose 1,6 bisphosphatase is lower in the dy^{2J} gastrocnemius compared to the normal gastrocnemius. Clearly, there
was an increase in the activities of the other gluconeogenic enzymes. One possible explanation for the observed decrease is the fiber type transformation occurring in the gastrocnemius as it becomes more oxidative (Hargroder et al. 1986). Fewer fibers would have high levels of this enzyme as it has been shown that more oxidative muscles have lower fructose 1,6 bisphosphatase activity than glycolytic muscles (Opie and Newsholme 1967). Therefore, whole muscle would show lower levels of fructose 1,6 bisphosphatase activity despite the presence of the few high glycogen fibers.

Glycogen phosphorylase, the enzyme responsible for glycogen breakdown, is reduced in total activity in the dy^2J gastrocnemius. A reduction in PHOS activity is expected due to the fiber type transitions occurring in this muscle resulting from the chronic activity. Typically, FG fibers have high PHOS activity and FOG fibers have low PHOS activity. As the oxidative capacity of the dy^2J gastrocnemius muscle increases (Hargroder et al. 1986), FOG fibers become more numerous. Thus, the number of fibers with high PHOS activity decreases as does the overall PHOS activity. The fiber type distribution of PHOS supports this idea. Muscle fibers from the normal superficial gastrocnemius muscle have high levels of PHOS activity. In the dy^2J muscle, the high glycogen fibers which maintain a low oxidative profile have high PHOS activity, whereas the low glycogen, typically high oxidative fibers, have reduced levels of PHOS activity. The methods used in this study were unable to show any differences in the percentage of PHOS in the active form.

Whole muscle biochemical analysis combined with histochemical
methods were able to demonstrate fiber type specific differences in ME, MDH, PHOS, and GS enzyme activities in response to chronic activity. Those fibers responsible for the increased glycogen content in the pseudomyotonic muscle were shown to have high activities of GS, PHOS and ME and to maintain a low oxidative profile. We hypothesize that the high glycogen fibers are a population of less active fibers within an actively contracting muscle (Talmadge et al. 1989). These fibers take advantage of lactate produced locally by the active fibers in the muscle and utilize it as a substrate for glycogen synthesis. The net result is an increased glycogen content within the whole muscle. Finally, the observation that the dy^{2J} triceps appeared normal in all aspects provides evidence that the observed changes are due to the chronic muscular activity imposed upon dy^{2J} GAST and are not due to any inherent genotypic defect in the muscle of these animals.
CHAPTER IV: VARIATION IN GLYCOGEN ALONG THE LENGTH OF SINGLE MUSCLE FIBERS IN NORMAL AND CHRONICALLY ACTIVE SKELETAL MUSCLE
Muscle fiber typing is often accomplished by examining serial cross-sections histochemically reacted for a number of substances or enzyme activities. Such fiber typing has been used to describe dynamic changes occurring in muscle fibers following experimental procedures such as denervation, immobilization, and artificial stimulation. Of course, discrete type grouping, while useful, does tend to obscure the continuous nature of most of the variables to be classified. Further, interpretation of fiber typing results is based on the assumption that a fiber is uniform in all characteristics along its entire length. However, during fast to slow fiber type transformation, induced by chronic stimulation, some fibers show characteristic staining as type II fibers in some regions and type I in other regions (Staron and Pette 1987). Thus, muscle fibers may not be homogeneous along their entire length under all circumstances, particularly during events which appear to cause dynamic alterations in state such as chronic contractile activity and chronic stimulation.

Similarly, the levels of muscle fiber metabolites and storage products, such as glycogen and/or lipid stores, have the potential to vary along the length of the fiber (Hintz et al. 1982). Thus, the validity of using a single cross-section to identify the "type characteristics" for a fiber must be carefully evaluated, particularly when the muscle fiber has undergone experimental treatments leading to dynamic changes in fiber characteristics.

The hindlimb muscles of the C57Bl6J dy^{2J}/dy^{2J} (dy^{2J}) mouse suffer
from a chronic stimulation via spontaneous firing of hindlimb motor neurons (pseudomyotonia). The pseudomyotonia originates due to ephaptic transmission at the spinal roots which are associated with abnormal myelination (Rasminsky 1978). This abnormal activity results in the expected increase in oxidative activity in most of the fast-twitch fibers in the gastrocnemius muscle (Silverman and Atwood 1980, Hargroder et al. 1986) Other fast-twitch fibers in this muscle have been shown to contain elevated levels of glycogen (Younger and Silverman 1984, Talmadge et al. 1989), similar to what is seen under artificial chronic stimulation (Hudlicka et al. 1977, Maier and Pette 1987). Autoradiography and enzyme histochemistry show that these high glycogen fibers appear to have an increased capacity for glycconeogenesis (Talmadge et al. 1989, Talmadge and Silverman 1991). The gastrocnemius muscle of the dy2J, therefore becomes an interesting model of chronic muscular activity which can be studied with reference to muscle fiber adaptation. In particular, the enzymatic adaptations responsible for observed increases in glycogen levels by some muscle fibers can be analyzed.

These studies as well as future studies require fiber-typing based on muscle fiber cross-sections. The current study documents the variability of glycogen content across the length of both normal and chronically active fast-twitch muscle fibers. In general, fibers from chronically active muscle show more variability in glycogen content along their length than fibers from control muscle, but the level of variability does not cause fibers to be misclassified with reference to glycogen content analyzed using single cross-section analysis.
MATERIALS AND METHODS

Normal C57Bl6J and C57Bl6J dy^{2J}/dy^{2J} (dy^{2J}) mice were housed in the Life Sciences Vivarium at Louisiana State University. This breeding colony has been maintained for approximately 11 years. All mice used in the following experiments were between 3 and 6 months of age. These represent mature adult mice.

Gastrocnemius muscles were removed from normal and dy^{2J} mice following cervical dislocation. The muscles were mounted at rest length on cryostat chucks in either tragacanth gum or Tissue Tek O.C.T. mounting medium, frozen in liquid nitrogen or Freon cooled by liquid nitrogen and placed in an American Optical Cryo-Cut cryostat cooled to -20° C. The time from cervical dislocation to tissue freezing was less than 1 minute. Serial 22 μm thick cross sections were taken at spaced intervals along the muscles' length (up to 3mm), mounted on glass slides, air dried for 30 min and stained for glycogen by the periodic acid Schiff (PAS) technique (Maier and Pette 1987). Briefly, sections were fixed for 1 hour in a mixture of 25% glacial acetic acid, 75% absolute ethanol, washed briefly in 70% ethanol. The sections were treated for 1 hour in 1% periodic acid in 90% ethanol, 5 min. in 90% ethanol, 1 min in distilled water, 30 min in Schiff's reagent (Luna 1968), two 3 min. washes in 0.5% sodium metabisulfite, and a final 5 min wash in distilled water. The sections were then dehydrated in a graded alcohol series, cleared in xylene and coverslips mounted with Poly-mount (Polysciences Inc. Warrington PA). Occasionally, serial sections were stained for myosin ATPase according to the acid
preincubation protocol of Staron et al. (1983).

Individual muscle fibers were first subjectively classified to be either high (dark staining), intermediate (intermediate staining) or low (light staining) in glycogen based on staining intensity of the PAS reaction. The glycogen content (PAS staining intensity) of classified single muscle fibers was then objectively quantified. Sections were viewed on a Nikon Optiphot microscope equipped with a Nikon halogen bulb (5.5 volts), NCB-10 filter, N-plan achromatic 60x objective lens, and a Nikon spot-meter photometer. The meter was set to ASA 25 and a background light intensity reading of 16.22 seconds exposure time. The staining intensity of single fibers was measured by placing the spot-photometer over the center of a given fiber and using the resulting exposure time as a determinant of staining intensity. Staining intensities ranged from 17 to 95 seconds exposure time. Some sections were also viewed using a Nikon Microphot-FXA microscope equipped with a lux meter to assess light transmittance and to verify the previous method. Exposure time measurements and lux measurements were shown to have a second order polynomial relationship and a coefficient of correlation of 0.965 (Fig. 1). Thus, the exposure time readings are a reliable determinant of staining density.
CHAPTER IV.

Figure 1. Relationship between camera exposure time readings and illuminance (lux) readings using spot photometry of the same individual fibers from normal and dy²J gastrocnemius muscle. The curve is significant at p < 0.01.
RESULTS

All fibers classified for glycogen content were fast-twitch according to ATPase histochemistry. Figure 2 shows a set of serial sections through the superficial region of normal gastrocnemius muscle spanning 858 μm. By visual observation there is no difference in the staining intensity along the examined lengths of single identified muscle fibers in the normal muscle. Also, the variation between separate individual fibers is quite low. In contrast, Figure 3 shows serial sections from the superficial region of dy²J gastrocnemius muscle, in this case spanning 1,430 μm. Clearly, there is much greater variation in the PAS staining intensities between individual fibers in this muscle. Subjective staining intensities (dark, intermediate, and light) can be assigned to the individual fibers of this muscle. The staining intensities along the lengths of individual fibers, however, appear to be rather invariable based on visual assessment of this muscle.

Quantified staining intensities are given in Table 1. In this case, 20 fibers of each subjectively assigned fiber type were evaluated according to the exposure time method. The visual assignments are supported by the quantitative data. First, the normal fibers show an extremely low variability either within single fibers (mean of SEM of single fibers) or between individual fibers (SEM of fiber means). Second, fibers from dy²J muscle show a much greater between fiber variation as seen by the difference in exposure time means between light, intermediate, and dark staining fibers. The high
CHAPTER IV.

Figure 2. Serial sections from normal gastrocnemius muscle stained for glycogen using the PAS technique. Section B is taken 396 μm and section C is 858 μm distal to section A. Note the lack of apparent variation in PAS staining intensity (glycogen content) along the length of the individual fibers. All fibers stain relatively light with very little variation between separate individual fibers. Fibers a, b, c, and d can be used for orientation. The bar = 50 μm for all sections.
CHAPTER IV.

Figure 3. Serial sections from dy²J gastrocnemius muscle stained for glycogen using the PAS technique. Section B is taken 330 μm, section C is 1,122 μm, and section D is 1,430 μm distal to section A. Section A was used to initially classify the fibers based on the subjective visual analysis of the fibers. Fibers a, b, and c are high glycogen (dark) fibers. Fibers d, and e are intermediate in glycogen, and fibers f, and g are low (light) in glycogen. Note how dark fibers a, b, and c change in staining intensity along their length compared to each other. In section A fiber b stains more intense than a or c. In section B fibers a, b, and c are all similar in staining intensity. In section C fiber b is only slightly more intense than a or c. In section D fiber a is more intense than b or c. Despite the changes in intensity of the three fibers, they remain dark fibers throughout the length examined. Also note the change in position of the intermediate fiber marked with the asterisk in relation to the three dark fibers, as well as the change in diameter of fiber f. The bar = 50 μm for all sections.
CHAPTER IV.

TABLE 1.

Individual Fiber Statistics

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Light</th>
<th>Intermediate</th>
<th>Dark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exposure Time Means</td>
<td>19.551</td>
<td>23.308</td>
<td>28.795</td>
<td>36.811</td>
</tr>
<tr>
<td>S. E. of Fiber Means</td>
<td>0.068</td>
<td>0.216</td>
<td>0.492</td>
<td>0.870</td>
</tr>
<tr>
<td>Tukey's Grouping</td>
<td>A</td>
<td>A</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>Mean of Fiber S. E.</td>
<td>0.260</td>
<td>0.596</td>
<td>1.207</td>
<td>2.507</td>
</tr>
<tr>
<td>Number of Fibers</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Number of Muscles</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

Exposure time means for the four classes of fibers, 1 normal class and 3 dy²J classes (Light, Intermediate and Dark). The exposure time is in seconds and is a quantitative measure of staining intensity. S.E. is the standard error. Tukey's grouping followed an analysis of variance.
between fiber variation is supported by the fact that light, intermediate, and dark staining fibers are statistically different from one another based on Tukey's groupings. Third, the within fiber variation is low enough in the three fiber types of the dy²J muscle such that statistically significant groups can indeed be identified.

The PAS staining intensity along the lengths of four typical fibers of the three classes in the dy²J and the single class in the normal superficial gastrocnemius muscle is shown in figure 4. Dark staining fibers from the dy²J muscle have the highest exposure times and fibers from normal muscle the lowest. The variation in PAS staining intensity along the length of a single dark fiber is higher than for any of the other groups, as indicated by the standard errors (Table 1). The higher the amount of glycogen staining in a single fiber the greater is the variation in glycogen along the length of that fiber.

Figure 5 shows one fiber from each class on the same y-axis scale and demonstrates that some fibers (see dark fiber) have relatively long segments in which the glycogen staining intensity is different from the remainder of the fiber. Although there is some overlap between dy²J dark and intermediate, and intermediate and light fibers, the overlap is not enough to lead to misidentification of fibers in most sections.
CHAPTER IV.

Figure 4. PAS staining intensity (exposure time reading in seconds) along the lengths of four representative fibers from each class. One fiber is represented per line. A) Dark staining fibers from dy²J gastrocnemius muscle, B) Intermediate fibers from dy²J gastrocnemius muscle, C) Light fibers from dy²J gastrocnemius muscle, and D) Fibers from normal muscle. Note the change in y-axis between B and C.
CHAPTER IV.

Figure 5. PAS staining intensity (exposure time readings in seconds) along the length of a single representative fiber from each of the four classes of fibers presented on the same graph. One fiber is represented per line. The ◊ represents a dark staining fiber from dy^{2J} gastrocnemius muscle, the □ an intermediate fiber from dy^{2J} gastrocnemius muscle, the ◦ a light fiber from dy^{2J} gastrocnemius muscle, and the △ a fiber from normal gastrocnemius muscle.
DISCUSSION

Myosin ATPase and SDH staining have long been used as a means for classifying muscle fiber types. Typically, fast-twitch glycolytic fibers have a relatively homogeneous PAS-staining intensity. However, in some muscles following chronic contractile activity, there exist distinct fiber types based on PAS staining intensity (glycogen content). The fiber types can be classified as high, intermediate and low glycogen containing fibers.

The presence of high glycogen fibers in the pseudomyotonic muscles of the C57B16J $dy^{2J}/dy^{2J}$ was initially reported by Younger and Silverman (1984). The metabolic processes by which high glycogen fibers accumulate high levels of glycogen in the pseudomyotonic muscle of the $dy^{2J}$ mouse have been studied (Talmadge et al. 1989, Talmadge and Silverman 1991). These fibers have an elevated capacity for glycogen synthesis from lactate as determined both histochemically and biochemically. However, it has not been shown that these fibers contain high glycogen along their entire length. In order for serial section histochemistry to be a valid tool for describing the enzymatic potentials of these fibers, the glycogen content of these fibers must be relatively uniform along the entire length of the fiber and not just in the form of local deposits of glycogen. This study clearly shows that the glycogen content of the fibers in both the $dy^{2J}$ and normal gastrocnemius muscle are relatively uniform along their length. Despite an increase in variability with increased glycogen content, any
fiber initially placed in a specific glycogen staining class based on a single section was never variable enough to be misclassified.

Our finding that there is some variation in the glycogen content along the length of normal and dy²J fast-twitch glycolytic muscle fibers (type IIa in mouse and type IIb in other mammals, Reichman and Pette, 1982) is similar to the findings of Hintz et al. (1982), who demonstrated that in rat muscles there is some local variation in muscle fiber glycogen. However, the variation noted by Hintz et al. (1982) in resting normal FG fibers in rats is greater than the variability noted here in mouse muscles (standard error of the mean of approx. 3-4% of fiber means by Hintz vs. 1% in our study). Some important differences must be noted between the two studies. Hintz et al. (1982) utilized approx. 50 μm lengths of freeze dried muscle fibers for each assay compared to 22 μm thick frozen sections in this study. Hintz et al. (1982) made use of microbiochemical procedures compared with histochemical procedures used in this study. Hintz et al. (1982) also noted that there are considerable swings in glycogen content along relatively long segments (millimeters) of fibers. Our results, especially those of the dy²J PAS intense staining fibers, appear to confirm that there are swings in glycogen content along relatively long segments of muscle fibers, but the variability is not enough to lead to misclassification of fibers in the dy²J muscles.

A somewhat surprising finding was the fact that the lowest glycogen staining fibers in the dy²J muscle were similar in PAS staining intensity to the fibers from the normal muscle. It was initially
thought that the lightest staining dy^2J fibers were depleted of glycogen, due to the relative lack of staining based on visual analysis, and therefore, lower in glycogen content than fibers from normal muscle. The surrounding PAS-intense staining fibers enhance the apparent lightness of the PAS-light fibers making them appear less intensely stained. The present results demonstrate that the lightest staining (PAS) fast-twitch fibers in the dy^2J gastrocnemius muscle are not glycogen depleted and indeed contain similar amounts of glycogen on average as normal fast-twitch glycolytic fibers.

The presence of high glycogen fibers in chronically active fast-twitch muscles was first noticed by Hudlicka et al. (1977), when they subjected rabbit fast-twitch muscle to chronic indirect stimulation. At the time, it was speculated that the presence of high glycogen in some fibers might provide these fibers with some additional resistance to fatigue (Hudlicka et al. 1977). This hypothesis is reasonable, as it has been shown that muscular endurance is related to the sustained glycogen content of the muscle (Terjung et al. 1985). A more recent study designed to study the time necessary for depletion of muscle fiber glycogen also demonstrated the presence of high glycogen fibers in fast-twitch muscles subjected to chronic, indirect muscle stimulation (Maier and Pette (1987).

It appears that the presence of high glycogen fibers in the dy^2J gastrocnemius is a consequence of the chronic activity imposed upon the muscle by the pseudomyotonia. It was found that removal of the chronic pseudomyotonia by either treatment with the anti-epileptic drug
phenytoin (Younger and Silverman 1984) or cutting the sciatic nerve innervating the pseudomyotonic gastrocnemius muscle (Silverman et al. 1983) caused a reversion to a more normal PAS staining pattern of the fast-twitch muscle fibers in the gastrocnemius muscle of dy^{2J} mice. This implies that the increased neuromuscular activity caused the production of high glycogen fibers.

The increase in glycogen content of a fast-twitch muscle fiber could be an initial step in the adaptation of muscle to chronic contractile activity. Alternatively, the presence of such fibers may be related to the characteristics of stimulus application. In all of the artificial stimulation experiments that produced fibers with abnormally high glycogen levels, the chronic stimulation was interspersed with hourly rest periods. A study performed by Henriksson et al. (1988) in which the muscle was not given any rest periods during the chronic motor nerve stimulation revealed no fibers storing abnormally high levels of glycogen. Thus, the superimposed rest periods appear to play a role in the supercompensation of glycogen by some fibers in a chronically active muscle. The dy^{2J} gastrocnemius muscle endures bouts of contraction and relaxation continuously throughout the life of the animal. Thus, the adaptations necessary to maintain the continued contractile activity have occurred and may have included an increase in glycogen content by fast-twitch fibers.

In this study, we demonstrate that the variation in glycogen content along the lengths of single muscle fibers in normal and chronically active muscles is low enough to allow for classification of
a muscle fiber (based on glycogen content) using a single section. The finding is important to single fiber studies, where one section of muscle fiber is identified by glycogen content and another section of the same fiber analyzed for enzymatic activities.
CHAPTER V: GLUTOSE UPTAKE AND GLYCOGEN SYNTHESIS IN NORMAL AND CHRONICALLY ACTIVE SKELETAL MUSCLE
INTRODUCTION

The C57Bl6J dy^2J/dy^2J mouse (dy^2J) suffers from pseudomyotonia, an abnormal chronic and repetitive stimulation of the hindlimb musculature (Rasminsky 1978), associated with a lack of myelination at the spinal roots innervating these muscles (Jaros and Jenkison 1983). The hindlimb muscles of the dy^2J mouse can be used as a model for chronic contractile activity (Talmadge et al. 1989, Talmadge and Silverman 1991).

We previously documented that the superficial region of the gastrocnemius muscle (GAST) from the dy^2J mouse: a) has an oxidative capacity greater than normal controls (Hargroder et al. 1986), b) contains higher amounts of glycogen (Talmadge et al. 1989, Younger and Silverman 1984), c) shows increased activities of some key glyconeogenic and glycogen synthetic enzymes (Talmadge and Silverman 1991), and d) has a population of fast-twitch glycolytic muscle fibers with an increased capacity to synthesize glycogen from lactate and store high amounts of glycogen (Talmadge et al. 1989). This muscle region does not show degenerative changes at the ages used in this study.

It was shown that exercise and contractile activity of a skeletal muscle can lead to metabolic changes, including increased glucose uptake (James et al. 1985, Wallberg-Henriksson 1987, Wallberg-Henriksson 1988), an increased sensitivity to insulin (Cartee et al. 1989, Cartee and Holloszy 1990), recruitment of the cytochalasin B inhabitable and insulin responsive glucose transporter (GLUT-4) into
the sarcolemma (Douen et al. 1990, Hirshman et al. 1988) and an increase in glycogen synthesis from glucose (glycogenesis) (Hutber and Bonen 1989). These changes have been demonstrated in muscles which have been either exercised or stimulated to contract in vitro for relatively short time periods. However, effects of long term contractile activity on these metabolic pathways have not been evaluated.

In this study, we have evaluated the glycogenesis rates in normal and dy²J hindlimb (GAST) and unaffected forelimb muscles (triceps, TRI), as well as the diaphragm muscle (DIA), which is chronically active under normal circumstances. We also evaluated the glucose uptake capacity of these muscles using 2 different glucose analogs (2-deoxyglucose, 2-DG and 3-O-methylglucose, 3-O-MG). All of these variables were determined in vivo and some by in vitro incubation experiments, including the capacity for insulin to stimulate glucose analog uptake and glycogen synthesis from glucose. The in vitro experiments were performed in order to determine if the muscle tissue was directly affected by the chronic activity as opposed to a humoral effect.

In order to perform the in vitro experiments, a relatively thin muscle with long tendons at each end was needed to compensate for diffusion limitations and to obtain muscle fibers which are not compromised by dissection. Therefore, in vitro experiments were performed using the soleus (SOL) and the extensor digitorum longus (EDL) muscles. The normal SOL contains a mixture of fast oxidative and slow fibers, with the dy²J SOL having a similar profile yet showing some
degeneration at the ages studied. The normal EDL contains primarily fast-twitch fibers with a mixed oxidative profile in the normal animal. The dy^{2J} EDL shows an increase in oxidative capacity and some high glycogen fibers, yet shows few degenerative changes at the ages used. Briefly, the chronically active muscles have an increased capacity for glucose uptake and glycogen synthesis as well as an enhanced response to insulin in comparison to non-chronically active muscles.
MATERIALS AND METHODS

Animal Care. Normal C57Bl/6J and C57Bl/6J dy^{2J}/dy^{2J} (dy^{2J}) mice are housed in the Life Sciences Animal Care Facility at Louisiana State University. This breeding colony has been maintained for approximately 11 years. All mice were between 3 and 6 months of age and represent mature adult mice.

In vivo Experiments. Blood glucose was determined on plasma samples taken from anesthetized normal and dy^{2J} mice using the hexokinase, glucose-6-phosphate dehydrogenase reaction according to Sigma Kit #16-10.

To assess the rates of glucose uptake in vivo, 2-deoxyglucose (2-DG) and 3-O-methylglucose (3-O-MG) were used as glucose analogs. Normal and dy^{2J} mice were given an intraperitoneal (IP) injection of $^{3}$H-2-deoxyglucose (0.16 μCi/g animal wt.) in 0.5 ml of 0.9% NaCl. Unlabeled 2-DG was not added to the injection so as not to decrease the specific activity of the label. Following a 30 minute incubation period, the animal was sacrificed by cervical dislocation, and muscle tissues (GAST, TRI, and DIA) were removed for analysis of $^{3}$H content. The isolated tissues were solubilized in 0.5 ml of 0.5 N NaOH. A 0.25 ml aliquot was assayed for $^{3}$H by liquid scintillation procedures using a Beckman LS8000 liquid scintillation counter.

In vivo experiments using 3-O-MG as a glucose analog were similar to above except that 0.16 μCi of $^{3}$H-3-O-MG and 0.016 μCi of $^{14}$C-methoxy-inulin were injected IP in 0.5 ml of 0.9% NaCl per g animal wt. In this case, $^{14}$C-methoxy-inulin served as an extracellular fluid marker,
and corrections for \(^{3}H\)-3-O-MG in the extracellular space were made. Tissues were not washed following removal from the animal to avoid loss of intracellular \(^{3}H\)-3-O-MG (since 3-O-MG is not phosphorylated as is 2-DG, it can diffuse out of the cell). Instead, tissues were immediately frozen in liquid nitrogen and then solubilized as for 2-DG. Radioactivity was counted using dual channel liquid scintillation procedures to determine both \(^{3}H\) and \(^{14}C\) total activities.

In vivo glycogenesis was determined by injecting normal and dy\(^{2}J\) mice IP with 0.1 \(\mu\)Ci \(^{14}C\)-glucose per g animal body weight in 0.5 ml 0.9% NaCl. Following a 30 minute incubation period, the animal was sacrificed by cervical dislocation and the tissues removed for glycogen isolation and analysis.

Tissue glycogen was isolated according to Hutber and Bonen (1989). Briefly, skeletal muscles (GAST, TRI, and DIA) were digested in screw cap tubes containing 0.5 ml of 30% KOH saturated with sodium sulfate. The tubes were placed for 20 minutes in a boiling water bath. Glycogen was precipitated by adding 0.6 ml of ice-cold 95% ethanol and the tubes placed on ice for 20 minutes. The tubes were centrifuged at 2,000g for 10 minutes and the supernatant was discarded. The glycogen pellet was washed 3 times by repeated resuspension in 66% ethanol and centrifugation. Finally, the glycogen pellet was air dried, by draining tubes over filter paper, and resuspended in 1.0 ml of distilled H\(_{2}\)O. A 0.2 ml aliquot of the isolated glycogen was assayed for \(^{14}C\) by liquid scintillation (Talmadge et al. 1989). A second aliquot (0.1 ml) was used for assaying total glycogen according to a phenol-sulfuric acid procedure of Lo et al. (1970).
Autoradiography, following in vivo administration of $^{14}$C-glucose, was performed according to Talmadge et al. (1989). Following the injection of the label and the 30 min incubation period, GAST were removed, mounted on cryostat chucks with O.C.T. (Tissue Tek) and frozen in liquid nitrogen. Serial tissue sections, 20 µm thick, were taken on an American Optical Cryo-Cut cryostat and stained for glycogen, using a variation of the periodic acid Schiff (PAS) technique (Talmadge et al. 1991), succinic dehydrogenase (Troyer et al. 1980), and myosin ATPase (Staron et al. 1983). Fiber types were then classified according to glycogen content in the dy2d gastrocnemius muscle (Talmadge et al. 1991). Some sections stained by the PAS technique were dipped in photographic emulsion (Kodak NTB2), placed in light tight boxes for 4 weeks, and developed according to Talmadge et al. (1989). The sections were viewed and photographed using a Nikon Microphot-FXA microscope equipped with an image analysis system (Image-1/AT, Universal imaging) and a Sony UP-5000 video printer.

In vitro Experiments. Skeletal muscle incubations were performed according to Henriksen et al. (1986), with some modifications. Briefly, the muscles (EDL and SOL) were incubated in 25 ml flasks containing 4 ml of oxygenated (95% $O_2$: 5% $CO_2$) Krebs-Henseleit bicarbonate (KHB) buffer (Krebs 1950) with 7 mM HEPES (pH 7.4), 1% BSA, and substrate at 37 °C. Two fifteen minute preincubations preceded a final fifteen minute incubation which contained labeled substrate, either $^3$H-2-DG (300 µCi/mMol) and $^{14}$C-inulin (20 nCi/ml) as an extracellular space marker for glucose uptake or $^{14}$C-glucose (2 µCi per bath) for glycogenesis. In glucose uptake experiments the pre-
incubation contained 1 mM glucose and in the final incubation the substrate was 1 mM 2-DG. In glycogenesis experiments, the concentration of glucose was 5 mM in both the pre-incubations and final incubation. In experiments utilizing insulin, the final concentration of insulin in the incubation buffer was from 1 to 10,000 µUnits per ml. An increase in insulin sensitivity is defined as a shift in the dose response curve to the left and responsiveness as a greater response at maximal insulin stimulation (Wallberg-Henriksson 1988). Tissues were analyzed for $^{14}$C and $^3$H as specified above for the in vivo experiments.

Statistical analysis. All values are presented as means ± standard error of the mean (SEM). For multiple group comparisons, analysis of variance (ANOVA) was performed. Bonferroni's post-ANOVA tests for selected group comparisons were performed to determine significant differences. For comparisons between two treatments, Student's t-tests were performed. In all cases, the level for significant differences was set at the p < 0.05 level.
RESULTS

The blood glucose concentration was similar between the normal and dy²J mice (Table 1). Thus, the in vivo experiments with labeled glucose and glucose analogs do not suffer from specific activity differences between groups. All of the muscles analyzed for uptake of 2-DG contained ³H-2-DG (Fig. 1). The chronically active DIA, both normal and dy²J, showed the highest amounts of 2-DG uptake (p < 0.05 versus normal GAST). The chronically active dy²J GAST took up 2-DG at levels about 2 times that of the normal GAST (p < 0.05). The non-chronically active dy²J TRI was not significantly different from normal TRI.

Similarly, the DIA also showed the highest rates of 3-O-MG uptake in vivo (p < 0.05 versus normal GAST, Figure 2). The dy²J GAST again showed increased uptake rates above normal (p < 0.05) and the dy²J triceps showed no difference from normal TRI.

The in vivo glycogenesis experiments followed the same pattern as the glucose uptake studies (Fig. 3). Normal and dy²J DIA had the highest amount of ¹⁴C incorporation into glycogen (p < 0.05). The dy²J GAST showed increased glycogen synthesis rates over normal GAST (p < 0.05), and the non-chronically active dy²J TRI showed no difference versus normal TRI.

A linear regression analysis comparing glycogenesis rates in vivo and glycogen contents within the muscles (Fig. 4A) shows that in all muscles unaffected by pseudomyotonia there is a negative correlation (p
CHAPTER V.

TABLE 1. Blood Glucose in normal and dy^{2J} mice.

<table>
<thead>
<tr>
<th>Animal</th>
<th>n</th>
<th>mM</th>
<th>mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>12</td>
<td>11.3 ± 0.5</td>
<td>203 ± 9</td>
</tr>
<tr>
<td>dy^{2J}</td>
<td>12</td>
<td>11.7 ± 0.7</td>
<td>210 ± 12</td>
</tr>
</tbody>
</table>

No significant differences were observed between the two groups following Student's t-test. All animals were analyzed following ad libitum access to food.
CHAPTER V.

Figure 1. Distribution of $^3$H in normal and chronically active muscles following in vivo administration of $^3$H-2-deoxyglucose. N = 6 animals per group. The chronically active diaphragm muscles (DIA) show the highest amounts of 2-DG uptake. The chronically active dy$^{2J}$ (dystrophic) gastrocnemius muscle (GAST) also shows significantly higher uptake than control GAST ($p < 0.05$). The unaffected forelimb triceps muscle (TRI) shows no significant difference between normal controls and dy$^{2J}$ animals. The * signifies significantly different from normal GAST at $p < 0.05$ using the Bonferroni method.
CHAPTER V.

Figure 2. Distribution of $^3$H in normal and chronically active muscles following in vivo administration of $^3$H-3-O-methylglucose. $N = 8$ animals per group. Normal and dy$^{2J}$ DIA took up the greatest amounts of 3-O-MG during the incubation period. The chronically active dy$^{2J}$ GAST showed a significant ($p < 0.05$) increase in uptake vs. normal controls. The TRI showed no significant differences (control vs. dy$^{2J}$). The * signifies significantly different from normal GAST at $p < 0.05$ using the Bonferroni method.
CHAPTER V.

Figure 3. Glycogen synthesis from $^{14}$C-glucose in normal and chronically active muscles following IP injection of $^{14}$C-glucose. $N = 8$ animals per group. The DIA show the highest rates of glycogen synthesis from the $^{14}$C-glucose injection. The dy$^{2J}$ GAST shows a significant ($p < 0.05$) increase over normal controls and the TRI shows no significant difference between normal and dy$^{2J}$ animals. The * signifies significantly different from normal GAST at $p < 0.05$ using the Bonferroni method.
CHAPTER V.

Figure 4. A) Linear regression of glycogen synthesis rates vs. glycogen contents in dy²J gastrocnemius muscles vs. all other muscle groups studied. The linear regression for the dy²J gastrocnemius muscle shows an increase in glycogen synthesis rate as glycogen content is increased ($r^2 = 0.2750$, $p < 0.05$). This is quite different from other muscles which show decreases in glycogen synthesis rates with increasing glycogen contents ($r^2 = 0.1169$, $p < 0.01$). B) Linear regression of glycogen synthesis rates vs. glycogen contents in chronically active muscles. The diaphragm muscles from control and dy²J animals show no dependence of glycogen synthesis rates on tissue glycogen levels ($r^2 = 0.0403$, $p > 0.05$). The dy²J gastrocnemius shows an increase in glycogen synthesis rates with increasing glycogen content ($r^2 = 0.2750$, $p < 0.05$). Perhaps, this aids in driving the glycogen concentration of the dy²J gastrocnemius muscle to the abnormally high levels. D = dystrophic, N = normal.
< 0.05) between glycogenesis rates and glycogen content. The dy^{2J} GAST, however, shows a positive correlation (p < 0.05). If only the DIA is plotted there is no significant relationship.

Autoradiography (Fig. 5A-F) demonstrates that only some of the high glycogen fibers in the dy^{2J} GAST incorporate glucose into glycogen at higher rates than other fibers. Normal GAST shows no fibers which incorporate label at higher rates than any other fibers, resulting in a homogeneous labeling of the muscle (Fig. 5G, and H).

In vitro incubation experiments. The dy^{2J} SOL and EDL muscles showed different types of responses when compared to control SOL and EDL. Basal 2-DG uptake was enhanced in the dy^{2J} EDL compared to control (Fig. 6A), while the dy^{2J} SOL was not different from control (Fig. 6B). The response to insulin treatment was also different for the two muscles. Normal EDL showed little response to insulin treatment, whereas dy^{2J} EDL showed a rather large response (Fig. 6A), resulting in increases in both sensitivity (curve shifted to left) and responsiveness (increase at maximal insulin). The normal SOL showed a typical increase in 2-DG uptake with an increase in insulin concentration (Fig. 6B), while the dy^{2J} SOL showed an increase in both sensitivity and responsiveness to insulin compared to control SOL (Fig. 6B).

Glycogen synthesis measured in vitro demonstrated that normal muscles, both EDL and SOL, showed increases in glycogenesis rate with increasing insulin concentration from 1 to 10,000 μU/ml (Fig. 7A and 8A). The dy^{2J} EDL had an increased basal glycogenesis rate as well as an increased response to insulin even at low (10 μU/ml) insulin
CHAPTER V.

Figure 5. Serial sections from dy²J and normal GAST, taken following in vivo administration of $^{14}$C-glucose. Sections A-F are from dy²J GAST, and sections G and H are from normal GAST. All sections are stained by the PAS reaction for glycogen. Autoradiographs from dy²J GAST (sections B,D,and F) were photographed using a Kodak # 25 filter to remove PAS staining intensity. Section B demonstrates the increased capacity for some high glycogen fibers in the dy²J GAST to take up and synthesize glycogen from glucose (fibers labeled 1, 2, 3, and 4). Sections C, and D are high magnification views of the above sections. Note, not all high glycogen fibers (fiber 5 in sections A-D, and fibers 6-8 in sections E and F) show an enhanced deposition of $^{14}$C. The low and medium glycogen containing fibers (sections A-F) do not show any appreciable deposition of $^{14}$C. Normal GAST (sections G and H) show no fibers staining high for glycogen or incorporating $^{14}$C. Bar equals 100 µm in all sections. Also note that if the $^{14}$C was converted to some form other than glycogen (ie. some small MW glycolytic intermediate) it would have been washed out of the tissue during the fixation and staining procedure.
CHAPTER V.

Figure 6. Insulin stimulated 2-deoxyglucose uptake rates by EDL (A) and SOL (B) during in vitro incubation experiments. N = 6 muscles for each point. All in vitro incubations were gassed with 95% O₂ : 5% CO₂. The concentration of DG was 1 mM, and a 30 min preincubation preceded the 15 min incubation period with ³H-labeled 2-DG. ¹⁴C-Inulin was used to correct for extracellular ³H-2-DG. The * signifies significantly different (p < 0.05) from normal controls at the same insulin concentration (Student’s t-test).
CHAPTER V.

Figure 7. Insulin stimulated glycogen synthesis from glucose by the EDL muscle during in vitro incubations. N = 6 muscles for each point. A) Glycogen synthesis rates at the various insulin concentrations. At all insulin concentrations tested glycogen synthesis was higher in the dy2J EDL vs. normal controls. B) Glycogen content following the incubations. The dy2J EDL contains higher amounts of glycogen following incubation than normal controls. The * signifies significantly different (p < 0.05) from normal controls at the same insulin concentration (Student's t-test).
CHAPTER V.

Figure 8. Insulin stimulated glycogen synthesis from glucose by SOL during in vitro incubation experiments. N = 10 muscles for each point. Glucose concentration was 5 mM. A) Glycogen synthesis rates at the various insulin concentrations. Only at the highest insulin concentration did dy²J SOL show a glycogen synthesis rate greater than normal. B) Glycogen contents following the incubation. Clearly, the dy²J SOL contains higher amounts of glycogen than normal SOL following incubation. The * signifies significantly different (p < 0.05) from normal controls at the same insulin concentration (Student's t-test).
concentrations, in comparison to control EDL muscle (Fig. 7A). Thus, both responsiveness and sensitivity to insulin were increased in the dy\textsuperscript{2J} EDL. The dy\textsuperscript{2J} SOL muscle showed no difference in basal or insulin stimulated glycogenesis in comparison to normal SOL muscles except at the highest insulin concentration tested (10,000 μU/ml) (Fig. 8A). Thus, only insulin responsiveness increased in the dy\textsuperscript{2J} SOL. The concentration of glycogen found in the muscle following the \textit{in vitro} incubation procedure was always higher in the dy\textsuperscript{2J} muscle compared to the normal muscle. This is shown for both the EDL and the SOL muscles (Figs 7B and 8B).
DISCUSSION

This is the first study, to our knowledge, that documents the effects of long-term contractile activity on the metabolism of glucose by skeletal muscle. We analyzed blood glucose levels, the capacity for chronically active skeletal muscles to take up glucose in vivo and in vitro (with and without insulin stimulation), as well as the capacity for chronically active skeletal muscles to synthesize glycogen from glucose in vivo and in vitro (with and without insulin stimulation).

The in vivo experiments demonstrated an increased capacity for chronically active skeletal muscles to take up glucose from the blood. One assumption for measurement of glucose uptake in vivo is that the blood glucose levels are similar between the treatment groups (James et al. 1985). In this study, blood glucose concentrations between normal and dy²J animals were nearly identical. The DIA, a muscle that is chronically active under normal conditions, demonstrated the highest levels of glucose uptake. The high level of glucose uptake exhibited by the DIA is expected, for the DIA has a persistent high energy requirement due its chronic contractile activity. Blood glucose is expected to be used at very high rates to maintain a constant production of ATP in order to sustain contractile activity. The high glucose uptake observed by the DIA is similar to previous results obtained by James et al. (1985), who demonstrated nearly a four-fold higher glucose uptake rate by the DIA in comparison to the GAST in vivo in resting rats. The dy²J GAST, another chronically active muscle, also showed high rates of glucose uptake, which were significantly higher.
than control GAST. Apparently, the energy demands of this muscle also lead to an increased uptake and usage of glucose as an energy source to supply the chronically contracting fibers.

The glucose uptake rates were demonstrated using two different glucose analogs, 2-DG, which is phosphorylated upon entering the cell, but is not further metabolized, and 3-O-MG, which is neither phosphorylated nor metabolized (Wallberg-Henriksson 1987). We observed a similar trend in glucose uptake regardless of which analog was used. The DIA showed the highest incorporation rates, with no observed difference between normal and dy²J DIA. The dy²J GAST showed the next highest glucose uptake rate. The non-chronically active muscles (normal GAST and TRI, and dy²J TRI) showed the lowest rates of uptake. We did observe differences in the absolute uptake rates between 3-O-MG and 2-DG. The lower amount of uptake observed with 3-O-MG may result from 3-O-MG being able to diffuse out of the cell. In which case, 3-O-MG distribution in vivo may reflect the intracellular concentration of glucose following equilibration. Since 2-DG is phosphorylated and remains in the cell, it may be a more reliable indicator of glucose transport than 3-O-MG in vivo. If 3-O-MG actually represents the intracellular glucose concentration, then there are higher concentrations of glucose in the chronically active muscles. A second possible explanation for the apparently lower 3-O-MG uptake in comparison to 2-DG is that some extracellular ³H-2-DG remained in the muscle despite the washout period, contributing to higher ³H in the tissue.

The rates of glycogenesis were higher in the chronically active
muscles, even though contractile activity normally results in a decrease in muscle glycogen content. The DIA shows the highest incorporation rates, followed by the dy^{2J} GAST and the non-chronically active muscles. It appears that those muscles with the highest rates of glucose uptake and perhaps the highest concentrations of intracellular glucose have the highest rates of glycogenesis. It was recently reported that in human skeletal muscle during hyperglycemia there is an increase in glucose uptake, muscle glucose content and glycogen synthase activity (Katz et al. 1991). The dy^{2J} GAST has been shown to have both increased fractional activity and total activity of glycogen synthase (Talmadge and Silverman 1991) in addition to the increase in glucose uptake reported here, thus the controlling factors may be similar in the two preparations.

Exercise also increases the rates of glycogen synthesis by skeletal muscles in vivo (Hutber and Bonen 1989). One difference between normal muscles, either resting (this study) or exercising (Hutber and Bonen 1989), and the chronically active dy^{2J} GAST is that in normal muscle there is an inverse relationship between glycogenesis and glycogen content, whereas in the dy^{2J} GAST this relationship shows a positive correlation. Similarly, it was previously shown that glycogen synthase (GS) activity in the dy^{2J} GAST is increased despite an increased glycogen content (Talmadge and Silverman 1991) and that the high glycogen containing fibers in the dy^{2J} GAST have the highest levels of GS activity. Thus, in the dy^{2J} GAST, high levels of glycogen are associated with high GS activity and increased glycogenesis. Therefore, the normal inhibitory effect of glycogen content on
glycogenesis is apparently lost in the dy^{2J} GAST (Talmadge and Silverman 1991). In comparison, the chronically active DIA showed a non-significant correlation between glycogen content and glycogenesis.

Autoradiography demonstrated that some, but not all, of the high glycogen fibers in the dy^{2J} GAST synthesize glycogen from glucose at increased rates, which may be related to the pattern of motor unit activity in the dy^{2J} muscle. Due to the pseudomyotonia, different motor units may be active at different times. The increased glycogenesis observed in the few high glycogen fibers may have resulted from either recent activity of a particular motor unit or activity of the motor unit concurrent with ^{14}C-glucose administration.

Although the in vivo studies demonstrate an increase in glucose uptake rates and glycogenesis, they do not demonstrate that it is the muscle itself which is adapting to the increased activity. It is possible that the whole animal adapts by regulating the amounts and types of humoral factors in the blood which in turn regulate muscle metabolism. In order to establish if the muscle itself was adapting, in vitro experiments were performed on both chronically active and control muscles. These experiments demonstrate that there is a direct effect of chronic activity on the muscle, and that there is a differential response dependent upon muscle type. The differential response appears to be related to the fiber type characteristics of the muscle. The SOL, which in the normal mouse contains primarily oxidative fibers of both fast and slow-twitch types, responded to chronic contractile activity in a much different way than the EDL, which contains a mixture of oxidative and glycolytic fibers of
primarily fast-twitch type. The dy²J SOL showed no change in basal glucose uptake or glycogenesis. Glucose uptake by the dy²J SOL showed an increased insulin sensitivity (defined as a shift in the dose response curve to the left, Wallberg-Henriksson 1988) and insulin responsiveness (defined as a greater response at maximal insulin stimulation, Wallberg-Henriksson 1988). Glycogenesis was only increased at non-physiologically high insulin levels (10,000 µU/ml), therefore only responsiveness and not sensitivity was increased. The EDL, on the other hand, showed increases in basal glucose uptake and glycogenesis, as well as insulin sensitivity, and insulin responsiveness for both glucose uptake and glycogenesis.

Perhaps the SOL, due to its high oxidative capacity, is near its limit regarding glucose uptake and insulin sensitivity; since, glucose uptake by oxidative muscles is more insulin sensitive than glucose uptake by glycolytic muscles (James et al. 1985, Wallberg-Henriksson 1987). Oxidative muscles also contain higher quantities of the insulin regulatable glucose transporter GLUT-4 (Henriksen et al., Kern et al. 1990). It is also possible that some degeneration is occurring in the dy²J SOL and may contribute to the difference in response observed between SOL and EDL. The normal EDL, a highly glycolytic muscle, shows only a low sensitivity to insulin. The dy²J EDL, subjected to the chronic activity, increases in oxidative capacity and may concurrently become more insulin sensitive with respect to glucose transport.

This experiment supports the hypothesis that glucose transport is not related to cellular glycogen levels (Richter et al. 1988). Glycogen levels are elevated in the dy²J GAST, EDL and SOL, but glucose
transport is also elevated. It was previously thought that the post-exercise increase in insulin responsiveness of glucose uptake is dependent upon glycogen depletion (Zorzano et al. 1986). It was also suggested that increases in muscle glycogen following exercise are responsible for the eventual decrease in glucose transport observed during recovery (James et al. 1985). In the case of the chronically active muscle, glucose transport is elevated despite increased glycogen levels.

It appears that the disposal of glucose by oxidation could be rate limiting for glucose catabolism, which may explain the concomitant increase in glycogen synthesis and lactate production in the dy^2J GAST (Talmadge et al. 1989). The data showing that an enhanced glucose uptake rate coincides with an increased rate of glycogen synthesis in an actively contracting highly oxidative muscle (dy^2J GAST) provide some evidence that glucose catabolism is rate limiting for glucose metabolism. Lactate also accumulates in the dy^2J GAST (Talmadge et al. 1989), suggesting that oxidation is the limiting factor in glucose catabolism. If glucose transport were rate limiting, then increased glucose transport would merely be a reflection of the rate of utilization. A muscle engaged in active contractions and therefore glucose oxidation would not be expected to have enhanced glycogenesis or accumulate lactate. The glucose moved across the sarcolemma would be phosphorylated, shuttled to the glycolytic pathway, and oxidized in the mitochondria. Glucose-6-phosphate would not be available for glycogen synthesis and lactate would not accumulate. Clearly this is not the case. If glycolytic flux were limiting, then lactate would not
accumulate for all of the pyruvate formed would be oxidized in the mitochondria.

An alternative explanation is that glucose metabolism is regulated at more than one point including both transport and utilization (Wallberg-Henriksson, 1987). It has been shown that there is an increase in the sarcolemmal associated glucose transporter, GLUT-4, with exercise (Douen et al. 1990). Thus, there is a definitive point of regulation for glucose metabolism at the transport step. Enzymes involved in the catabolism of glucose are also regulated by contractile activity and insulin (Leighton et al. 1989). If during the initial phase of contractions both glucose transport and enzymatic metabolism are up regulated in synchrony, then no glucose-6-phosphate would be available for glycogen synthesis. However, at longer stimulation times, the metabolic pathways of the muscle may switch to utilizing alternative fuels, such as lipid and lactate, for oxidation to supply contractile energy requirements. If at this point glucose transport remains stimulated, then glucose in the form of glucose-6-phosphate is now available for glycogen synthesis. The oxidation of alternative fuels by some fibers despite an increased glucose uptake would then lead to an increase in lactate production in fully oxygenated muscle, which may explain the increased lactate found in the dy^2^/GAST (Talmadge et al. 1989).

In summary, chronically active muscles have higher rates of glucose uptake and glycogenesis. Insulin stimulation of glucose uptake and glycogen synthesis is enhanced in these muscles and shows a different response dependent on the muscle being analyzed.
Autoradiography suggests that not all high glycogen fibers in the dy²J muscle use glucose for glycogen synthesis to the same extent and may be dependent upon motor unit activity. Finally, at least some chronically active muscles appear to have a dissociation of the regulation of glucose transport and glycogenesis by cellular glycogen levels, resulting in increased storage of glycogen by the muscle.
CHAPTER VI: GLYCOGENESIS BY SKELETAL MUSCLE:

POSSIBLE INVOLVEMENT OF PEPCK
INTRODUCTION

The synthesis of glycogen from lactate (glyconeogenesis) by skeletal muscle has long been debated, but it has become accepted as an important pathway for the resynthesis of glycogen in reptilian and fish muscle after exercise (Gleeson and Dalessio 1990, Batty and Wardle 1979). However, glyconeogenesis is not as well accepted in mammalian skeletal muscle (Gaesser and Brooks 1984). Recently, glyconeogenesis was shown to be functional in rabbit (Pagliasotti and Donovan 1990), rat (Shiota et al. 1984, Stevenson et al. 1987), and mouse (Bonen et al. 1990, Talmadge et al. 1989). The primary fiber type making use of glyconeogenesis is the fast-twitch glycolytic (FG) type (Shiota et al. 1984, Pagliasotti and Donovan 1990, and Bonen et al. 1990). This conclusion is based on assessment of whole muscle data with the interpretation related to varying fiber type percentages in the muscles being studied. One study, using autoradiographic techniques (Talmadge et al. 1989), specifically identified muscle fibers undergoing glyconeogenesis. In a chronically active muscle, FG fibers have an increased capacity to synthesize glycogen from lactate (Talmadge et al. 1989).

The biochemical pathway for glycogen synthesis from lactate by skeletal muscle has not been elucidated. It is known that the pathway is not merely a reversal of the glycolytic pathway, because two enzymatic steps of this pathway are essentially irreversible (Stryer 1989). The enzymes are 1) phosphofructokinase (PFK), and 2) pyruvate kinase (PK). The bypass reaction for circumventing PFK is thought to
occur via fructose-1,6-bisphosphatase, an enzyme that is found in abundance in FG muscle fibers (Opie and Newsholme 1967). The pathway for the formation of phosphoenolpyruvate from pyruvate (i.e. the reversal of PK) is not as well established. It was suggested that this pathway may occur via 3 enzymes (malic enzyme, ME; malate dehydrogenase, MDH; and phosphoenolpyruvate carboxykinase, PEPCK) and 2 intermediates (malate, MAL; and oxaloacetate, OAA) (Bendall and Taylor 1970, Connett 1979). The three enzymes are found in the cytosol of FG muscle (Nolte et al. 1972, Pette 1968), implying that mitochondria may not be involved. An alternative view is that this bypass series is not needed and that PK can be reversed under certain conditions (Dyson et al. 1975). Studies were performed aimed at establishing the involvement of PEPCK by using the PEPCK inhibitor, 3-mercaptopicolinic acid (3-MPA). This inhibitor was shown to inhibit glyconeogenesis in liver at relatively low concentrations (DiTullio et al. 1974) and to work at the PEPCK step (Kostos et al. 1975). It was shown to inhibit gluconeogenesis in liver in vivo (Turcotte et al. 1990) and in perfusion experiments (DiTullio et al. 1974). Studies on muscle have yielded conflicting results; one study demonstrated inhibition of skeletal muscle glyconeogenesis by 3-MPA (Connett 1979). Perfusion studies with 3-MPA showed no inhibitory effect on glyconeogenesis in muscle (Pagliasotti and Donovan 1990, and Shiota et al. 1984).

In a chronically active muscle, that contains some fibers showing enhanced glyconeogenesis, there is an increase in PEPCK activity (Talmadge and Silverman 1991). The increase in PEPCK activity by chronically active muscle is circumstantial evidence that PEPCK is
involved in glyconeogenesis, at least in the chronically active muscle.

The model for chronic activity is the C57Bl6J dy^{2J}/dy^{2J} (dy^{2J}) mouse. The hindlimb muscles of the dy^{2J} mouse suffer from pseudomyotonia, a chronic and repetitive neural stimulation (Rasminsky 1978), associated with abnormalities in myelination at the spinal roots (Jaros and Jenkison 1983). Certain muscle fibers in the superficial region of the gastrocnemius muscle (GAST) of the dy^{2J} mouse store high amounts of glycogen and remain glycolytic despite the chronic activity of the whole muscle. It is suggested that the high glycogen fibers are less active than surrounding fibers that show an increase in oxidative capacity (Hargroder et al. 1987, Talmadge and Silverman 1991). The high glycogen fibers were demonstrated to have an increased capacity for glyconeogenesis from lactate (Talmadge et al. 1989).

In this study, we analyze the capacity for normal and dy^{2J} muscles to synthesize glycogen from lactate in vitro. In order to perform in vitro experiments, relatively thin muscles were used to limit adverse effects of long diffusion distances. Therefore, the soleus (SOL) and extensor digitorum longus (EDL) muscles were used in place of the GAST. The conditions of the in vitro preparations were manipulated in an effort to 1) determine the optimal conditions for glyconeogenesis by skeletal muscle; 2) to establish the effects of previous chronic (dy^{2J} muscles) and short-term (swim exercised) activity; and 3) to determine the involvement of PEPCK by utilizing two PEPCK inhibitors, 3-MPA and quinolinic acid (Robinson and Oie 1975).
MATERIALS AND METHODS

In vitro incubation experiments were performed, at 37°C, according to Bonen et al. (1990) using a Dubnoff metabolic shaker with the following modifications. The extensor digitorum longus (EDL) and soleus (SOL) muscles were incubated in a non-bicarbonate buffered Krebs-phosphate buffer (KPB) (Krebs 1950). Krebs-Henseleit bicarbonate buffer (KHB) and KPB have been used for the manometric determination of tissue respiration with similar results (Krebs 1950). KPB offers the advantage that 5% CO₂ is not needed in the gas phase to maintain proper pH.

Briefly, muscles were pre-incubated for two 15 min periods each without radioactive label in 4 ml of KPB supplemented with lactic acid as the sole substrate at various concentrations, and 1% BSA. A 15 min incubation followed the pre-incubation. The incubation buffer also contained radiolabeled ¹⁴C-lactic acid (0.615 μCi/ml). The gas phase was room air (ATM), or as specified. The pH of the pre-incubation and incubation buffers was set after warming to 37°C and equilibrating with the appropriate gas mixture. The pH was measured prior to and following each pre-incubation and incubation, and did not change during the course of the experiments. The addition of inhibitors of PEPCK, quinolinic acid and 3-MPA, were at various concentrations and the pH of the pre-incubation and incubation baths was adjusted accordingly.

Following incubation the EDL and SOL were weighed using a Cahn electrobalance, rinsed in isotonic saline, and solubilized in 0.5 ml of 30% KOH with saturated sodium sulfate in a boiling water bath for 15
min (Hutber and Bonen 1989, Talmadge and Silverman 1991). Glycogen was precipitated with 95% ethanol, washed with 66% ethanol according to Hutber and Bonen (1989), and the glycogen pellet was resuspended in 0.5 ml of dH₂O. A fraction (0.2 ml) of the glycogen was used for the determination of ¹⁴C-carbon according to standard liquid scintillation procedures. A second fraction was used for the determination of total glycogen according to the phenol-sulfuric acid method (Lo et al. 1970).

In some experiments, the effects of previous contractile activity were analyzed. Short term exercise consisted of a 15 min swim in 35°C water. The muscles were taken from the animals immediately following the exercise protocol. The effects of chronic muscular activity were also evaluated using the dy² mouse as a model for chronic muscle contractile activity. A colony of these mice has been maintained at the L.S.U. Basic Sciences vivarium for approximately 11 years. This mouse suffers from a chronic and intermittent neural stimulation of the hindlimb muscles (Rasminsky 1978). Thus, both short bouts of exercise and chronic activity are evaluated for their effects on glycogen synthesis from lactate.

Statistical analysis. All values are presented as means ± standard error of the mean (SEM). For multiple group comparisons, analysis of variance (ANOVA) was performed. Bonferroni’s post-ANOVA tests for selected group comparisons were performed to determine significant differences. For comparisons between two treatments, Student’s t-tests were performed. In all cases, the level for significant differences was set at the p < 0.05 level.
RESULTS

Glycogen synthesis from lactate was demonstrated to occur under in vitro conditions for both the EDL and the SOL muscles from normal control mice. The EDL demonstrated approximately a two-fold greater incorporation rate than SOL (Fig. 1). The rate of glycogen synthesis from lactate was pH dependent for both the SOL and the EDL with a pH of 6.6 being optimal for both (Fig. 1). Therefore, all successive experiments were performed at pH 6.6. There was also a linear concentration dependence (Fig. 2) ($r^2 = 0.982$, $p < 0.05$ for EDL and $r^2 = 0.972$, $p < 0.05$ for SOL). Due to the concentration dependence all remaining experiments were performed at 10 mM lactate concentration.

The glyconeogenic rates were similar under atmospheric gassing of the bathing fluid and gassing with 100% $O_2$ (Fig. 3); however, when 95% $O_2$: 5% $CO_2$ or 100% $N_2$ were used to gas the bathing medium, the rate was significantly reduced for both the EDL and SOL (Fig. 3). A gas phase of 100% nitrogen resulted in a complete inhibition of glycogen synthesis from lactate. In all successive experiments atmospheric gassing of the medium was used unless otherwise specified.

The effects of previous exercise are displayed in Fig. 4. A short term bout of exercise, 15 min of swim exercise, did not significantly affect the rates of glyconeogenesis from lactate for both EDL and SOL. Chronic muscle activity, as demonstrated by the dy2J hindlimb muscles, resulted in a significant decrease in glyconeogenesis in the EDL and no difference in the SOL (Fig. 4).
CHAPTER VI.

Figure 1. Effect of pH on in vitro glyconeogenic rates in mouse extensor digitorum longus (EDL) and soleus (SOL) muscle. Each data point represents the mean and standard error of the mean (SEM). N = 8 for all points except at pH 6.6 for which n = 10 for both EDL and SOL. Krebs-phosphate buffer (KPB) was the incubation medium and was gassed with room air (ATM). Lactate concentration was 10 mM in both pre-incubation and incubation and was the sole substrate. The * denotes significantly different from all other points for EDL. The ** denotes significantly different from all points except pH 7.0 for SOL. Significant differences are based on Bonferroni's post-ANOVA significance tests with significance set at p < 0.05.
CHAPTER VI.

Figure 2. Effect of lactate concentration on glyconeogenic rates for mouse EDL and SOL. $N = 8$ for each point except 10 mM lactate for which $n = 10$ for both EDL and SOL. The pH was maintained at 6.6; all other conditions were the same as in Fig. 1. For EDL $r^2 = 0.982$, $p < 0.05$, slope = 0.241, and $y$-intercept = 0.029. For SOL $r^2 = 0.972$, $p < 0.05$, slope = 0.118, and $y$-intercept = 0.013. See Fig. 1 for abbreviations.
CHAPTER VI.

Figure 3. Effect of various gases (ATM; 100% O₂, 100%; 95% O₂:5% CO₂, 95:5; and 100% N₂, Nitr.) on glyconeogenic rates for mouse EDL and SOL. Bars represent mean and SEM. Each bar represents an n = 8, except for ATM = 10 for EDL and SOL. The * denotes significantly different from ATM for that muscle type, based on Bonferroni's post-ANOVA significance test, p < 0.05. Lactate concentration was 10 mM and pH 6.6 in KPB for all gas mixtures.
CHAPTER VI.

Figure 4. Effect of various contractile activities on glyconeogenic rates in mouse EDL and SOL. Exercised animals were swim exercised for 15 min and represent short term activity, dy2J are dy^{2J}/dy^{2J} animals and represent chronic activity. Each bar represents the mean and SEM with an n = 8, except for control for which n = 10. The * denotes significantly different from control, based on Bonferroni's post-ANOVA significance test at p < 0.05. Lactate concentration was 10 mM, pH 6.6 in KPB with ATM.
The effect of two PEPCK inhibitors was also evaluated. 3-Mercaptopyruvate inhibited glyconeogenesis in a dose dependent manner (Fig. 5). For the EDL, 3-MPA inhibits glyconeogenesis at relatively low concentrations (0.5 mM). The SOL is not significantly inhibited until 3-MPA is added at 1 mM concentration. A maximal inhibition of glyconeogenesis is achieved for both EDL and SOL at approximately 3 mM 3-MPA (Fig. 5). An increase in 3-MPA up to 30 mM results in no significant difference in comparison to 3 mM (Fig. 6). Quinolinate, another inhibitor of PEPCK, was less effective at inhibiting glyconeogenesis from lactate in EDL and ineffective in SOL (Fig. 6).

At rest EDL and SOL had glycogen contents of approximately 1.25 mg/g wet wt (Fig. 7A). After the bout of swim exercise the EDL showed a marked reduction in glycogen content (Fig. 7A). The SOL, did not show a depletion in glycogen. After exercise and incubation in the 10 mM lactate, pH 6.6 KPB medium for a total of 45 min (two 15 min pre-incubations and one 15 min incubation), the glycogen content of the EDL showed no change, while the glycogen content of the SOL was decreased to approximately half of the non-incubated muscle, suggesting that glycogenolysis occurred (Fig. 7A). Addition of 3-MPA into the medium resulted in a decrease in EDL glycogen and no change in SOL glycogen content (Fig. 7A).

The dy^2J muscles, EDL and SOL, had higher glycogen contents than normal muscle prior to incubation (Fig. 7B). Upon incubation the glycogen content of dy^2J SOL and dy^2J EDL were decreased to about half that of the non-incubated muscles (Fig. 7B). Addition of 3-MPA had no
CHAPTER VI.

Figure 5. Dose dependent response of glyconeogenic rates to 3-mercaptopicolinic acid (3-MPA) in mouse EDL and SOL. Each point represents the mean and SEM of 8 values, except for 0 mM 3-MPA for which n = 10. The * denotes significantly different from no 3-MPA (i.e. no 3-MPA in incubation) for that muscle, according to Bonferroni's post-ANOVA significance test at p < 0.05. 3-MPA was present in pre-incubations and incubations at the specified concentration. The conditions were 10 mM lactate, pH 6.6 in KPB with ATM.
CHAPTER VI.

Figure 6. Maximal response of glyconeogenic rates to 3-MPA and quinolinic acid (Quin) in mouse EDL and SOL. Each point represents the mean and SEM of 8 values except for control for which n = 10. The * indicates significantly different from control for that muscle, according to Bonferroni's post-ANOVA significance test at p < 0.05. 3-MPA or Quin was present in the pre-incubations and incubations at the specified concentration. The conditions were 10 mM lactate, pH 6.6 in KPB with ATM.
CHAPTER VI.

Figure 7. Glycogen content of muscles prior to and following incubation. A) Glycogen content following exercise and incubation. Displayed are glycogen contents at rest with no incubation (Rest), immediately after exercise with no incubation (Ex), after exercise and 45 min incubation (two 15 min pre-incubations and one 15 min incubation) (Inc), and after exercise and 45 min incubation with 3 mM 3-MPA (MPA). The * denotes significantly different from rest, the ** denotes significantly different from exercise alone, and the *** denotes significantly different from exercise and incubation. B) Glycogen content of dy²J muscle compared to normal and after incubation. Displayed are resting values for normal muscle without incubation (Norm), resting values for dy²J muscle without incubation (dy), values for dy muscles following 45 min incubation (Inc), and values for dy muscle following 45 min incubation with 3 mM 3-MPA. The * denotes significantly different from normal, and the ** denotes significantly different from dy²J without incubation. C) Glycogen content following treatment of the incubation with various gases. Displayed are values following incubation with ATM, 100% oxygen (100), 95% O₂: 5% CO₂ (95:5), and 100% N₂ (Nitr). The * denotes significantly different from ATM. All significant differences are according to Bonferroni's post-ANOVA significance test for that muscle type with significance set at p < 0.05. N = 8 for all groups.
effect on glycogen content in the dy²J muscles (Fig. 7B).

The gas used with the incubation medium effected the glycogen content of the muscles of mice following swim exercise. Gassing with 100% O₂ resulted in a higher glycogen content in both EDL and SOL muscles (Fig. 7C). The glycogen content approximated the control non-incubated values suggesting a net synthesis of glycogen under conditions of 100% O₂ gassing. Gassing with 95% O₂:5% CO₂ resulted in a decreased glycogen content of the EDL muscle and no difference for the SOL compared to gassing with atmospheric conditions. Gassing with 100% nitrogen resulted in decreased glycogen for both EDL and SOL.

The effect of 3-MPA on glycogen content is more pronounced in the EDL muscle, as demonstrated by 1.0 mM 3-MPA resulting in a significantly lower glycogen content than no 3-MPA. For the SOL, glycogen content is not affected until 3-MPA is at 30 mM in the incubation medium.
CHAPTER VI.

TABLE 1. Effect of 3-MPA on glycogen contents of EDL and SOL after exercise and incubation with lactate as the sole substrate.

<table>
<thead>
<tr>
<th>3-MPA concentration (mM)</th>
<th>Glycogen Content (mg/g wet wt)</th>
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<th></th>
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<tbody>
<tr>
<td></td>
<td>EDL</td>
<td>SOL</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.640 ± 0.044</td>
<td>0.847 ± 0.079</td>
<td></td>
</tr>
<tr>
<td>0.3</td>
<td>0.792 ± 0.097</td>
<td>0.869 ± 0.030</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0.476 ± 0.016</td>
<td>0.691 ± 0.088</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>0.343 ± 0.041**</td>
<td>0.613 ± 0.034</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>0.338 ± 0.021**</td>
<td>0.828 ± 0.085</td>
<td></td>
</tr>
<tr>
<td>3.0</td>
<td>0.301 ± 0.059**</td>
<td>0.569 ± 0.033</td>
<td></td>
</tr>
<tr>
<td>30.0</td>
<td>0.183 ± 0.031***</td>
<td>0.492 ± 0.105*</td>
<td></td>
</tr>
</tbody>
</table>

The values are means ± SEM. The * represents a glycogen content that is significantly different (p < 0.05) from the glycogen content when no 3-MPA is present in the bath, ** p < 0.01, *** p < 0.001. Statistically significant differences based on Anova and Bonferroni's post anova tests. N = 8 for each group. All animals were swim exercised for 15 min. Incubation was for a total of 45 min, two 15 min pre-incubations followed by one 15 min incubation in KPB with 10 mM lactate, pH 6.6, and ATM.
DISCUSSION

Mammalian skeletal muscle has the capacity to synthesize glycogen directly from lactic acid (Bonen et al. 1990, Pagliasotti and Donovan 1990, Shiota et al. 1984, Stevenson et al. 1987, and Talmadge et al. 1989). This in vitro study confirms that glyconeogenic rates are higher in predominantly fast-twitch glycolytic EDL muscle compared to the slow-twitch oxidative SOL muscle. The pH optimum for glyconeogenesis being is in the 6.5 to 6.6 range. Bonen et al. (1990) observed a pH optimum spanning from pH 6.5 to pH 7.0.

We determined the maximal glyconeogenic rate for the EDL muscle to be approximately 0.5 nMol/mg wet wt/15 min at pH 6.6 and 10 mM lactate. This is equivalent to approximately 2.5 nMol/mg protein/15 min, based on a protein content for rat fast-twitch muscle of 0.2 mg/mg wet wt (Kelso et al. 1987). Bonen et al. (1990) observed a maximal rate of 4.5 nMol/mg protein/15 min at pH 6.5 and 10 mM lactate for mouse EDL. For the SOL, we observed a maximal rate of approximately 0.25 nMol/mg wet wt/15 min under the same conditions. This is equivalent to 1.5 nMol/mg protein/15 min, based on a protein content for rat soleus of approximately 0.17 mg/mg wet wt (Kelso et al. 1987). Bonen et al. (1990) observed a maximal rate of 1.0 nMol/mg protein/15 min for mouse SOL. Thus our observed maximal rates are in general agreement with Bonen et al. (1990). The only differences in experimental protocol between the two studies are: 1) Bonen et al. (1990) used the KHB medium and 95% O2:5% CO2 for buffering and oxygenation, while we used the KPB buffer and ATM for buffering and
oxygenation; and 2) Bonen et al. (1990) used palmitate as a substrate for oxidative metabolism during the pre-incubations, while in our study only lactate was used as a substrate during both pre-incubations and incubations.

The glyconeogenic rates observed in this study using ATM for oxygenation of the medium are in agreement with other studies. Increased oxygenation of the medium by gassing with 100% O\textsubscript{2} resulted in no change in the maximal rates of glycogen synthesis from lactate for both the EDL and SOL. Increased oxygenation did, however, result in the net accumulation of glycogen from lactate as sole substrate by the EDL during the 45 min of pre-incubation and incubation. Thus, a cycle involving glyconeogenesis and glycogenolysis occurs within the muscle during incubation. The synthesis of glycogen from lactate appears to be less sensitive to O\textsubscript{2} than is glycogenolysis. At high O\textsubscript{2} tensions in the incubation medium, such as when 100% O\textsubscript{2} is provided, glycogenolysis is reduced to a point where a net synthesis of glycogen from lactate is observed in muscles previously depleted of their glycogen by swim exercise. However, at lower O\textsubscript{2} tensions (i.e. ATM) glyconeogenesis is matched by the increased glycogenolysis, resulting in no net change in glycogen content. This is one possible explanation for the observed increase in glycogen in the EDL muscle during incubations with 100% O\textsubscript{2}. The finding that glyconeogenesis and glycogen content are both reduced when anoxia is simulated by gassing with N\textsubscript{2} provides evidence that glyconeogenesis is at least indirectly dependent upon some oxidative metabolism occurring in the cell. Oxidative metabolism may supply NADH from the Krebs cycle and ATP from the electron transport chain to the
glyconeogenic enzymes.

The decreased glyconeogenic rate with 95% O₂:5% CO₂ was unexpected in light of the use of this gas by Bonen et al. (1990) for in vitro glyconeogenesis experiments. It is possible that this is due to a shift in pH due to the addition of more CO₂ resulting in a shift in the equilibrium of the carbonic anhydrase reaction and an increased acidity. However, there was no measurable change in the pH of the medium during any of the incubations and the pH of the medium was set while the bathing fluid was at 37°C and being gassed with the appropriate gas or mixture of gases. An alternative could involve PEPCK formation of CO₂ as a product in converting oxaloacetate to PEP in the PK bypass steps. An increase in the cytosolic CO₂ due to gassing the medium with 5% CO₂ could have an inhibitory effect on the formation of PEP due to mass action for reversible reactions resulting in a net decrease in glyconeogenesis.

The hypothesis that PEPCK is involved in skeletal muscle glyconeogenesis becomes more likely in light of the results obtained using 3-MPA and quinolinic acid to inhibit PEPCK. Low concentrations of 3-MPA inhibited glyconeogenesis in the EDL muscle. Slightly higher concentrations of 3-MPA were effective in the SOL muscle. For both muscles, there was a dose-dependent relationship with 3-MPA. Quinolinic acid was a less effective inhibitor of PEPCK. Similar results were observed in the kidney of guinea pigs and rats (Robinson and Oei 1975). It was previously shown that 3-MPA affects primarily the cytosolic form of PEPCK as opposed to the mitochondrial form (Robinson and Oei 1975). Thus, our results are in agreement with the
proposed cytosolic bypass pathway for circumventing PK. The finding that perfusion studies were unable to demonstrate that 3-MPA inhibits glyconeogenesis in skeletal muscle (Pagliasotti and Donovan 1990, and Shiota et al. 1984), but were effective for demonstrating inhibition of liver PEPCK (DiTullio et al. 1974), may be due to the anatomical differences in capillary morphology of muscle and liver. The capillaries in the liver are discontinuous sinusoids providing the blood and blood borne chemicals with a direct contact with the hepatocyte plasmalemma; however, in skeletal muscle the blood and blood borne elements must cross two membranes (the inner and outer endothelial cell membranes) prior to reaching the extracellular space and contacting the myofiber sarcolemma. The difference in diffusional barriers could directly effect the inhibitor concentration in the cytoplasm and effect cytosolic PEPCK activity. In vitro experiments overcome this problem due to the direct accessibility of the bathing medium to the muscle fiber membrane, because delivery of the inhibitor occurs via the extracellular space as opposed to the vascular system. Thus, it appears that cytosolic PEPCK is involved in the synthesis of glycogen from lactate by skeletal muscle.

There was no affect of prior muscle contractile activity on glycogen synthesis from lactate. Indeed, the EDL muscle appears to be involved in the swim exercise due to the depletion in glycogen observed after the relatively short duration swim. Despite the previous activity and the depletion in muscle glycogen, there was no effect on the maximal rate of glycogen synthesis from lactate. Chronic activity, such as observed in the dy²J muscles, resulted in a decrease in the
maximal rate despite the presence of some muscle fibers that showed an enhanced capacity for glyconeogenesis from lactate (Talmadge et al. 1989, Talmadge and Silverman 1991). The lower glyconeogenic rate observed in dy\textsuperscript{2J} muscles is most likely due to the fiber type transition that occurred in these muscles. The EDL of the dy\textsuperscript{2J} becomes more oxidative with time due to the chronic pseudomyotonic activity (Dribin and Simpson 1977). Thus, there are fewer FG, high glyconeogenic fibers present in the dy\textsuperscript{2J} EDL and the whole muscle becomes less able to engage in glyconeogenesis as chronic activity persists and oxidative capacity increases.

The slightly acidic pH optimum may be related to two separate phenomena. First, phosphofructokinase (PFK), the enzyme that forms fructose 1,6-bisphosphatase (FBP) from fructose 6-phosphate (F6P), is inhibited by reduced pH (Stevenson et al. 1987). A reduction in PFK activity would result in an increase in the net formation of F6P from FBP via fructose 1,6-bisphosphatase, resulting in a decreased glycolytic flux and enhanced glyconeogenic flux. Second, it was shown that rat renal PEPCK is activated during lactic acid induced acidification of the blood, resulting in tissue accumulation of malate, phosphoenolpyruvate and F6P (Vargas et al. 1981). Therefore, there may also be regulation at the level of three and four carbon sugars. Thus, at reduced cellular pH and increased tissue lactate, such as occurs during vigorous exercise (Sahlin et al. 1976), the conditions are optimal for glycogen synthesis from lactate.

In summary, we have determined that mammalian skeletal muscle has the capacity to synthesize glycogen from lactate and that fast-twitch
muscles have greater capacities for glyconeogenesis than do slow-twitch muscles. We also demonstrate that there is a linear dependence of glyconeogenic rate on lactate concentration and a pH optimum that is slightly acidic. Glyconeogenesis appears to occur via a pathway that involves PEPCK due to the inhibitory influences of 3-MPA and quinolinic acid. Previous muscle contractile activity and glycogen depletion do not enhance the glyconeogenic rate. Thus, glyconeogenesis appears to be regulated primarily by the availability of the substrate and pH.
The hindlimb muscles of the C57Bl/6J dy2J/dy2J (dy2J) mouse suffer from a chronic neural stimulation (pseudomyotonia), that results in increased contractile activity. The chronically active muscles adapt to the increased activity by altering metabolic characteristics, including an increased oxidative capacity and the appearance of a small population of fibers that store high amounts of glycogen. In this dissertation, the capacities for chronically active muscle to synthesize and store glycogen were examined. An emphasis was placed on establishing the presence and utilization of the glyconeogenic pathway (synthesis of glycogen from lactate) for glycogen synthesis.

Chapter II demonstrated that the gastrocnemius muscle (GAST) of the dy2J mouse, a chronically active muscle, stored approximately twice as much glycogen as control mice. The triceps muscle (TRI), a non-chronically active muscle in the dy2J, showed no differences in glycogen content between normal and dy2J mice. The dy2J GAST also contained twice as much lactic acid as normal GAST. An intraperitoneal injection of 14C-lactate resulted in an increased incorporation of 14C into the glycogen fraction of dy2J GAST as compared to control GAST. To determine if skeletal muscles from normal and dy2J mice were synthesizing glycogen from lactate directly without other body organ input, an in situ preparation was designed whereby the GAST, with intact neural and arterial supplies, but with impaired venous return, was placed in a saline bath containing 10 mM 14C-lactate. Both normal and dy2J GAST incorporated 14C into glycogen, indicating direct glycogen synthesis from lactate. Autoradiography revealed that the high glycogen containing muscle fibers in the dy2J GAST had the highest
capacity for glyconeogenesis. This is the first study to document the specific fiber types utilizing lactate as a substrate for glycogen synthesis.

The enzymes necessary for glycogen synthesis from lactate were analyzed in Chapter III for both normal and dy^{2J} GAST and TRI. Glycogen synthase (GS), malic enzyme (ME) and phosphoenolpyruvate carboxykinase (PEPCK) were all elevated in dy^{2J} GAST, but not in dy^{2J} TRI in comparison to control GAST and TRI. Histochemical analysis demonstrated that the high glycogen fibers in the dy^{2J} GAST had higher activities of GS and ME than any of the other fibers. Thus, high glycogen fibers in chronically active muscles have a higher enzymatic capacity for glyconeogenesis.

The variation of glycogen along the length of single muscle fibers in both normal and dy^{2J} GAST was analyzed in Chapter IV. The variation increased with increasing glycogen content. The variation was sufficiently low in all fibers to allow for a single histochemical section to be a good predictor of glycogen in specific fibers.

In Chapter V, glucose uptake and glycogen synthesis from glucose (glycogenesis) were evaluated in chronically active and normal muscles. This is the first study to analyze the effects of chronic contractile activity on muscle glucose metabolism. Both glucose uptake and glycogenesis were elevated in chronically active muscles, both in vivo and in vitro. The diaphragm muscle, a chronically active muscle under normal conditions, had the highest rates of glucose uptake and glycogenesis in vivo. Insulin stimulation of glucose uptake and glycogenesis was enhanced in chronically active muscles in vitro from
An *in vitro* analysis of glyconeogenesis demonstrated that two specific inhibitors of PEPCK, 3-MPA and quinolinic acid, inhibited glyconeogenesis in skeletal muscle, demonstrating quite clearly the involvement of PEPCK in glyconeogenesis. These data were presented in Chapter VI and are the first studies documenting that PEPCK is involved in glyconeogenesis in mammalian skeletal muscle. Prior contractile activity had no effect on maximal glyconeogenic rates. Maximal rates of glyconeogenesis were linearly dependent on substrate concentration and had a pH optimum of 6.6.

Thus, it was determined that glycogen synthesis from lactic acid occurs in skeletal muscle and that specialized high glycogen fibers in chronically active muscles have higher capacities for glyconeogenesis. The biochemical pathway involved was characterized in normal and chronically active muscles, and the results indicate that the enzymatic pathway involves PEPCK. Chronic contractile activity also results in an enhanced glycogenesis and glucose uptake. Thus, chronically active muscles show enhanced utilization of both pathways for glycogen synthesis resulting in an increased glycogen content of the whole muscle. The increase in glycogen content may provide the muscle with some additional resistance to fatigue.


APPENDIX I: GLYCOGENESIS FROM ALANINE BY DIAPHRAGM AND OTHER
CHRONICALLY ACTIVE MUSCLES
INTRODUCTION

Skeletal muscle has the capacity to synthesize glycogen from three carbon metabolites, such as lactate. Following strenuous exercise in humans, skeletal muscle glycogen is synthesized partially from lactate (Hermansen and Vaage 1977). Amphibian and lizard muscle can synthesize glycogen directly from lactate (Connett 1979, Gleeson 1985). Glyconeogenesis from lactate can occur at a maximal rate which is 32% of glycogenesis (glycogen synthesis from glucose) in the extensor digitorum longus muscle (EDL) of mice (Bonen et al. 1990). Thus, three carbon sources are important substrates for the direct synthesis of glycogen by skeletal muscle.

The gastrocnemius muscle of the C57Bl6J dy^{2J}/dy^{2J} (dy^{2J}) mouse is chronically active due to pseudomyotonia (Rasminsky 1978). The spinal roots leading to the hindlimbs of dy^{2J} mice exhibit an abnormal demyelination (Jaros and Jenkison 1983). The spinal roots are also associated with the spontaneous generation and ephaptic transmission of action potentials (Rasminsky 1978). The increased neural activity results in abnormal increases in increased contractile activity. The dy^{2J} gastrocnemius muscle also contains fibers which exhibit elevated glycogen and glyconeogenic capacity (Talmadge et al. 1989, Talmadge and Silverman 1991). Thus, chronic activity appears to be associated with an enhanced glyconeogenic capacity by some muscle fibers in the dy^{2J} gastrocnemius muscle.

Increased levels of alanine are found within actively contracting muscles (Nie et al. 1989). Actively contracting fibers may dispose of
excess pyruvate by converting it to alanine via the alanine aminotransferase (AlaAT, EC 2.6.1.2) reaction (Pardridge and Davidson 1979). Inactive fibers may use alanine as a glyconeogenic substrate. During extreme exercise, the venous effluent of the diaphragm shows no accumulation of glycolytic end-products such as alanine and lactate (Manohar and Hassan 1990); however, during in vitro stimulation, the diaphragm produces these substances (Pope et al. 1989). Since neither alanine nor lactate accumulate within the blood during in vivo contractions, it is hypothesized that alanine and lactate may be produced by some fibers within the diaphragm muscle and consequently used by neighboring fibers for glyconeogenesis.

In this study, we examined whether chronically active muscle could synthesize glycogen from alanine. The diaphragm muscle, a chronically active muscle under normal conditions, was used to study glyconeogenesis from alanine. This muscle was used because it is not associated with muscle fiber degeneration, as are portions of the gastrocnemius muscle of the dy²J mouse (Silverman and Atwood 1980). Therefore, the effects of chronic activity can be dissociated from effects of muscle fiber degeneration.
MATERIALS AND METHODS

Animal Care. Normal C57Bl/6J and C57Bl/6J dy2J/dy2J (dy2J) mice were housed in the Life Sciences Animal Care Facility at Louisiana State University. The mice used in this study were between three and six months of age and represent mature adult mice.

AlaAT activity. The method used for determination of AlaAT activity was that of Horder and Rej (1986). Briefly, gastrocnemius (hindlimb) and triceps (forelimb) muscles, along with diaphragm and liver were homogenized in 9 volumes of 10 mM Tris-HCl, 1 mM EDTA, pH 7.2 on ice using a ground glass tissue homogenizer. The homogenate was centrifuged at 4,000g for 10 minutes at 4°C. The resulting supernatant was assayed for enzymatic activity.

AlaAT activity was determined using standard NADH spectrophotometric procedures in an assay buffer consisting of 500 mM L-alanine, 100 mM Tris, pH 7.2, 0.18 mM NADH, 0.11 mM pyridoxal phosphate, and 1200 U/l LDH. Fifty microliters of the supernatant fraction of the tissue homogenate was added to 2 ml of assay buffer. Two-hundred microliters of 15 mM 2-oxoglutarate was added to start the reaction and the absorbance change was recorded using a Beckman model 35 recording spectrophotometer.

*In vivo* ^14^C-alanine administration. An injection of 0.048 μCi of ^14^C-alanine (3 mCi/mMol alanine) per gram body wt was administered by intraperitoneal injection. After a 3 hour incubation period the animal was sacrificed by cervical dislocation. Tissues (gastrocnemius, triceps, and diaphragm muscle) were removed and analyzed for glycogen
and $^{14}$C according to Hutber and Bonen (1990). The tissues were weighed and immersed in 0.5 ml of 30% KOH saturated with sodium sulfate and boiled for fifteen minutes. The tubes were cooled on ice, and 0.6 ml of 95% ethanol was added to precipitate the glycogen. After 20 minutes, the samples were centrifuged at 2200g at 4 °C. The supernatant was discarded, and the precipitated glycogen was washed in 1.0 ml of 66% ethanol and recentrifuged. The washing was repeated and the glycogen pellet was resuspended in 1 ml of distilled water. A 100 μl aliquot of the sample was counted in a Beckman LS8000 liquid scintillation counter. A 200 μl aliquot of the sample was used for glycogen determination by a phenol-sulfuric acid procedure (Lo et al. 1970). Briefly, the sample was diluted to 1 ml with distilled water and 1 ml of 5% phenol and 5 ml of 95% sulfuric acid were added in rapid succession. The tubes were gently shaken, and cooled in a water bath at room temperature. The absorbance was measured at 490 nm using a Beckman model 35 spectrophotometer to determine the total glycogen content of the muscle.

**In vitro** diaphragm preparations. The animals were anesthetized with pentobarbital (60 mg/Kg body wt.). After making a ventral incision through the skin, the pectoral and abdominal muscles were trimmed. The ligamentous connection to the liver and gall bladder was severed and the diaphragm and supporting ribs were removed by cutting anterior and posterior to the diaphragm.

The diaphragm preparation was incubated at 37°C in flasks containing 4 ml of oxygenated (95% O$_2$: 5% CO$_2$) Krebs-Henseleit bicarbonate (KHB) buffer, with 7 mM HEPES (pH 7.4), 1% BSA, and glucose
(5 mM), or alanine at various concentrations. Two 15 min preincubations without \(^{14}\)C-labeled substrate preceded a final 15 min incubation which contained labeled substrate, either \(^{14}\)C-glucose (2 \(\mu\)Ci per bath), or \(^{14}\)C-alanine (2 \(\mu\)Ci per bath). In experiments utilizing insulin, the final concentrations of insulin varied from 1 to 10,000 \(\mu\)Units per ml. Extensor digitorum longus (EDL) and soleus (SOL) muscles were also incubated as above for analysis of glyconeogenesis from alanine.

For analysis of a pH effect, the procedure was modified slightly by using Krebs-sodium phosphate (KPB) buffer (Krebs 1950) supplemented with 7 mM HEPES (pH 5.5, 6.0, 6.3, 6.6, 7.0, 7.5, and 8.0), 1% BSA, and 1 mM \(^{14}\)C-alanine (2 \(\mu\)Ci \(^{14}\)C-alanine per bath). The incubations were equilibrated with room air. This method yielded similar results to the KHB method at 1 mM alanine, and pH 7.5 (0.092 ± 0.010 nMol alanine incorporated into glycogen per gram wet wt per minute using KHB method vs. 0.100 ± 0.004 nMol/g wet wt/min using KPB method n = 5 for each, not significantly different at \(p < 0.5\) level).

Following incubation, the ribs, excess connective tissue and fat were carefully trimmed away, and the diaphragm, EDL or SOL muscle weighed, glycogen content measured, and \(^{14}\)C determined as stated above.

2-Deoxyglucose (2-DG) incubations. The incubations for 2-DG uptake were similar to \(^{14}\)C-alanine experiments with the following modifications. The KHB incubation buffer was used. The two 15 min preincubations contained 1 mM glucose. The final 15 min incubation contained 1 mM 2-DG with \(^{3}\)H-2-DG (300 \(\mu\)Ci/mMol), and \(^{14}\)C-inulin (20 nCi/ml) as an extracellular space marker. Insulin was added in some
experiments to 10,000 μUnits per ml of incubation buffer.

Utilization of alanine by the diaphragm. To determine the percentages of $^{14}C$ that were oxidized to $CO_2$ and converted to protein and glycogen from $^{14}C$-alanine, a similar KHB incubation procedure was performed with the following modification. During the final incubation the flask was capped with a $CO_2$ trap of 50% methanol and 50% phenylethylamine. After the incubation, the diaphragm was blotted dry and carefully split into equal sized hemi-diaphragms. Perchloric acid (4%) was added to the bath to liberate the remaining $CO_2$ in the incubation fluid, and the flask was recapped and shaken for one additional hour. An aliquot of the trapped $^{14}CO_2$ was counted using the previously stated liquid scintillation procedures.

One hemi-diaphragm was homogenized in 2.0 ml of 0.5 mM tetraethylamine (TEA) and 5 mM EDTA for protein determination. Protein was precipitated from an aliquot of the homogenate by addition of 200 μl of 10% TCA and centrifugation at 5000g in a Beckman microfuge for 10 min at room temperature. The precipitate was resuspended, washed twice with TEA, and counted using liquid scintillation procedures. Protein content was determined according to Bradford (1976). The second hemi-diaphragm was used to measure glycogen content as previously described.

Values are presented as means ± standard error of the mean (SEM). Student’s t-tests with significance set at $p < 0.05$ were used for comparisons between two group means. Analysis of variance (ANOVA) and Fisher’s (protected) least significant difference tests (FLSD) were used for multiple group comparisons and significance level set at $p < 0.05$. 


RESULTS

Alanine aminotransferase activity was greatest in the diaphragm muscle compared to the other muscles in the normal mouse (p < 0.05) (Fig. 1). However, AlaAT activity was not significantly different in dy\(^{2J}\) diaphragm compared to normal. The dy\(^{2J}\) gastrocnemius showed a significantly greater AlaAT activity than normal gastrocnemius (p < 0.05). There was no significant difference in AlaAT activity between normal and dy\(^{2J}\) triceps muscles. The triceps muscle serves as a useful comparison to the gastrocnemius since it is not pseudomyotonic in the dy\(^{2J}\) mouse, therefore it may be treated as an internal control.

The in vivo administration of \(^{14}\)C-alanine resulted in higher levels of \(^{14}\)C incorporation into glycogen in both normal and dy\(^{2J}\) diaphragm muscle in comparison to other muscle groups (Fig. 2). The dy\(^{2J}\) gastrocnemius muscle incorporated significantly greater amounts of \(^{14}\)C than did normal controls. The amounts of \(^{14}\)C incorporation into glycogen by dy\(^{2J}\) triceps and dy\(^{2J}\) diaphragm were not different from normal.

The diaphragm showed significantly higher level of 2-DG uptake (p < 0.05) in the presence of insulin (10,000 \(\mu\)Units/ml) compared to no insulin (Fig. 3). The diaphragm exhibited an increased incorporation rate of glucose into glycogen as insulin levels were increased between the levels of 10 and 1,000 \(\mu\)Units/ml, reaching a maximum at 1,000 \(\mu\)Units/ml (Fig. 4). No insulin response was detected for glycogen synthesis from alanine by the diaphragm muscle (Table 1). The glucose incorporation rate into glycogen was 250 times greater at 1 \(\mu\)Unit
APPENDIX I.

Figure 1. Alanine aminotransferase (AlaAT) activity in normal and dy²J muscle homogenates. The values are presented as means ± standard error of the mean (SEM) for 6 individual muscles. The * denotes significantly different (p < 0.05) from normal gastrocnemius muscle, according to Fisher's protected least significant difference test (FLSD) following analysis of variance (ANOVA).
APPENDIX I.

Figure 2. In vivo $^{14}$C-alanine incorporation into skeletal muscle glycogen by normal and dy²J mice. The values are presented as means ± SEM for 6 individual muscles. The * denotes significantly different (p < 0.05) from normal gastrocnemius muscle, according to FLSD following ANOVA.
APPENDIX I.

Figure 3. In vitro 2-Deoxyglucose (2-DG) uptake by the diaphragm muscle. A significantly higher level of uptake (p < 0.05), according to Student's t-test, was measured in the presence of insulin in the diaphragm muscle. The n = 6 for each point.
APPENDIX I.

Figure 4. In vitro diaphragm $^{14}$C-glucose incorporation rates into glycogen with varying insulin levels. Significant increases ($p < 0.05$) in the rates $^{14}$C-glucose incorporation into glycogen were observed with increasing insulin concentration between 10 and 1,000 μUnits/ml. The n = 6 for each value.
APPENDIX I.

TABLE 1. INSULIN EFFECTS ON GLYCOGENESIS AND GLYCOMEONEGENESIS FROM ALANINE

<table>
<thead>
<tr>
<th>INSULIN µUnits/ml</th>
<th>GLUCOSE 5mM</th>
<th>ALANINE 0.5mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>23.0 ± 5.2</td>
<td>0.092 ± 0.010</td>
</tr>
<tr>
<td>10000</td>
<td>115.3 ± 36.3</td>
<td>0.087 ± 0.026</td>
</tr>
</tbody>
</table>

Values represent nMol of substrate converted into glycogen per gram wet tissue wt. per minute. Each point represents the mean and standard error of the mean for 8 individual diaphragm muscles.
insulin per ml and over 1,000 times greater at 10,000 μUnits insulin per ml compared to alanine.

Alanine incorporation into glycogen is a linear function of alanine concentration ($r^2 = 0.986$, slope = 0.134, $p < 0.05$) (Fig. 5). A 10-fold increase in incorporation rate was observed between alanine concentrations of 0.25 mM and 5.0 mM. These concentrations span the physiological range of blood alanine levels (0.20 - 0.50 mM). The soleus muscle incorporated alanine into glycogen at rates similar to the diaphragm muscle (Fig. 5), and the rates were linear with increasing alanine concentration ($r^2 = 0.996$, slope = 0.103, $p < 0.05$). There was a peak incorporation rate of $^{14}$C-alanine into glycogen by in vitro diaphragm preparations at a pH of 6.3 (Fig. 6).

Normal and dy^{2J} EDL, fast-twitch muscles, showed significantly higher ($p < 0.05$) rates of glyconeogenesis from alanine than either normal SOL or DIA (Fig. 7), which contain higher percentages of slow-twitch fibers. The dy^{2J} SOL also showed a significantly higher ($p < 0.05$) rate of glycogen synthesis from alanine than normal SOL.

The diaphragm oxidized the major portion of the alanine which it metabolized (Table 2). A small percentage was used for protein synthesis and little was used for glyconeogenesis. Increasing bath alanine concentration led to increased alanine incorporation rates into protein and glycogen (Table 2, Fig. 5).
APPENDIX I.

Figure 5. The effects of varying [alanine] on $^{14}$C-alanine incorporation rates into glycogen using \textit{in vitro} diaphragm and soleus muscle preparations. Each point represents 6 individual muscles for both diaphragm and soleus muscles.
APPENDIX I.

Figure 6. The effects of incubation medium pH on $^{14}$C-alanine incorporation into glycogen by in vitro diaphragm preparations. The n = 6 for each value.
Figure 7. Glyconeogenesis by normal and chronically active muscles in vitro. The * denotes significantly different (p < 0.05) from normal SOL and normal DIA according to FLSD after ANOVA. N = 10 for each value.
APPENDIX I.

TABLE 2. UTILIZATION OF ALANINE BY THE DIAPHRAGM MUSCLE

<table>
<thead>
<tr>
<th>ALANINE CONCENTRATION (mM)</th>
<th>0.25</th>
<th>0.50</th>
<th>1.00</th>
<th>2.50</th>
</tr>
</thead>
<tbody>
<tr>
<td>%CO₂</td>
<td>98.57 ± 0.36</td>
<td>98.94 ± 0.36</td>
<td>97.16 ± 1.28</td>
<td>92.07 ± 1.99*</td>
</tr>
<tr>
<td>%Prot</td>
<td>1.35 ± 0.33</td>
<td>0.97 ± 0.34</td>
<td>2.73 ± 1.29</td>
<td>7.71 ± 1.91*</td>
</tr>
<tr>
<td>%Glyc</td>
<td>0.08 ± 0.04</td>
<td>0.09 ± 0.03</td>
<td>0.10 ± 0.03</td>
<td>0.22 ± 0.11</td>
</tr>
</tbody>
</table>

Values represent the relative distribution of $^{14}$C incorporated into three measured end products from $^{14}$C-alanine following *in vitro* incubations. The values represent the mean and standard error of the mean for 5 individual diaphragm muscles. The * denotes significantly different ($p < 0.05$) from 0.25 mM and 0.50 mM alanine, according to FLSD following ANOVA.
DISCUSSION

The high activity of AlaAT in the chronically active dy^{2J} gastrocnemius and the diaphragm muscles provides evidence that chronically active skeletal muscle metabolizes alanine at higher rates than less active muscle. We determined the AlaAT activity in the normal gastrocnemius muscle of the mouse to be 2.9 µMol/g/min at 25°C, which is similar to the previously reported value of 3.5 µMol/g/min at 38°C in normal rat skeletal muscle (Krebs 1972). The lowered activity we measured may be attributed to the temperature difference and the fiber type differences in the muscles.

It is of interest that the high activity of AlaAT determined in this study contrasts with studies of aspartate aminotransferase in dy^{2J} mouse muscle (Reichmann and Pette 1984). It was found that the activities of several enzymes of anaerobic metabolism, including aspartate aminotransferase, were reduced in dy^{2J} animals (Reichmann and Pette 1984). However, activities of some key glyconeogenic enzymes are elevated in the dy^{2J} gastrocnemius muscle compared to normal (Talmadge and Silverman 1991). Also, the dy^{2J} gastrocnemius shows an increase in oxidative enzymes with age (Hargroder et al. 1986). Therefore, high AlaAT activities in chronically active muscles are appropriate, since alanine may be used for both oxidation and glyconeogenesis.

During increased contractile activity, alanine levels increase in skeletal muscle because of the increased availability of pyruvate and amino groups within the muscle (Felig and Wahren 1971). Furthermore, an increase in muscle transaminase activity was observed within
exercising skeletal muscle (Felig and Wahren 1971), supporting our finding of increased AlaAT activity in the chronically active muscles. The increased production of alanine in exercised muscle was shown to exceed the rate of alanine uptake by the liver, resulting in alanine accumulation within the muscle (Felig and Wahren 1971). If alanine is present in excess in the exercised muscle, alanine could be used as a substrate for glyconeogenesis in skeletal muscle (Fig. 8). This hypothesis is similar to that proposed for lactate metabolism in the dy²3 gastrocnemius muscle, where some active fibers produce lactate and adjacent less active fibers utilize lactate as a glyconeogenic substrate (Talmadge et al. 1989, Talmadge and Silverman 1991).

Alanine is suggested to be a major gluconeogenic substrate during recovery from exercise, contributing to increases in glycogen resynthesis in both the liver and skeletal muscle after exercise (Favier et al. 1987). Alanine is a glyconeogenic substrate in muscle, but it is of greater importance to gluconeogenesis in the liver. The carbon skeleton of alanine is a significant endogenous precursor for hepatic glucose production (Felig and Wahren 1971). A glucose-alanine cycle is proposed to occur, in which alanine is taken up by liver, where its carbon skeleton is converted to glucose (Felig and Wahren 1971). Thus, we hypothesize that during in vivo administration of ¹⁴C-alanine, the ¹⁴C-alanine was subsequently used for gluconeogenesis by the liver, resulting in ¹⁴C-labeled glucose. The muscles were able to utilize the ¹⁴C-glucose for glycogen synthesis. This may explain the increased amounts of ¹⁴C incorporation into glycogen we observed within the diaphragm and the dy²³ gastrocnemius muscle in comparison to normal,
APPENDIX I.

Figure 8. A schematic representation of pathways involved in glycogen synthesis and insulin (+) stimulation.
non-chronically active muscles.

In order to identify a direct pathway for alanine use in glyconeogenesis by skeletal muscle, *in vitro* incubations were performed. The diaphragm exhibited a typical increase in glycogen synthesis from alanine in response to an increasing alanine concentration. The soleus muscle (SOL), another highly oxidative muscle with a high percentage of slow-twitch fibers, also synthesizes glycogen from alanine at rates similar to that of the diaphragm muscle. The normal EDL muscle, primarily fast-twitch with a low oxidative capacity (Bonen et al. 1990), showed a higher glyconeogenic rate than did normal SOL. In studies using lactate as a substrate for glyconeogenesis, it was demonstrated that the EDL had higher *in vitro* glyconeogenic capacities than SOL from normal mice (Bonen et al. 1990). The dy^2J^ SOL also showed an increased glyconeogenesis from alanine than normal SOL, but this may be due to the degeneration of fibers. Therefore, the muscles with the highest oxidative capacities appear to have the lowest glyconeogenic capacities.

A pH of 6.3 was observed as the optimum for glycogen synthesis from alanine. This pH is similar to the intracellular pH of 6.33 measured within single muscle fibers of the diaphragm post fatigue (Metzger & Fitts 1987). Cytosolic pH is important in muscle fatigue, and intense muscular activity causes intracellular pH to decline. The reduced pH inhibits glycolytic enzymes such as phosphofructokinase (PFK) (Walsh and Milligan 1988, Stevenson et al. 1987). The inhibition of PFK would reduce glycolytic flux within the muscle, which would favor the reverse reaction and result in an increased glycogen
synthesis from 3-carbon sources. Also, a reduction in plasma pH brought on by lactic acidosis was shown to activate phosphoenolpyruvate carboxykinase in the kidney resulting in an increased glyconeogenesis (Vargas et al. 1981).

Since insulin was previously shown to increase the rates of amino acid transport across the sarcolemma (Kipnis and Noall 1958) and glycogen synthesis (Leighton et al. 1989) (see Fig. 8), it was hypothesized that insulin would stimulate the utilization of alanine for glycogen synthesis. Insulin did stimulate glycogen synthesis from glucose by the diaphragm, but not glycogen synthesis from alanine. Thus, insulin may not play a role in regulating glyconeogenesis from alanine in skeletal muscle. In addition, insulin is known to stimulate glycolysis (Leighton et al. 1989), converting glucose to pyruvate, but a reversal of glycolysis is needed for glyconeogenesis from alanine. Since these two pathways share many of the same controlling enzymes, the reaction cannot be favored in both directions, providing a possible explanation for the lack of an increase in glycogen synthesis from alanine upon insulin addition.

We demonstrate that glucose is the primary substrate for muscle glycogen synthesis, with a small contribution from alanine. Furthermore, lactate appears to be a much better substrate for glyconeogenesis than alanine (Table 3). Also, the greater production of lactate as a metabolic end-product than alanine, results in higher tissue levels of lactate than alanine following muscle contraction. Therefore, more lactate is available for glyconeogenesis than alanine.

We demonstrate that the majority of the metabolized alanine was
APPENDIX I.

TABLE 3. Comparison of alanine and lactate as substrates for 
glycogenogenesis at physiological substrate concentrations.

<table>
<thead>
<tr>
<th>Muscle Type</th>
<th>Alanine (nMol/g/min)</th>
<th>Lactate (nMol/g/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDL</td>
<td>0.385 ± 0.030</td>
<td>34.7 ± 1.9*</td>
</tr>
<tr>
<td>SOL</td>
<td>0.079 ± 0.014</td>
<td>15.5 ± 2.1*</td>
</tr>
<tr>
<td>DIA</td>
<td>0.092 ± 0.010</td>
<td>-------</td>
</tr>
<tr>
<td>dy^3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDL</td>
<td>0.371 ± 0.038</td>
<td>25.5 ± 2.6*</td>
</tr>
<tr>
<td>SOL</td>
<td>0.365 ± 0.030</td>
<td>17.8 ± 2.4*</td>
</tr>
</tbody>
</table>

Glycogenogenesis from lactate rates are from Talmadge and Silverman (1991). For alanine the conditions were 0.5 mM alanine, pH 7.4. For lactate conditions are 10 mM lactate at pH 6.6. The * denotes significantly different from corresponding alanine value at p < 0.05 (Student's t-test). The n = 10 for all lactate and 6 for all alanine values.
converted to CO₂, indicating that the substrate was oxidized via the tricarboxylic acid (TCA) cycle (Fig. 8). A significant portion of the alanine was used for protein synthesis. As the concentration of alanine supplied to the diaphragm was increased, the rates of incorporation into protein and glycogen increased.

In conclusion, skeletal muscle has the capacity to utilize alanine as a substrate for glyconeogenesis. Chronically active, and highly oxidative muscles appear to utilize alanine primarily as an oxidative substrate. Less active and lower oxidative muscles (EDL) appear to have higher potentials for glyconeogenesis from alanine. Thus, glyconeogenesis from alanine may be important only in muscles of low oxidative potential.
REFERENCES


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ABSTRACTS


ABSTRACTS (Continued)


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Title of Dissertation: Metabolic Pathways associated with glycogen storage in chronically active and normal skeletal muscle

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