Proglycogen and Macroglycogen in Equine Skeletal Muscle

Response to Exercise in Standardbred Trotters and in Horses with Polysaccharide Storage Myopathy

Johan Bröjer
Faculty of Veterinary Medicine and Animal Sciences
Department of Clinical Sciences
Uppsala

Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 2006

Acta Universitatis Agriculturae Sueciae
2006: 41

ISSN 1652-6880
ISBN 91-576-7090-0

Tryck: SLU Service/Repro, Uppsala 2006

Abstract

Bröjer. J. 2006. *Proglycogen and macroglycogen in equine skeletal muscle. Response to exercise in Standardbred trotters and in horses with polysaccharide storage myopathy*. Doctoral dissertation.
ISSN 1652-6880, ISBN 91-576-7090-0

The first aim of the present thesis was to establish a method for determination of proglycogen (PG) and macroglycogen (MG) in equine skeletal muscle. The measurement technique for determination of PG and MG was accurate and gave reproducible results. There was an excellent correlation for total glycogen determined in muscle biopsy specimens between the new PG and MG technique and the traditional acid hydrolysis method.

A further aim was to examine the degradation of PG and MG in Standardbreds during a maximal exercise test. Horses performed an incremental maximal treadmill exercise test in one-minute steps until they no longer could keep pace with the treadmill. The exercise test induced an anaerobic metabolic response as demonstrated by decreased glycogen, ATP and creatine phosphate and increased lactate in muscle after exercise. The two fractions of glycogen contributed equally to glycogenolysis, 48% and 52% of total glycogen degradation for PG and MG respectively.

A study was also performed to determine the degradation and resynthesis of PG and MG in skeletal muscle after intermittent exercise on a slope. At a training camp well trained Standardbreds performed a training session comprising a warm up period, seven repeated 500 m bouts of uphill exercise and a recovery period. Muscle biopsies were taken at rest, at the end of exercise and 1, 4, 8, 24, 48 and 78 hours post exercise. The exercise caused degradation in MG that was twice as large as for PG. The rate of glycogen resynthesis during 1 to 24 hours post exercise was higher for MG than PG. The rate of muscle glycogen synthesis thereafter was slower and did not differ between MG and PG up to 72 hours.

The last study was conducted in order to determine PG, MG, glucose and glucose-6-phosphate (G-6-P) in skeletal muscle of horses with polysaccharide storage myopathy (PSSM) before and after light exercise. Horses with PSSM completed repeated intervals of 2 minutes of walking followed by 2 minutes of trotting until muscle cramping developed. Untrained control horses performed a similar exercise test for up to 20 minutes. Before exercise muscle glycogen concentrations were 1.5, 2.2 and 1.7 times higher for PG, MG and total glycogen respectively in PSSM horses compared to control horses. No changes in total glycogen, PG, MG, G-6-P and lactate were found after exercise. However, free glucose concentration increased in skeletal muscle in PSSM horses after exercise indicating that glucose uptake in skeletal muscle is augumented after light exercise.

Keywords: glycogen, proglycogen, macroglycogen, horse, muscle, metabolism, polysaccharide storage myopathy.

Author's address: Johan Bröjer, Department of Clinical Sciences, P.O. Box 7054, Faculty of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences, S-750 07 UPPSALA, Sweden. Johan.Brojer@kv.slu.se

The perfect computer has been developed.
You feed in your problems, and they never come out again.

Al Goodman

To my family Caroline, Rebecka and Kristoffer
– the most important part of my life

Contents:

Abbreviations

AC	Acid hydrolysis
ATP	Adenosin triphosphate
CK	Creatine kinase
CP	Creatine phosphate
CR	Coefficient of repeatability
dw	Dry weight
G-6-P	Glucose-6-phosphate
GP	Glycogen phosphorylase
GS	Glycogen synthase
G_t	Total glycogen
HPLC	High performance liquid chromatography
MG	Macroglycogen
phe	Phenylalanine
PG	Proglycogen
PCA	Perchloric acid
PSSM	Polysaccharide storage myopathy
TCA	Trichloroacetic acid
tyr	Tyrosine
UDP-glucose	Uridine-diphosphate-glucose

Appendix

Papers I-V

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Bröjer, J.T., Stämpfli, H.R. and Graham, T.E. 2002. Effect of extraction time and acid concentration on the separation of proglycogen and macroglycogen in horse muscle samples. *Canadian Journal of Veterinary Research* 66, 201-206.

II. Bröjer, J.T., Stämpfli, H.R. and Graham, T.E. 2002. Analysis of proglycogen and macroglycogen in muscle biopsy specimens obtained from horses. *American Journal of Veterinary Research* 63, 570 – 575.

III. Bröjer, J., Jonasson, R., Schuback, K. and Essén-Gustavsson, B. 2002. Pro- and macroglycogenolysis in skeletal muscle during maximal treadmill exercise. *Equine Veterinary Journal, Supplement* 34, 205-208.

IV. Bröjer, J., Holm, S., Jonasson, R., Hedenström, U. and Essén-Gustavsson, B. 2006. Synthesis of proglycogen and macroglycogen in skeletal muscle of Standardbred trotters after intermittent exercise. Submitted manuscript.

V. Bröjer, J.T., Essén-Gustavsson, B., Annandale, E.J. and Valberg, S.J. 2006. Proglycogen, macroglycogen, glucose and glucose-6-phospate concentrations in skeletal muscles of horses with polysaccharide storage myopathy performing light exercise. *American Journal of Veterinary Research*. Provisionally accepted.

Front cover photo:

Introduction

General background

The horse is an impressive athlete with a unique physical capacity. The equine athlete is able to run at speeds up to 10 to 12 m/s for long distances and reach peak speeds of 18 m/s due to its high heart weight to body weight ratio, large cardiac output and substantial capacity for oxygen carriage resulting from erythrocyte release during exercise (Rose & Hodgson 1994). In addition, the muscular system of the horse has evolved to produce powerful, efficient movements at high speeds and to have a large capacity to perform muscular work.

Carbohydrate, such as muscle glycogen and plasma glucose, as well as fat derived from adipose tissue and intramuscular sources are the predominant energy sources during exercise for contracting muscle. The relative contribution of carbohydrate and triglycerides as energy sources are dependent on factors such as exercise intensity, level of fitness and nutritional status (Jose-Cunilleras & Hinchcliff 2004). Horses have as much as 20 – 25 times more energy stored in the form of triglycerides than carbohydrate in their bodies (Harris 1997). Compared to lipid stores, the total energy stored in the form of glycogen is limited but glycogen is essential as it is a key determinant of exercise duration, performance and the onset of fatigue in both humans (Bergström *et al*. 1967) and horses (Lacombe *et al*. 1999; Lacombe *et al*. 2001). The metabolism of muscle glycogen has been intensely studied. The introduction of the percutaneous muscle biopsy technique by Bergström (1962) has expanded our understanding of both equine and human muscle tremendously over the last 40 years.

Decrease in muscle glycogen concentration during exercise is dependent on factors such as exercise intensity, type of exercise and duration. Substantial muscle glycogen depletion is observed in horses performing long distance, low- to moderate-intensity exercise (Snow *et al*. 1981; Essén-Gustavsson *et al*. 1984). The rate of glycogen utilization increases with work intensity (Nimmo & Snow 1983; Essén-Gustavsson *et al*. 1984; Harris *et al*. 1987; Snow *et al*. 1987; Snow & Harris 1991; Schuback & Essén-Gustavsson 1998). In addition, the rate of glycogenolysis is most pronounced during the initial stages or during the first bouts of high intensity exercise (Lindholm & Saltin 1974; Harris *et al*. 1987; Hodgson *et al*. 1987). During exercise glycogen depletion in different fibers occurs progressively following the recruitment of muscle fiber types. The initial depletion occurs in type I fibers followed by depletion of glycogen in type IIA and the type IIB fibers (Snow *et al*. 1981; Valberg 1986).

Depletion of glycogen stores in muscle has been associated with fatigue in horses performing an endurance ride (Snow *et al*. 1981). The impact of carbohydrate availability and oxidation on exercise performance in horses has been studied in two situations. First as an increase in time to fatigue in horses administered supplemental glucose by intravenous infusion during prolonged treadmill exercise (Farris *et al*. 1995) and second as lower exercise performance as

a consequence of depletion of muscle glycogen prior to exercise (Lacombe *et al*. 1999 and Lacombe *et al*. 2001). These findings suggest that glucose availability limits performance in horses during moderate prolonged exercise and that decreased muscle glycogen availability decreases anaerobic capacity in the horse.

In general, muscle glycogen resynthesis after exercise is dependent on factors such as carbohydrate substrate availability and time from completion of the exercise to access to carbohydrate substrate. Compared to humans, glycogen resynthesis in the horse is a slow process and supercompensated muscle glycogen stores do not occur in horses (Lacombe *et al*. 2003). Horses fed a high carbohydrate diet or supplemented with a glucose polymer did not have an enhanced muscle glycogen resynthesis over the first 24 hours post exercise (Snow *et al*. 1987; Davie *et al*. 1994). One potential explanation is that the horse has a limited ability to digest starch in the small intestine compared to other monogastric animals due to slower rate of amylase secretion (Comline *et al*. 1969). This is in agreement with the findings that oral glucose supplementation does not increase muscle glycogen resynthesis during the first 24 hours post exercise whereas intravenous administration does (Davie *et al*. 1994; Davie *et al*. 1995). Unlike other animals, horses fed increasing amounts of digestible carbohydrate have only a minimal increase in the rate of muscle glycogen synthesis (Lacombe *et al*. 2004).

The glycogen content in skeletal muscle of well-trained horses is generally on the order of 500 to 650 mmol glucosyl units per kg dw (Snow & Valberg 1994). Training of horses has been shown to increase the glycogen stores in their muscles (Lindholm & Piehl 1974; Hodgson *et al*. 1985). Several authors have reported an increase in muscle glycogen content in response to several weeks of exercise conditioning as a consequence of increased muscular oxidative capacity and progressive decreased rate of glycogenolysis during submaximal exercise (Hodgson *et al*. 1985; Gansen *et al*. 1999). One study showed that glycogen levels decreased after 1 – 2 weeks of controlled intensive daily treadmill training and remained low during the 5 week training period (Essén-Gustavsson *et al*. 1989). However, after one week of cessation of training glycogen levels had increased to pre-training levels.

There are a variety of diseases that are associated with defects in glycogen metabolism in both humans and horses. Glycogen accumulation and usage are closely linked to the metabolism of individual cells as well as to whole body glucose utilization. Aberrant glycogen storage has the potential to be both a result and a cause of disease (Roach 2002). A recently recognized disease in Quarter horses and related breeds is equine polysaccharide storage myopathy (PSSM). Equine polysaccharide storage myopathy is an inherited disorder characterized by the accumulation of glycogen and abnormal polysaccharide in skeletal muscle with normal glycogenolytic enzyme activites (Valberg *et al*. 1998; Valberg *et al*. 1999; Annandale *et al*. 2004).

Carbohydrate structure

Glycogen is a very large branched polymer of glucose residues that is built upon a protein core. The primary function of glycogen is to provide a readily mobilized storage form of glucose in most animal cell types. The liver and muscle serve as the two main sites of glycogen storage. The liver has the highest glycogen concentration and this organ has an important role in the maintenance and regulation of blood glucose. Despite the higher relative glycogen concentration in the liver, skeletal muscle contains a larger amount of glycogen due to its greater mass. The primary purpose of the glycogen stores in skeletal muscle is to provide readily accessible energy for muscle work (Lehninger 1982).

Glycogen consists of glucose residues linked together by two types of glycosidic bonds, the α-1,4 linkage which joins glucose units in straight linkages and accounts for 93% of all bonds and α-1,6 branched linkages that account for the remaining 7% of bonds. The α-1,4 glucosidic bonds are formed by glycogen synthase while the α-1,6 glycosidic bonds are formed by branching enzyme. The branched structure of glycogen has several advantages compared to a non-branched structure: 1) it creates a more compact structure, 2) it increases the rate of glycogen synthesis and degradation by increasing the number of terminal residues - the action sites of glycogen synthase and phosphorylase, 3) it increases the solubility of glycogen (Stryer 1988).

A theoretical model for glycogen structure has been proposed by Goldsmith and colleagues (Goldsmith *et al*. 1982). According to this model the glycogen molecule is built on a protein core and consists of approximately 4200 glucose chains with an average length of 13 glucose units each. The number of chains increases by a factor 2 for each new layer in the glycogen molecule. After 12 layers the surface density of the chains has increased to the level where further branching is limited and the growth of the molecule is self-limiting.

It has previously been demonstrated that the distribution of glycogen within the muscle fiber is not random but organized in distinct subcellular locations including subsarcolemmal, intra- and intermyofibrillar regions (Fridén *et al*. 1989). Recently, Marchand and coworkers (2002) quantified the intracellular location of glycogen in muscle fibers of humans. They demonstrated that the highest densisty of glycogen was in the subsarcolemmal location and that the diameter of the glycogen granules followed a normal distribution with an average range of 10 – 44 nm.

Glycogenin

The biosynthesis of glycogen involves both a specific initiation phase and a phase of elongation. The enzyme glycogen synthase (GS) cannot transfer glucosyl residues from uridine-diphosphate-glucose (UDP-glucose) without a disaccharide already present and an initiating stage is therefore required (Calder 1991). Krisman and Barengo (1975) suggested that there was a protein backbone to which glucosyl units are transferred at the initiation stage by the activity of a

”glycogen initiator synthase” and that the further elongation of the glycogen particle was catalyzed by glycogen synthase and branching enzyme. Later, Whelan and co-workers (Rodriquez & Whelan 1985) identified a protein that was covalently attached to glycogen via tyrosine (tyr) residues. This protein backbone of glycogen was named glycogenin (Pitcher *et al*. 1987). Further work performed by Whelan (Lomako *et al*. 1988) and Cohen (Pitcher *et al*. 1987) showed that glycogenin was not only the protein backbone on which glycogen is synthesized but that it also had self-glucosylating activity. Via self-glucosylation glycogenin forms an oligosaccharide primer of 7-11 glucosyl units, which then serves as an effective substrate for glycogen synthase (Alonso *et al*. 1995b).

The protein glycogenin in skeletal muscle has a molecular mass of 37 kDa and consists of 332 amino acids (Campbell & Cohen 1989). The discovery by the group of Cohan (Pitcher *et al*. 1988) and Whelan (Lomako *et al*. 1988) that glycogenin had enzymatic activity by catalyzing a self-glucosylation reaction with UDP-glucose as a donor, was a landmark in understanding glycogenin function. Glycogenin is now classified as a hexosyltransferase (EC 2.4.1.186). The self-glucosylation of glycogenin is stimulated by Mn^{2+} and requires UDP-glucose as a substrate (Alonso *et al*. 1995b). The glycogen is attached to the protein glycogenin by a single glucose-1-O-tyrosyl linkage. The tyrosine (Rodriquez & Whelan 1985) was identified by protein chemical methods as tyr-194 (Campbell & Cohen 1989). Alonso and coworkers found that when tyr was replaced with phenylalanine (phe) the glycogenin would no longer self-glucosylate, which showed the importance of this site for the activity and function as well as for the anchor of glucose (Alonso *et al*. 1995a). During the formation of an oligosaccharide primer the glucose residues are attached to each other by α-1.4-glycosidic bonds as in glycogen (Pitcher *et al*. 1988). Because of the different chemical nature of these linkages between protein and the first glucose residue and the rest of the glucose residues, there was initially a debate as to whether a separate glucosyltransferase was needed to mediate the initial step. It seemed unlikely at the time that one enzyme could be capable of catalyzing both carbohydrate-protein and carbohydrate-carbohydrate bonds (Roach & Skurat 1997). Later research showed, however, that glycogenin indeed was able to catalyze both linkages and the role of glycogenin in the biosynthesis of glycogen is now fairly well established (Roach & Skurat 1997.

Pro- and macroglycogen

In the traditional model of glycogen synthesis glycogenin, glycogen synthase, branching enzyme and the substrate UDP-glucose are sufficient to account for glycogen synthesis. Recently, Lomako *et al*. (1991) proposed the existence of an additional discrete intermediate form in the synthesis and degradation of glycogen, termed proglycogen (PG).

Using fresh muscle extracts incubated with UDP[^{14}C]glucose and subjected to SDS-PAGE they identified a well defined band with high molecular weight of approximately 400 kDa (p400) but no bands with smaller molecular weights. The p400 was glycogen-like and could be broken down to glycogenin by amylase

treatment at 37°C (Lomako *et al*. 1990). Under normal conditions glycogenin does not exist in a free, detectable state in skeletal muscle (Lomako *et al*. 1990; Smythe *et al*. 1990). Based on these and other findings Lomako, Whelan and Alonso (Lomako *et al*. 1991, Lomako *et al*. 1993, Alonso *et al*. 1995b) proposed the existence of two forms of glycogen; proglycogen and macroglycogen. According to this model the p400 (proglycogen molecule) was suggested to be a stable intermediate in the synthesis of mature glycogen (macroglycogen) and under normal conditions never broken down to glycogenin (Figure 1). Lomako, Whelan and coworkers showed that the formation of proglycogen and macroglycogen by the addition of glucosyl units using UDP-glucose as a substrate were both stimulated by glucose-6-phosphate (G-6-P), which is characteristic for glycogen synthase (Lomako *et al*. 1991). However, the Km (Michaelis constant) for UDP-glucose was three orders of magnitude lower for the synthesis of proglycogen compared to the synthesis of macroglycogen and the formation of macroglycogen but not the formation of proglycogen was inhibited by ammonium ions (Lomako *et al*. 1991, Lomako *et al*. 1993). Thus, the reactions responsible for adding glucosyl units in the formation of proglycogen and macroglycogen showed remarkable differences. They therefore suggested that the synthesis of glycogen from glycogenin to mature glycogen via the formation of proglycogen is catalyzed by three different enzyme activities (Alonso *et al*. 1995b). The first step in the glycogen synthesis is the self- glucosylation by glycogenin. The second step is the formation of proglycogen by proglycogen synthase and the final step is the formation of macroglycogen by macroglycogen synthase. However, this hypothesis has not been confirmed in subsequent studies (Shearer & Graham 2004).

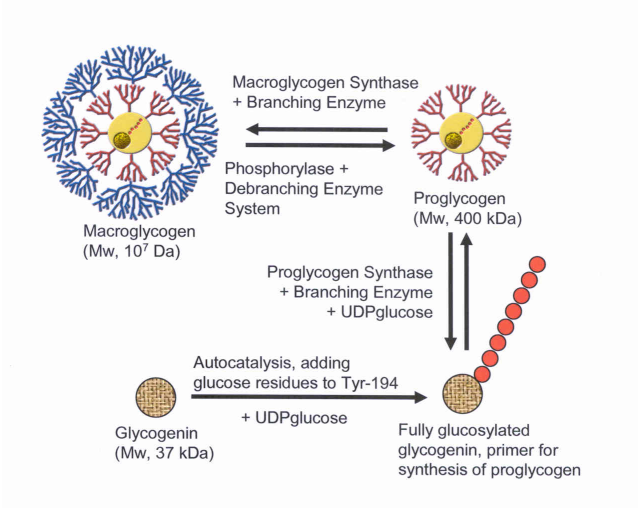

Figure 1. The proposed model of biogenesis of glycogen. Glycogenin autocatalytically adds glucose from UDP-glucose to its tyr-194 and then an average of 7 residues to form a fully glucosylated glycogenin that serves as a primer for the synthesis of proglycogen by proglycogen synthase and branching enzyme. Proglycogen was suggested to be a stable intermediate in the synthesis of mature glycogen (Macroglycogen). The priming chain of 8 glucose residues corresponds to the average length of elongation but in actuality ranges from 7 to 11 glucose residues (Lomako et al. 1993).

According to the model proposed by Lomako, Whelan and coworkers proglycogen is a smaller intermediate in the synthesis of mature glycogen (macroglycogen) with a molecular weight of approximately 400kDa. Macroglycogen is the larger glycogen molecule which can reach a molecular weight of 10,000 kDa. Both molecular forms contain identical amounts of protein but different amounts of associated carbohydrates. Therefore, the two forms differ in the relative proportion of protein to carbohydrate. Proglycogen contains approximately 10% protein and is precipitable in 10% trichloroacetic acid (TCA) whereas macroglycogen (MG) is soluble in TCA due to its very small relative amount of protein (approximately 0.4%) (Alonso *et al*. 1995b).

The hypothesis of the existence of two forms of glycogen is not by any means novel. Authors of earlier studies have also discussed the possibility of two forms of glycogen (Kits Van Heijningen & Kemp 1955). Willstaetter and Rhodewald (1934) demonstrated the existence of two forms of glycogen separable by TCA solubility; lyo- and desmoglycogen. Lyoglycogen was soluble in TCA and was believed to be protein-free whereas desmoglycogen was believed to contain protein and was therefore insoluble in TCA. Stetten *et al*. (1958) found that fractionalization of glycogen by extraction in 5% TCA or by alkaline digestion in KOH resulted in glycogen with different mean molecular weights. In 1981 Jansson reported that there were two portions of glycogen in human skeletal muscle; one was acid soluble in 1.5 M perchloric acid (PCA) and one was insoluble. Jansson also demonstrated that the relationship between acid soluble and insoluble glycogen in human skeletal muscle was not influenced by the type of acid (PCA vs. TCA) or by the strength of PCA in the range between 0.5 and 3 M (Jansson 1981). The theory of the existence of two glycogen pools was supported in 1989 by Fridén *et al*. who identified two different sizes of glycogen particles in human skeletal muscle by using electron microscopy (Friden *et al*. 1989).

In 1998 Adamo and Graham validated a separation technique for MG and PG for human and rat skeletal muscle where freeze dried muscle biopsies were extracted for 20 minutes in 1.5 M PCA. The separation technique was based on the findings from both the work performed by Jansson (1981) and Lomako, Whelan and coworkers (Lomako *et al*. 1993, Alonso *et al*. 1995b). Total glycogen analyzed by three different methods: enzymatic hydrolysis, acid hydrolysis with 1M HCl and by the separation technique for pro- and macroglycogen, yielded comparable results with regard to both total glycogen concentration and repeatability (Adamo & Graham 1998).

However, subsequent studies on PG and MG have failed to confirm that discrete PG molecules exist. Rather, glycogen molecules consist of a range of molecular sizes (Roach *et al*. 1998; Marchand *et al*. 2002). Whether or not PG can be defined as descrete molecules remains a topic of controversy. Regardless, acid separation of glycogen into two fractions is a useful way to divide smaller and larger glycogen granules. This is of interest as the two glycogen fractions have different rates of synthesis, degradation and sensitivity to carbohydrate supplementation (Adamo *et al*. 1998; Graham *et al*. 2001; Shearer *et al*. 2001; Battram *et al*. 2005).

Storage of glycogen in skeletal muscle – the relationship between pro- and macroglycogen

Human and rat muscle glycogen is composed of pro- and macroglycogen and the relationship between these two forms of glycogen is dependent on the total glycogen concentration. In muscle with very low total glycogen concentration almost all glycogen is present as proglycogen. When the total glycogen concentration increases the relative proportion of proglycogen decreases whereas the relative proportion of macroglycogen increases. Despite the decrease in the relative proportion of proglycogen the absolute proglycogen concentration in fact increases (Jansson 1981, Adamo *et al*. 1998, Hansen *et al*. 2000). Jansson (1981) demonstrated that for human skeletal muscle with normal glycogen concentrations (300-350 mmol glucosyl units/kg dry dw) the acid soluble fraction (macroglycogen pool) comprised approximately 25%. At total glycogen concentrations over 300-350 mmol glucosyl units/kg dw the increase in total glycogen was predominantly due to increase in the acid soluble pool. Similar results were obtained by Adamo and Graham (1998) in a study performed on human skeletal muscle. As glycogen concentration increases above 300 – 400 mmol glucosyl units/kg dw (supercompensation), additional glucose was added to MG but there was no corresponding decline in PG. Based on these results it is not clear whether the increase in MG is the result of the addition of glucose to all existing glycogen molecules, with some then becoming acid soluble (MG) or whether there is an increase in glycogen molecule numbers as well. Using a myotube cell culture and labeled glucose Elsner and coworkers (2002) showed that there was an increase in both granule size and numbers when the cell synthesized glycogen. In addition, glycogenin activity is proportional to muscle glycogen concentration (Shearer *et al*. 2000). Taken together, this information suggests that increase in glycogen stores is achieved not only by increasing glycogen molecules but also by increasing the number of glycogen granules.

Metabolism of pro- and macroglycogen

Adamo *et al*. (1998) studied the resynthesis of the two pools of glycogen after exhaustive exercise in humans with either a low or high carbohydrate diet. They showed that proglycogen and macroglycogen differed in both timing and magnitude during resynthesis. Proglycogen was re-synthesized to a greater extent compared to macroglycogen in the early phase of recovery. The synthesis of macroglycogen was slower and more constant over a recovery period of 48 hours. When the total glycogen concentration in the muscle reached a concentration over 300-350 mmol glucosyl units/kg dw (high carbohydrate group) the resynthesis of glycogen was mainly in the macroglycogen pool. Similar resynthesis pattern for PG and MG were obtained in a recent study by Battram *et al*. (2004) after glycogen depleting exercise in humans.

Asp *et al*. (1999) studied the resynthesis of proglycogen and macroglycogen in human athletes after a marathon race. The major finding from this study was that the proglycogen returned more rapidly back to the pre-race concentration than did

the macroglycogen fraction. These findings were in agreement with the aforementioned studies by Adamo *et al*. (1998) and Battram *et al*. (2004).

Glucose can be incorporated into glycogen molecules during glycogen resynthesis in different ways. It can be incorporated into newly formed glycogen granules, where glucose is added to glycogenin, or glucose can be added to existing PG or MG molecules. If resynthesis of glycogen in the aforementioned studies occurred predominantly in the MG fraction this would suggest that glucosyl units were added to existing PG molecules growing into MG or existing MG molecules growing larger. In the study by Adamo *et al*. (1998) and Battram *et al*. (2004) the initial and largest increase occurred in the PG fraction indicating that either new glycogen granules were formed or that existing PG grew larger. This finding is supported by work from Marchand (2001) who reported an increase in glycogen number rather than an increase in glycogen granule diameter during the first 4 hours of recovery from exhaustive exercise in humans. Interestingly, increase in glycogenin and glycogenin mRNA accompany glycogen resynthesis in human skeletal muscle during recovery of prolonged exhaustive exercise in humans (Shearer *et al*. 2005). This may facilitate rapid glycogen resynthesis by providing the protein primer (glycogenin) that catalyzes glycogen granule formation in skeletal muscle.

Graham *et al*. (2001) studied the effect of exercise intensity and duration as well as the effect of repeated bouts of exercise on the two fractions of glycogen in human skeletal muscle. This study demonstrated that proglycogen is more dynamic than macroglycogen and that the net rate of glycogenolysis in most exercise situations is greater for the proglycogen fraction. When the exercise intensity was increased this caused an increase in the net rate of glycogenolysis of both pro- and macroglycogen but this increase was more distinct in proglycogen. Repeated bouts of exercise caused a decrease in the net rate of glycogenolysis with time and this was predominately caused by a decreased net rate of catabolism in macroglycogen.

When Asp *et al*. (1999) studied the utilization of pro- and macroglycogen in skeletal muscle during a marathon race they concluded that a greater fraction of macroglycogen was utilized compared with proglycogen. These findings are in contrast to the work by Graham *et al*. (2001). However, the type and intensity of the exercise differs remarkably between the two studies. In another study performed by Shearer *et al*. (2001) it was demonstrated that proglycogen and macroglycogen were degraded at different rates depending on the initial glycogen concentration. Proglycogen was preferentially degraded over macroglycogen at higher initial glycogen concentrations whereas proglycogen and macroglycogen contributed equally to glycogenolysis at lower total glycogen concentrations. This led the authors to speculate over why proglycogen is preferentially degraded during catabolism. The outer branches of the smaller proglycogen molecule may be more readily accessible for glycogen phosphorylase (GP) compared to the more densely branched macroglycogen molecule. In addition, the number of proglycogen molecules by far outnumber the macroglycogen molecules. Another explanation could be the different subcellular location of pro- and macroglycogen

within the muscle, resulting in different susceptibility to degradation by GP. In two studies, Fridén *et al*. (1985, 1989) demonstrated that, for human skeletal muscle, the type and duration of exercise had implications on where glycogen degradation occurred within the muscle fiber. They found the subsarcolemmal glycogen fraction in the type II fibers to be depleted during short-term high intensity exercise whereas it remained largely unaffected in the type I fibers during long-term aerobic exercise. Aerobic exercise primarily resulted in degradation of glycogen from the intramyofibrillar regions within the muscle fibers. Friden *et al*. (1989).

In contrast, Derave *et al*. (2000) showed that macroglycogen was the major contributor of total glycogenolysis in rats when muscles were glycogen supercompensated. For rodents receiving a normal or low carbohydrate intake the proglycogen and macroglycogen fraction contributed almost equally to glycogenolysis. Furthermore, they showed that the initial concentration of macroglycogen but not the proglycogen concentration was significantly correlated to glycogenolysis.

The results of these experiments demonstrate that both proglycogen and macroglycogen are suitable substrates for GP during exercise in both human and rat muscle. The proglycogen pool, however, appears to be the most dynamic form in human skeletal muscle whereas in rodent skeletal muscle the macroglycogen pool is the major contributor of glycogenolysis at least at high glycogen levels. In human skeletal muscle PG appears to be the glycogen fraction readily metabolized upon demand while the more resistant MG fraction is degraded to a higher extent under extreme energy demands such as marathon races. A potential explanation may be the number of glycogen granules, glycogen granule surface area and/or location as well as the nature of associated enzymes and other proteins within each glycosome.

Aims of the Thesis

The principal aims of the present studies were to establish methods for studying PG and MG in equine skeletal muscle and to use these methods in more detailed studies of the metabolism of PG and MG in healthy horses during exercise and recovery. A further aim was to determine the metabolism of PG and MG in horses with equine polysaccharide storage myopathy performing light exercise that induced rhabdomyolysis.

The specific aims of the studies were:

- To establish and evaluate a method for determination of PG and MG in equine skeletal muscle (Studies I and II).
- To compare analysis of total glycogen in muscle biopsy specimens from horses using two different methods; acid hydrolysis (AC) and MG + PG determination (Study II).
- To compare the effect of different sampling depths in the *gluteal medius* muscle on the concentrations of PG and MG (Study II).
- To examine the degradation of PG and MG during intense exercise in Standardbred trotters performing a standardised incremental exercise test (Study III).
- To determine the resynthesis of PG and MG in skeletal muscle in racing Standardbred trotters after they had performed repeated bouts of exercise on a slope (Study IV).
- To determine PG, MG, free glucose and glucose-6-phosphate concentrations in muscle of PSSM horses at rest and following light submaximal exercise that induced rhabdomyolysis (Study V).

Materials and Methods

A brief summary of the materials and methods used and some additional information are given below. Fore more detailed accounts, see papers I – V.

Horses

Healthy horses were used in all the studies except in study V where the horses were affected with equine polysaccharide storage myopathy (Table 1). The horses in study I – II were untrained and owned by the department of Clinical Studies, Ontario Veterinary College, Guelph, Canada with the exception of seven privatly owned endurance ride horses. In study III trained horses owned by the Department of Large Animal Clinical Sciences, SLU, Uppsala, Sweden were used. The horses in study IV belonged to the National Equine Centre, Wången, Sweden and they were all in racing condition. Untrained Quarter horses or Quarter Horse crossbreeds owned by the University of Minnesota, St Paul, USA were used in study V.

All studies were approved by the Ethical Committees for Animal Experiments in the respective countries (Canada in study I – II, Sweden in study III – IV and USA in study V).

Table 1. Summarised data for the horses investigated in the respective studies.

Study	No	Breed	Age Years	Sex	Fitness
I	10	Standardbred horses	2 – 10	8 mares, 2 geldings	Untrained
	6	Standardbred horses	2 – 11	5 mares, 1 gelding	Untrained
II	25	Standardbred horses	2 – 17	21 mares, 4 geldings	Untrained
	7	Crossbred Arabian horses	8 – 17	1 mare, 6 geldings	Trained for endurance racing
	9	Standardbred horses	6 – 15	9 mares	Untrained
III	10	Standardbred horses	5 – 9	2 mares, 8 geldings	Regular training
IV	9	Standardbred horses	6 – 12	4 mares, 5 geldings	Racing condition
V	6	Quarter Horses	3 – 9	5 mares, 1 gelding	Untrained, affected with PSSM
	4	Quarter Horses or Quarter Horse crossbreed	10 – 16	4 mares	Untrained, healthy control horses

Protocol study I

Study I evaluated the influence of extraction time and PCA concentration on the amount of PG and MG recovered from freeze-dried muscle biopsy specimens.

Sixteen Standardbred horses were used to provide muscle biopsy specimens for two experiments. Two samples were obtained from the same biopsy site in the *gluteus medius* muscle from each horse.

Experiment 1 – Effect of extraction time

The experiment was conducted to evaluate the effect of extraction time on the recovery of PG and MG from muscle biopsy specimens using the PG and MG determination technique. Biopsy specimens were obtained from 10 horses. Ten individual freeze dried and powdered muscle biopsy specimens were divided into four equal portions each and randomly assigned to four different treatment combinations. To assess influence of extraction time the four parts were extracted in 1.5 M PCA for 10 min, 20 min, 60 min and 120 min respectively. Extraction time was measured from the time PCA was added until the commencement of centrifugation.

Experiment 2 − Effect of PCA concentrations and extraction times

The aim of this experiment was to evaluate the influence of different PCA concentrations and extraction times on the amount of PG and MG recovered from freeze dried muscle biopsy specimens using the PG and MG determination technique. Biopsy specimens were obtained from 6 horses. Six individual freeze-dried powdered muscle samples were divided into 24 pieces each. Each group of 24 pieces was randomly subjected to 12 different treatment combinations of varying extraction time (10, 20, 30 and 40 minutes) and PCA molarity (0.5 M, 1.5 M, and 3.0 M). Thus, for each treatment combination two pieces from the same horse were analyzed in duplicate. Extraction time was measured from the time PCA was added until the commencement of centrifugation.

Protocol study II

Forty-one clinically normal horses of various breeds were used to provide muscle biopsy specimens for two experiments.

Experiment 1 – Comparison between methods and within-biopsy variability

The experiment studied within-biopsy variability and compared results between AC and PG plus MG determinations for glycogen determination. Biopsy specimens (n = 45) from 32 horses were collected from the *gluteus medius* muscle and/or the *triceps* muscle during conditions ranging from rest to 12 hours after exercise to allow us to obtain specimens with a wide variation in glycogen content. Total glycogen content was measured in all 45 muscle biopsy specimens, using two methods (ie, AC and PG plus MG determinations). Error of the analytical method (variability within muscle biopsy specimens) for the AC and PG plus MG methods was calculated from duplicate analyses of each muscle biopsy specimen.

Experiment 2 – Effect of sampling depth and between-biopsy variability

The experiment evaluated the effect of biopsy depth in the *gluteus medius* muscle and between-biopsy variability for PG and MG content. Nine horses were biopsied at rest from the right and left gluteus medius muscle. Two repeated biopsies were obtained on the same occasion at a depth of 6 cm from the right *gluteus medius* muscle for evaluation of between biopsy variability. Two biopsies were collected from the left *gluteus medius* muscle; one at a depth of 40 mm and one at a depth of 80 mm. The first biopsy was taken at the 40 mm depth. Glycogen content was analyzed in these specimens with the PG plus MG determination technique.

Protocol study III

The degradation of PG and MG in skeletal muscle during intense exercise was examined in this study. Ten Standardbred horses performed a standardised incremental maximal exercise test to fatigue on a high speed treadmill (Säto, Sweden). After a warm-up period of 10 minutes (5 min walk and 5 min trot), the treadmill incline was increased from a horizontal level to an incline of 4.7° (8.2 %) and the speed was set to 7 m/s. The velocity of the treadmill was then increased by 1 m/s every 60 seconds until the horses could not keep pace with the treadmill despite mild encouragement. At this point the incline of the treadmill was lowered to a horizontal level and the horses were allowed to walk during the recovery period of 15 minutes, after which the treadmill was stopped. Biopsy specimens for measurement of the MG, PG, total glycogen, ATP (adenosine triphosphate), CP (creatine phosphate) and lactate concentrations were collected from the *gluteus medius* muscle at rest, immediately post exercise and after 15 minutes of recovery.

Protocol study IV

The resynthesis of PG and MG in skeletal muscle of 9 Standardbred racing trotters was determined after the horses had performed a common training regime composed of repeated bouts of exercise on a slope. The training session consisted of a warm up period of slow trot over 4000 m, 7 repeated 500 m bouts of exercise on an uphill slope at a speed of 9 m/s followed by a period of slow trotting over a 2000 m on a track. Between the bouts of exercise the horses were walked downhill. The slope ascended 24 m per 500 m. The horses performed the training session in pairs based on their training condition. Over the next 3 days the horses were stall rested and walked daily for 60 minutes in the morning and 40 minutes in the afternoon. Muscle biopsies for analysis of PG, MG, free glucose and G-6-P were taken percutaneuosly at a depth of approximately 6 cm from the left and right *gluteus medius* muscle. Samples were taken at rest prior to exercise, immediately post exercise and 1, 4, 8, 24, 48 and 72 hours after exercise. Blood samples were taken before exercise, immediately after the last bout of exercise on the slope, and at the time of each muscle biopsy for analyses of plasma lactate and glucose and serum insulin.

Protocol study V

This study was designed to determine PG, MG, free glucose and G-6-P concentrations in muscle of PSSM horses at rest and following short-term submaximal exercise that induced rhabdomyolysis. Six Quarter Horses with PSSM and four control horses (Quarter Horses or Quarter Horse crossbreeds) were used in this study. The horses performed a light submaximal exercise test on a flat treadmill consisting of repeated intervals of 2 min of walk (1.9 m/s) followed by 2 min of trot (3-4 m/s). The test duration was a maximum of 20 minutes in the control horses. In the PSSM horses the test lasted until horses exhibited evidence of a tucked up abdomen, stiffeness or shifting lameness, and/or muscle fasciculations. Based on previous studies in these horses it was anticipated that the exercise session would last 20 minutes or less. Blood samples were acquired by jugular venipuncture before exercise and 4 hr post exercise for serum CK (creatine kinase). Muscle biopsy samples were collected percutaneously from the *gluteus medius* muscle at rest prior to exercise and immediately after the exercise test through the same skin incision.

Methods

Heart rate

The heart rate in study III was monitored by a bipolar electrocardiogram (Siemens-Elema Minograph 804) whereas the heart rate in study IV was monitored with a pulsimeter (Polar Electron OY).

Blood chemistry

Venous blood samples were taken from the jugular veins (study IV – V) in heparinized tubes and in tubes without additive using a Vacutainer system (Becton Dickinson, Meylan). Vials containing no additive (serum vials) were used for measurement of CK activity and insulin concentrations, whereas vials containing heparin were used for measurement of plasma lactate and glucose concentrations. The heparinized tubes were kept on ice until centrifugation. Plasma and serum were harvested after centrifugation and stored at - 80ºC until analyzed. Analyses for glucose and CK were assayed according to routine methods used at the clinical pathology laboratory at the University of Minnesota or Swedish University of Agricultural Sciences respectively. Plasma lactate was measured enzymatically with a lactate analyser (Analox GM7, Analox instruments Ltd, London UK). Serum insulin concentration was analyzed using a soild-phase radioimmunoassay with a commercial kit (Coat-A-Count Insulin, Diagnostic Products Corporation, LA USA).

Muscle biopsy specimen sampling

Muscle biopsy specimens were collected percutaneously from the *gluteus medius* muscle (study I – V) and from the *triceps* muscle (study II) with the needle biopsy technique as described by Lindholm and Piehl (1974). When repeated biopsies specimens were obtained in study I – III and V the same incision were used for all biopsies. In study IV pre- and immediately post exercise biopsies were obtained

through the same incision whereas different skin incisions were used for the remainder of the repeated biopsies over the 72 hour period. All samples were immediately frozen in liquid nitrogen and stored at -80°C until further analysis. The samples were freeze-dried and dissected free from blood, connective tissue and fat before analyses.

Proglycogen, macroglycogen and glucose analyses

Proglycogen and MG was separated with the method described by Adamo and Graham (1998). Briefly, ice-cooled PCA (200 µL) was added to 1.5 – 3.0 mg of freeze dried muscle samples. The muscle samples were mixed with PCA using a small rod. The extraction was performed on ice for 20 minutes followed by centrifugation in 4º C at 4000 revolutions/min for 10 min, after which 100 and 75 µL of the PCA supernatant were removed and kept for determination of MG and free muscle glucose respectively. The remaining PCA was discarded and the pellet used for determination of PG. The PG and MG fractions were boiled for 2 hours in 1 M HCl. After boiling samples were neutralized by the addition of 2 M Trizma base, vortexed, and centrifuged at 4000 revolutions for 10 minutes. Subsequently, the glucosyl units formed in the supernatants from each fraction were measured fluorometrically with the hexokinase method (Lowry & Passoneau 1973). Free glucose was analysed after neutralization with 2 M KHCO3 according to the method of Harris *et al*. (1974). The obtained MG fractions were corrected for free glucose (study III – V) and G-6-P concentrations (study I – V).

The extraction times and PCA concentrations were modified in study I in order to evaluate the influence of these factors on the amount of PG and MG recovered. The PCA concentrations during the extraction in study II was 1.5 M and 0.5 M in studies III – V.

Muscle metabolites

Muscle metabolites were analyzed on freeze dried muscle samples. Total glycogen was analyzed in study II by the acid hydrolysis technique, which was adopted from the method described by Passonneau and Lauderdale (1974). The concentration of ATP, CP and lactate were analyzed in study III. Freeze dried muscle samples were weighed, extracted in ice-chilled 1.5 M PCA and neutralized with KOH followed by centrifugation. The analysis of ATP and CP were performed on the supernatants using modified high performance liquid chromatography (HPLC) techniques with a C:18 (250 x 4.6, 5 µm) column (Sellevold *et al*. 1986; Dunnet *et al*. 1991). Muscle lactate was assayed from the neutralised supernatants with a fluorometric method (Lowry & Passonneau 1973). In study V lactate was analyzed in aliquots from the PCA supernatants using a commercially available kit (L(+) lactate kit, Sigma Aldrich Sweden AB). Glucose-6-phosphate (study IV – V) was measured fluorometrically in aliquots from the PCA supernatants (Lowry & Passonneau 1973).

Calculations and statistical analyses

Total glycogen content (G_t) for the MG plus PG determination technique was calculated as the sum of the measured PG and MG contents. The percentage of PG and MG of total glycogen was determined by dividing the PG or MG concentration by total glycogen (PG + MG) concentration and multiplying by 100. Degradation of PG and MG in study III was expressed as both absolute decreases (ΔPG and ΔMG) and in relation to the degradation of G_t ($\Delta PG/\Delta G_t$ and $\Delta MG/\Delta G_t$). The net resynthesis rates for PG and MG in study IV were determined by taking the difference between two data points and dividing by the time interval in hours.

Minitab® Statistical software (Minitab Inc., State College PA USA) was used for statistical analysis in Studies I, II and V. Statistica® (Statsoft Inc., Tulsa OK USA) was used in study III and Sigma Stat® software (SPSS Inc., Chicago USA) was used in study IV.

Linear regression analysis was used to determine correlation among methods for total glycogen determination in study II. Agreement between analyses methods and assessment of repeatability (coefficient of repeatability, CR) in study II were calculated as described by Bland and Altman (1986). The limits of agreement were calculated as mean difference ± 2 SD. The CR values were obtained by taking twice the standard deviation of the differences of the duplicates.

A paired t-test was used in study I, II and V to analyze the effect of repeated biopsies or duplicate analyses on the concentration of G_t, PG and MG and in study III to compare degradation in glycogen fractions (ΔPG and ΔMG). An unpaired t-test was used to compare concentrations in muscle metabolites between control horses and horses with PSSM in study V. Analyses of variance (ANOVA) for repeated measures or by using the linear model (GLM) was performed to compare concentrations in blood parameters or muscle metabolites in study I, III and IV. When indicated, significant differences in means were compared with Tukey post hoc test. The null hypothesis was rejected at $P<0.05$ in all tests. All results are given as mean ± SD.

Results

Analysis of pro- and macroglycogen

Effect of extraction time and PCA concentration (Study I)

The effect of extraction time and PCA concentration on the separation of PG and MG was evaluated in two experiments. In the first experiment, where the PCA concentration was 1.5 M during all extractions, the PG and MG concentrations were not affected by extraction times between 10 to 60 min. With extraction for 120 min, the PG concentration decreased whereas the MG concentration was unchanged (Table 2).

Table 2. Mean PG and MG concentrations after different periods of extraction.

	Extraction time (min); concentration (mmol/kg)*			
Form of glycogen	10	20	60	120
PG	347 ± 35	348 ± 73	326 ± 44	310 ± 40[a]
MG	184 ± 46	190 ± 43	190 ± 46	201 ± 45

*Each concentration is a mean ± SD for 10 muscle biopsy specimens, in mmol glucosyl units/ kg dw (mmol/kg).
[a] Significantly different (P<0.05) from 10 and 20 min.

In the second experiment, the effect of the combination of different extraction times and PCA concentrations on the separation of PG and MG were studied. Similar concentrations for PG and MG were obtained with extraction in PCA at concentrations of 0.5 and 1.5 M at each extraction time (10 to 40 min). Extraction in 3.0 M PCA yielded lower PG concentrations and higher MG concentrations than did extraction in 0.5 or 1.5 M at each extraction time. In addition, extraction in 3.0 M PCA gave lower PG and higher MG concentrations at 40 min extraction compared to extraction between 10 – 30 min (Table 3). The mean free glucose concentrations in the PCA extracts were similar for the 12 treatment combinations of extraction time and PCA concentration, ranging from 0.45 to 0.54 mmol/kg dw. Thus, glycogen was not hydrolyzed into glucose residues during PCA extraction regardless of extraction time or PCA concentrations.

Table 3. Mean PG and MG concentrations for different combinations of extraction time and PCA concentration.

Extraction time	PCA concentration (M); PG (mmol glucosyl units/kg dw)*			MG (mmol glucosyl units/kg dw)*		
(min)	0.5	1.5	3.0	0.5	1.5	3.0
10	327 ± 40	326 ± 41	307 ± 39[a]	138 ± 63	142 ± 69	172 ± 91[a]
20	324 ± 38	328 ± 27	305 ± 42[a]	139 ± 63	147 ± 69	185 ± 94[a]
30	319 ± 39	322 ± 33	294 ± 39[a]	143 ± 67	152 ± 74	185 ± 90[a]
40	318 ± 35	318 ± 31	282 ± 44[a]	142 ± 65	150 ± 75	190 ±92[ab]

*Each data set contains values from 6 horses, 2 samples per horses (mean ± SD).
[a] Significantly different (P<0.05) from 0.5 M and 1.5 M for each extraction time.
[b] Significantly different (P<0.05) from values at 10 and 20 min within a column.

Comparison of total glycogen with different methods (Study II)

This study presents evidence that the PG + MG determination is comparable to the AC method of analysis of total glycogen content in muscle biopsy samples from the horse (Figure 2). There was an excellent correlation between the MG + PG and the AC determinations (r = 0.99). The line of identity with a slope equal to one and with the intercept of 0 was almost identical to the regression line (y = x – 9.2). The mean difference for the analyses of total glycogen between the PG + MG and AC determination was –7.1 mmol glucosyl units per kg dw with 95% confidence interval –11 to –3 (Figure 3). The limit of agreement was –34 to +20 mmol glucosyl units per kg dw.

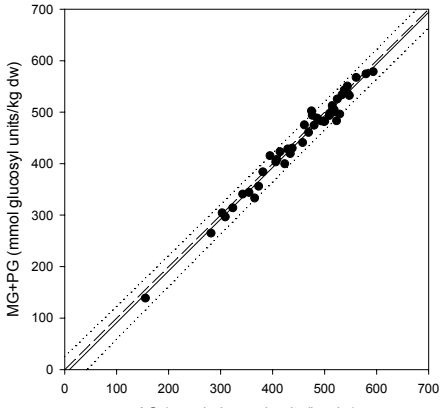

Figure 2. Comparison of total muscle glycogen content in equine muscle determined by use of acid hydrolysis (AC) and macroglycogen (MG) plus proglycogen (PG) determination. Each point represents results for a specific muscle biopsy specimen (n = 45). Solid line represents results of linear regression analysis (equation of line, y = x – 9.2; r = 0.99). Dashed line represents line of identity with slope equal to 1. Dotted line represents 95% prediction interval.

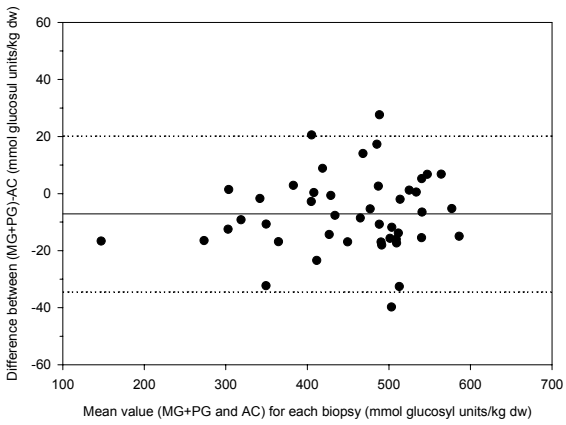

Figure 3. Scatter diagram (Bland-Altman plot) of paired differences between results for 2 methods of analysis of total glycogen content (AC and PG plus MG techniques) and mean value of the two measurements. Each point represents results for a specific muscle biopsy specimen (n = 45). Solid line represents mean difference between values determined by use of the PG plus MG and AC methods (–7 mmol glucosyl units/kg of dw. Dotted line represents limits of agreement (ie, mean difference ± 2 SD; –34 to 20 mmol glucosyl units/kg of dw).

Within-biopsy variability, reported as SD, CV, and coefficient of repeatability (CR), for analysis of total glycogen content determined with the AC or PG plus MG techniques was similar between methods (Table 4).

Table 4. Precision for results of total glycogen content determined in duplicate analyses of the same muscle biopsy specimen for the acid hydrolysis (AC) and proglycogen (PG) plus macroglycogen (MG) techniques.

Measurement technique	N	Mean*	SD	SEM	CV (%)	CR
MG	45	149	11	0.9	7.3	31
PG	45	298	12	0.7	4.0	33
PG + MG	45	447	16	0.6	3.6	45
AC	45	454	15	0.7	3.2	41

*Values reported are mmol glucosyl units/kg of dw.
CR = Coefficient of repeatability. CV = Coefficient of variation.

Effect of sampling depth and between biopsy variability (Study II)

Between-biopsy variability, reported as SD, CV, and correlation of repeatability, for analysis of PG, MG, and G_t (PG + MG) content was slightly higher, compared with values for within-biopsy variability (Table 5). There were no differences in glycogen content between the first and second biopsy specimen.

Table 5. Precision for results of duplicate biopsy specimens obtained at the same site in the gluteus medius muscle of 9 horses

Measurement Technique	Mean* Biopsy 1	Mean* Biopsy 2	SD	SEM	CV (%)	CR
MG	197	194	18	0.3	9.1	50
PG	285	288	20	0.3	7.0	56
PG plus MG	482	482	20	0.2	4.3	58

*Values reported are mmol glucosyl units/kg of dw.
CR = Coefficient of repeatability. CV = Coefficient of variation.

Depth at which samples were collected in the gluteus medius muscle affected the total glycogen and PG concentrations (Table 6). Biopsy specimens obtained more superficially had higher total glycogen and PG content, compared with content in specimens obtained from deeper in the muscle.

Table 6. Effect of depth at which biopsy samples were collected from the gluteus medius muscle of 9 horses on glycogen content (mean ± SD; mmol glucosyl units/ kg of dw)

Measurement technique	Depth for sample collection	
	40 mm	80 mm
MG	191 ± 17	184 ± 16
PG	284 ± 7[a]	267 ± 9[b]
PG + MG	478 ± 21[a]	456 ± 21[b]

[a,b]Within a row, values with different superscript letters differ significantly ($P<0.05$).

Degradation of pro- and macroglycogen during exercise

Degradation during a standardised incremental exercise test (Study III)

The heart rate increased with speed and reached a peak of 214 ± 7 beats/min (range 200 – 226 beats/min). The running time until fatigue varied among horses (246 ± 32 s; range 195 - 300 s) but all horses performed four speed steps (7, 8, 9 and 10 m/s). When fatigue occurred there was a decrease in all horses in muscle concentrations of ATP (Δ 6.2 ± 3.9 mmol/kg d.w.) and CP (Δ 39.7 ± 13.2 mmol/kg dw) as well as an increase in muscle lactate (Δ 78.3 ± 40.1 mmol/kg dw) compared to resting levels.

Before exercise the mean glycogen concentration in the muscle was 504 ± 60 mmol/kg dw and 57 % of this concentration was composed of MG. The exercise caused a decrease in muscle content of G_t (Δ 120 ± 63 mmol glucosyl units/kg dw), PG (Δ 57 ± 22 mmol glucosyl units/kg d.w.) and MG (Δ 63 ± 66 mmol glucosyl units/kg dw). The two fractions of glycogen contributed equally to glycogenolysis, 48 % and 52 % of G_t degradation for PG and MG, respectively (Figure 4).

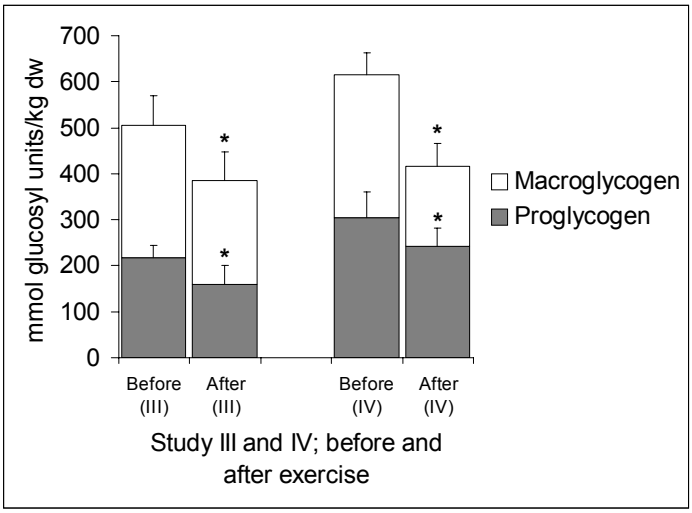

Figure 4. Proglycogen and macroglycogen levels before and immediately after exercise presented as means ± SD for study III and IV.
* Significant decrease ($P<0.05$) between before and immediately after exercise within a study.

Degradation during repeated bouts of exercise on a slope (Study IV).

The exercise was performed in the winter under good weather and track conditions between 9 and 12 pm. The speed for all horses was set at 9 m/s but was adjusted slightly in accordance with their training condition to achieve a HR between 210 and 220 beats per minute at the end of each exercise bout. At the end of the last exercise bout on the slope the plasma lactate concentration increased from a resting level of 0.5 ± 0.3 mmol/L to 15.3 ± 2.6 mmol/L.

The mean G_t in the muscle before exercise was 616 ± 49 mmol/kg d.w. and MG represented 50 % of Gt. A decrease in muscle content of Gt (Δ 200 ± 75 mmol/kg dw), PG (Δ 63 ± 26 mmol/kg dw) and MG (Δ 136 ± 68 mmol/kg dw) was observed after exercise. The MG fractions contributed significantly more to the glycogenolysis compared to PG; 68% and 32 % of G_t degradation for MG and PG, respectively (Figure 4).

Resynthesis of pro- and macroglycogen after exercise (Study IV)

Concentrations of G_t, PG and MG for all time points are shown in Figure 5. MG was the only form to have a significant increase over the first 24 hours. During the first hour post-exercise there was a further decrease in MG in 7 of 9 horses (Δ 46 ± 62 mmol/kg dw), however the decrease was not statistically significant (P=0.14). After 48 hours of recovery G_t, MG and PG reached their pre-exercise levels. During the first hour of recovery no resynthesis occurred in MG but during 1 – 24 hours of recovery there was a net resynthesis rate of 4.7 ± 1.7 mmol glucosyl units/kg dw (Figure 6). The rate of MG resynthesis exceeded that of PG over the first 1 to 24 hours. The rate of MG resynthesis thereafter declined whereas the rate of PG was low for all time periods and remained unchanged (Figure 6).

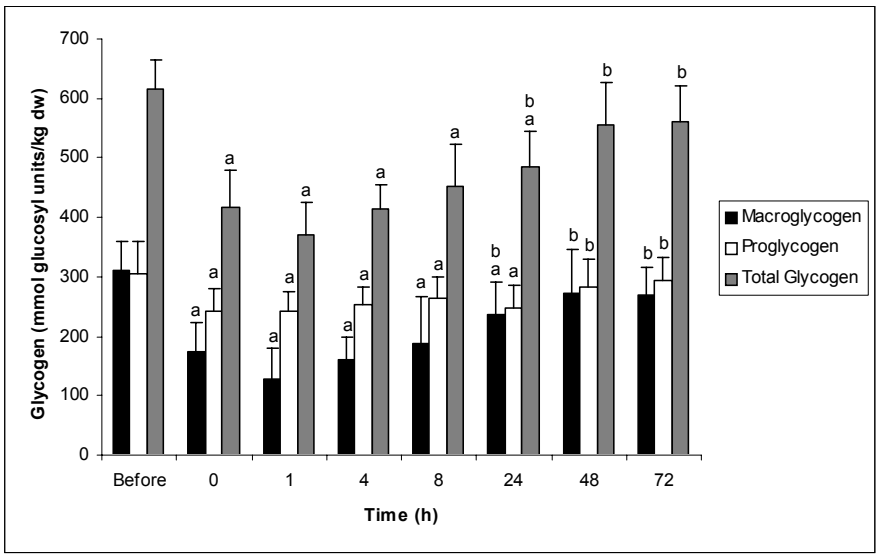

Figure 5. Macroglycogen (black bars), proglycogen (open bars) and total glycogen (gray bars) concentrations in skeletal muscle before and after intermittent exercise and during 72 hours of recovery. Values are means ± SD; n = 9 muscle biopsies per time point. Within a type of glycogen: [a]significantly different from before exercise; [b]significantly different during the recovery period from value 0 hr (P<0.05).

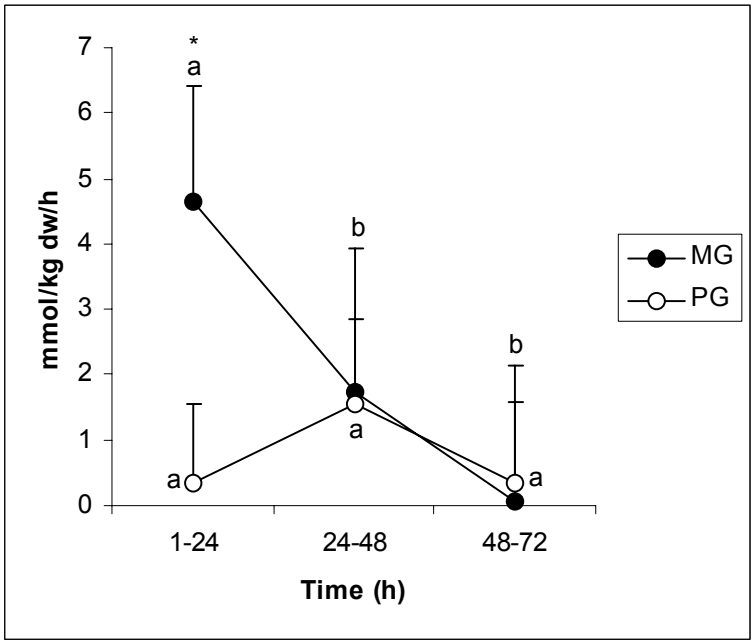

Figure 6. Net synthesis rates of PG and MG during the time intervals 1-24, 24-48 and 48 to 72 hours in muscle biopsy samples after intermittent exercise. Values represent means ± SD; n = 9 muscle biopsies per time point. Values with same letter are not significantly ($P<0.05$) different within a glycogen fraction. *Significant difference ($P<0.05$) between PG and MG within a time point).

Plasma lactate decreased from 15.3 ± 2.6 mmol/L immediately after the last exercise bout to 2.0 ± 0.8 mmol/L at the beginning of the recovery period. Plasma glucose concentration immediately after the last exercise bout was 8.1 ± mmol/L but decreased (Δ 2.7 ± 1.3 mmol/L) to pre exercise levels at 1 hour of recovery. The values for serum insulin increased significantly after completion of the exercise session (Δ 7.6 ± 6.8 mIU/L) and decreased to values before exercise at 1 hour of recovery.

Pro- and macroglycogen in horses with polysaccharide storage myopathy (Study V)

The serum CK activity was higher in PSSM horses 4 h post exercise (5607 ± 8441 U/L) compared to controls (206 ± 64 U/L). Muscle content of PG, MG and Gt was higher in the PSSM horses compared to controls both before and after exercise (Fig 6). Resting muscle glycogen concentrations were 1.5, 2.2 and 1.7 times higher for PG, MG and Gt respectively in PSSM horses than controls. There were no differences in pre exercise concentrations of the muscle metabolites free glucose, G-6-P and lactate between PSSM horse and controls (Table 7). However, resting G-6-P concentrations in PSSM horses were more variable (range 0.7 – 9.5 mmol/kg dw) compared to controls (range 2.2 – 4.8 mmol/kg dw).

With the small number of horses and with the light exercise conducted no differences were observed between pre and post exercise levels of PG, MG, Gt, G-6-P and lactate for PSSM and control horses respectively (Figure 7 and Table 7). However, the light exercise caused a significant increase in the concentrations of free muscle glucose in the PSSM horses (Table 7).

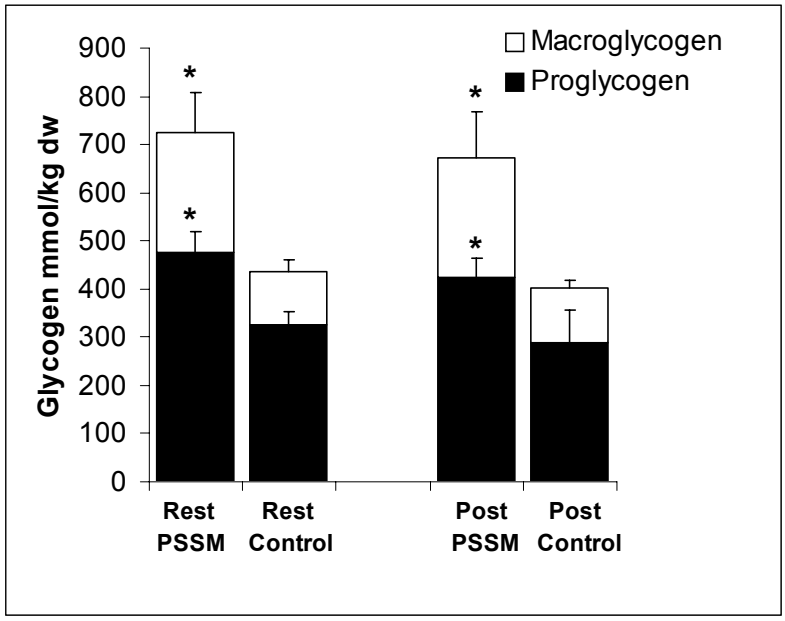

Figure 7. Proglycogen and macroglycogen levels at rest and immediately after exercise (Post) for PSSM and control horses. Data is presented as means ± SD. *Indicates a significant difference (P<0.05) between PSSM and control horses at rest or post exercise.

Table 7. Muscle metabolite concentrations pre exercise (Pre) and post exercise (Post) in 6 Quarter Horses with PSSM and 4 control horses.

	Free glucose		G-6-P		Lactate	
	Pre	Post	Pre	Post	Pre	Post
PSSM	1.8 ± 0.5	3.4 ± 0.9*†	5.1 ± 2.8	5.25 ± 2.5	39.1 ± 23.9	51.4 ± 25.1
Control	2.0 ± 1.1	1.3 ± 0.7	3.1 ± 1.2	2.9 ± 1.6	23.6 ± 11.9	32.2 ± 7.9

Data are reported as means ± SD. Values reported are mmol/kg of dry weight. *Indicates significantly different from pre exercise value. † Indicates significantly different from post exercise controls. P < 0.05.

General Discussion

Analysis of pro- and macroglycogen (Study I, II)

One purpose of this study was to determine whether the concentrations of PG and MG are stable during the extraction procedure with regard to extraction time and PCA concentration. If the relationship between the two forms of glycogen continues to change with extraction time the reliability of the separation technique should be questioned and the biological significance of the data would be limited. Our results showed that the concentrations of PG and MG were stable with extraction time. The stability seems to be more pronounced for extraction in 0.5 M and 1.5 M PCA compared to extraction in 3.0 M PCA (Study I). Extraction in 3.0 M PCA yielded lower PG values and higher MG concentrations ($P<0.05$) for all studied extraction times (10 – 40 minutes). The possibility that stronger acid concentration yielded higher MG concentrations due to pre-hydrolysis of glycogen into glucose residues by the PCA was excluded by analysis of glucose in the PCA extracts prior to acid hydrolysis in HCl. The mean glucose concentration in the PCA extracts for each of the treatment combinations of different PCA concentrations and extraction times were almost identical (range 0.45 - 0.54 mmol glucose per kg dry weight). Instead it is possible that the stronger acid (PCA 3.0 M) caused a limited amount of hydrolysis of small chains of polysaccharides from the PG fraction, which entered the PCA-soluble fraction (MG fraction). This theory is supported by the fact that the change in the PG and MG pool tended to be inversely related.

In the present study, there was a strong positive correlation between total glycogen measured by AC and by the PG + MG determination technique over a wide range of glycogen concentrations using linear regression analysis ($y = x - 9.2$; $r = 0.99$). Linear regression analysis is a common approach to compare two measurement techniques but the correlation coefficient and the regression analysis should be interpreted cautiously. The exclusive use of the correlation coefficient as a statistical tool in these situations can be misleading and inappropriate for several reasons. The correlation coefficient is a measure of association and it would be surprising if two methods designed to measure the same thing were not related. The correlation coefficient depends on the range of measurements used and could therefore be increased by choosing widely spaced observations (Campbell & Machin 1993). It is more appropriate to analyze how well two measurement techniques agree (Bland & Altman 1986). The linear regression line in our study was almost identical to the line of identity; which indicated that the two analysis methods were likely to agree very well. We confirmed the agreement between the two methods by using an alternative statistical method proposed by Bland and Altman (1986). The limit of agreement was -34 to 20 mmol glucosyl units per kg dry weight and these limits were smaller than the limits of agreement for duplicate analysis for total glycogen obtained by analysis with the AC and PG + MG determination technique.

Repeatability is also of importance in studies where two methods are compared because the variation within each method limits the amount of agreement. If one method has poor repeatability the agreement between the two methods will be poor (Bland & Altman 1990). Our results showed that the AC and PG + MG determination techniques had almost identical values for the coefficient of repeatability (Study II). The within and between biopsy variability for total glycogen gave CV values of approximately 4% for total glycogen analyzed by the AC and PG + MG determination technique. The results of repeatability (CV and SD) for total glycogen determined by AC and the PG + MG technique were in good agreement with previous studies on humans (Hultman 1967; Essen & Henriksson 1974; Harris *et al*. 1974; Adamo & Graham 1998) and horses (Lindholm & Piehl 1974). Together, the linear regression equation, the limits of agreement and the values of repeatability indicate a strong agreement between the AC and PG + MG determination techniques for total glycogen.

The present study evaluated repeatability between muscle biopsies exclusively from resting horses. The variation between muscle biopsies would probably have been higher if the study had been conducted on post-exercise samples. During exercise the fiber recruitment varies within the muscle, which results in differences in the glycogen depletion pattern (Essen 1978; Snow et al. 1981; Valberg 1986; Gottlieb 1989). This may potentially lead to higher variation in total glycogen for post-exercise samples. The results from this study illustrate the importance of a standardized sampling site and depth in the muscle. In the gluteal muscle the total glycogen and PG concentrations decreased with increased sampling depth (Study II). This may be related to an increased proportion of type I fibers with increasing depth in the *gluteaus medius* muscle (Karlström *et al*. 1994; Serrano et al. 1996).

A problem with the PG + MG determination technique is that endogenous tissue glucose and G-6-P are recovered in the MG fraction and these metabolites will therefore be incorrectly measured as MG with the hexokinase method. In resting muscle the concentrations of these metabolites are very small relative to the MG fraction and they could therefore be ignored. However, under conditions of intense exercise the intracellular concentration of glucose and G-6-P increases, which could cause a potential error in the measured MG fraction (Stetten *et al*. 1958; Essén & Kaijser 1978; Derave *et al*. 2000). For example, if not corrected for glucose in study III the decrease in MG would have resulted in an overestimate of up to 12 %. The error of G-6-P could be excluded in the hexokinase method by adding the enzyme glucose-6-phospate dehydrogenase already to the reagent rater than together with hexokinase later in the analysis procedure (Lowry & Passonneau 1973). Correction of glucose, however, requires separate analysis of free muscle glucose in nonhydrolysed samples (Passonneau & Lauderdale 1974).

Previous studies conducted on human and rat muscle have demonstrated that the relative proportions of PG and MG on a weight basis were dependent on the total glycogen concentration (Adamo & Graham 1998; Adamo *et al*. 1998; Hansen *et al*. 2000). As total glycogen concentration increased in the muscle, the proportion of MG increased whereas the proportion of PG decreased. Despite a decrease in

the relative proportion of PG, the absolute PG concentration increased as total glycogen increased. A similar relationship has been demonstrated in this study for equine skeletal muscle (Study II). The absolute increase in PG could be explained by an increase in the size of PG molecules, an increase in the number of glycogen particles or by a combination of theses mechanisms. Interestingly, Elsner *et al*. (2002) have reported that cultured myotubes synthesize glycogen by forming new granules together with increasing glycogen granule size.

There is controversial information in the literature regarding whether PG and MG exist as distinct molecular sizes or as a range of sizes (Lomako *et al*. 1991; Lomako *et al*. 1993; Alonso *et al*. 1995b; Roach & Skurat 1997). The group of Whelan and colleagues (Alonso *et al*. 1995b; Lomako *et al*. 1993) was the first to assign distinct sizes to glycogen. They showed that glycogen did not exist in a continuum of sizes from glycogenin upward. Instead, they found a discrete glycogen molecule of 400 kDa (PG), which was an intermediate between glycogenin and the mature glycogen granule (MG) (Lomako *et al*. 1993). However, subsequent studies have failed to confirm that PG exists as a discrete molecule and suggest that glycogen rather consist of a continuum of molecular sizes from low to high molecular masses (Skurat *et al*. 1997; Marchand *et al*. 2001). The work by Marchand *et al*. (2001) suggest that the smallest glycogen granules are much below the upper limit for PG and that the glycosomes are found in size ranges up to the 12-tier diameter. Regardless, this controversy does not exclude that separation of PG and MG is unimportant since the two glycogen entities (PG and MG) have different rates of synthesis and degradation in humans (Adamo *et al*. 1998; Graham *et al*. 2001; Shearer *et al*. 2001; Battram *et al*. 2004) and horse skeletal muscle (Essén-Gustavsson & Jensen-Waern 2002; Study III and IV) as well as different sensitivity to carbohydrate ingestion in humans (Adamo *et al*. 1998).

Degradation of pro- and macroglycogen during exercise (Study III, IV)

The catabolism of PG and MG appears to be dependent on factors such as exercise intensity, duration and total glycogen concentration in both horses and humans. In the present thesis the degradation of PG and MG were evaluated during two different types of exercise. In study 3, when horses performed an incremental exercise test on a treadmill until fatigue, glycogenolysis occurred both in the PG and MG fractions to a similar extent. Marked anaerobic metabolism near fatigue occurred as shown by the increase in muscle lactate and by the decrease in glycogen, ATP and CP concentrations immediately after exercise. In study IV the horses performed a training session comprising a warm up period, 7 repeated bouts of exercise on an uphill slope and a recovery period. Interestingly, during this type of exercise the glycogenolysis in the MG fraction was over twice as high compared to the magnitude of the glycogenolysis in the PG fraction. It is tempting to compare the glycogenolysis in the PG and MG fractions between the studies. However, the horses participating in the two studies had different levels of fitness, initial glycogen concentrations and percentage distribution of PG and MG in their muscles. Such comparisons should therefore be made with caution as these are

factors that could influence the relation as well as the magnitude of the glycogenolysis in the two glycogen fractions.

The results from horses in this thesis differ from earlier studies on degradation of PG and MG in human skeletal muscle where PG is found to be more dynamic under most exercise conditions. At low to moderate exercise intensities, up to 70 % of VO_2 max, PG and MG contribute equally to the glycogenolysis. However, as exercise intensity increases the glycogenolysis occurs predominantly in the PG fraction (Graham *et al*. 2001). When repeated exercise at 130 % of VO_2 max is performed, the catabolism of MG can be remarkably suppressed in comparison to the catabolism of PG (Shearer *et al*. 2001).

One explanation for the difference in glycogenolysis pattern between horses and humans may be that the glycogen content, especially the MG content, is higher in horse muscle compared to the previously mentioned human studies. A recent study on rats demonstrated that when the MG fraction increased in the muscle the relative rate of glycogenolysis for the MG pool also increased (Derave *et al*. 2000). However, it should be recognized that MG is not only degraded to a greater extent because of its higher concentration in horse skeletal muscle. If PG and MG were degraded proportionally to their initial glycogen concentrations, PG and MG would be degraded by 57 and 43 % respectively in study III and by 50% in both fractions in study IV, which was not observed. Thus, the type and duration of exercise likely has a strong impact on the degradation of the two glycogen fractions. During a marathon race human skeletal muscle utilizes more MG than PG for glycogenolysis (Asp *et al*. 1999). A recent study on horses performing endurance type of exercise indicates that horses have a near three times higher degradation in the MG compared to the PG pool (Essén-Gustavsson & Jensen-Waern 2002). It is therefore possible that MG is more readily degraded in the type I and IIa fibers as they have been shown to be preferentially glycogen depleted after exercise of endurance type (Snow et al. 1981; Sherman *et al*. 1983; Jensen-Waern *et al*. 1999).

Different regulation between PG and MG may also originate from the way glycogen synthase (GS) and glycogen phosphorylase (GP) interact with glucose residues on the outer tiers of the glycogen granule. The number of branches increase exponentially with increasing granule size, resulting in progressively more sites for GP degradation in the larger MG molecule compared to the PG molecule. This would suggest that the larger granule would be more dynamic in glucose turnover but this is not always the case. A potential explanation for this is that the outer chains may become too dense for easy movement of the enzyme GP between branches and the large glycosome volume may shift inhibiting protein interactions.

It is unknown whether the glycogenolysis of a MG molecule proceeds until a PG molecule results or if the degradation continues until all of the glucosyl units in the molecule are liberated. In the former scenario the degradation of the MG pool would supply molecules to the PG pool. The calculations of the PG degradation in

this thesis do not consider this issue and are therefore estimates of the true PG glycogenolysis.

Resynthesis of pro- and macroglycogen after exercise (Study IV)

The resynthesis of glycogen after repeated bouts of exercise on a slope occurred to a greater extent in the MG fraction compared to the PG fraction. The fraction most depleted during exercise (MG) had restricted resynthesis during the first hour of recovery but then had the highest rate of resynthesis over the remainder of the first 24 hours. The resynthesis of PG was a slow process compared to MG. The overall patterns of PG and MG resynthesis as well as the rates of total glycogen resynthesis in the present thesis seem to differ from previous work performed in humans during the recovery period. The general resynthesis pattern in human skeletal muscle after intense exercise is predominantly resynthesis of PG early in recovery and a slower more constant rate of MG synthesis over the next 24 hours (Adamo et *al*. 1998; Shearer *et al*. 2001; Battram *et al*. 2004). One explanation for differences may be related to the fact that the fraction of MG in horse muscle is greater compared to humans. Another perhaps more important explanation, may be the effect of the level of glycogen depletion. The horses in the present study decreased their total glycogen concentration in muscle to approximately 400 mmol glucosyl units per kg dw whereas the humans that participated in the aforementioned recovery studies had post-exercise concentrations between 50 and 100 mmol glucosyl units per kg dw. Such extreme glycogen depletion probably requires the formation of new glycogen granules rather than the addition of glucose to existing glycogen molecules, which is a more likely situation to occur when glycogen depletion is more modest. This hypothesis is supported by Shearer *et al*. (2005) who demonstrated that during glycogenolysis changes in glycogenin were not apparent until a more prominent decrease in glycogen had occurred. Therefore, resynthesis patterns of PG and MG should always be interpreted in light of the level of glycogen depletion.

During glycogen resynthesis glucose can either be added into existing PG or MG molecules or be incorporated into newly formed glycogen particles from glycogenin. If resynthesis of glycogen occurred predominantly in the PG fraction this would suggest that glucosyl units were added to existing PG molecules or that new glycogen granules were formed. In the present study, the largest increase in glycogen concentration after the first 24 hours of recovery occurred in the MG fraction. It is unknown whether this is a result of existing PG growing into MG or if existing MG particles grew larger by replenishment of the outer tiers.

In agreement with previous studies, the rate of glycogen resynthesis is found to be a slow process in the horse (Snow *et al*. 1991; Hyyppä *et al*. 1997; Lacombe *et al*. 2004). The relatively slower rate of glycogen resynthesis during the postexercise period compared to other species has not been explained. The horse limited ability to digest starch as well as local factors in the muscle such as decreased glucose transport across the membrane and low activity of GS have all been suggested mechanisms for this restricted capacity of glycogen resynthesis (Lacombe *et al*. 2004). Another limiting factor for glycogen resynthesis during

early recovery could be that glucose is used for oxidation rather than fat. As demonstrated in previous studies, plasma glucose and serum insulin were increased at the beginning of the recovery period (Lacombe *at al*. 2004; Hyyppä *et al*. 1997), which increases the potential for intramuscular glucose uptake and glycogen resynthesis. It therefore seems paradoxical that persistent glycogenolysis occurred in 7 of 9 horses during the early recovery period in the present study. However, this phenomenon in the horse post exercise has been reported previously by Hyyppä *et al*. (1997). The decrease in muscle glycogen stores in the present and previous studies indicates an inability of the skeletal muscle to shift from carbohydrate to fat oxidation during the early recovery period. In humans, plasma fatty acids, very low density lipoproteins and triacylglycerols have been shown to be important fuel sources for aerobic energy, particularly during the first hour of recovery (Kiens & Richter 1998). This increased fat oxidation during early recovery in humans is even present during elevated plasma concentrations of insulin and glucose. Thus, it appears that glycogen resynthesis is of high priority in humans but of low priority in the horse.

Pro- and macroglycogen in horses with polysaccharide storage myopathy (Study V)

In agreement with previous studies, the total resting muscle glycogen concentrations were 1.7 times higher in PSSM horses than in controls (Valberg *et al*. 1999, Annandale *et al*. 2004). This difference was due to both higher PG and MG concentrations in PSSM horses. Interestingly, the mean concentration of the PG fraction in the PSSM horses was nearly 500 mmol glucosyl units per kg dw which is higher than the total glycogen content of the control horses, i.e. the sum of PG and MG. To our knowledge, the values for PG in the PSSM horses are the highest values reported in the literature.

Glycogen stores in muscle can theoretically rise by either increasing the size of existing glycogen granules or by increasing the number of glycogen granules by adding glucose to newly formed glycogenin. In the first scenario, the number of granules in skeletal muscle remains constant as existing granules grow larger requiring no new additional glycogenin protein, which would result in a decreased PG/MG ratio. Conversely, the latter situation in which new glycogen granules are formed would result in an increase in PG and require additional glycogenin protein. This scenario would result in an increased PG/MG ratio. Earlier studies in both man (Adamo et al. 1998) and horses (Study II) have demonstrated that with increasing glycogen concentrations in skeletal muscle there is an increase in the MG pool without a corresponding decrease in the PG pool. These findings suggest that increase in glycogen stores in skeletal muscle is not only obtained by increasing the size of glycogen granules but also by increasing the number of glycogen granules. With increasing glycogen concentrations there is a decline in the PG/MG ratio and when G_t is approximately 600 mmol glucosyl units per kg/dw the PG/MG ratio is around 1 (study II and IV) in horses. As a comparison a PG/MG ratio of 1 occurs at glycogen levels over 500 mmol glucosyl units/kg dw in humans and this distribution between PG and MG even persists in McArdle´s patients who lack GP despite G_t exceeding 800 mmol/kg dw (Shearer & Graham

2002). It is therefore surprising that the PSSM horses in the present study, with an average glycogen concentration of 724 mmol glucosyl units per kg dw, had a mean PG/MG ratio over 2:1. These findings suggest that the high glycogen content in PSSM horses is achieved to a much higher extent by increasing the number of glycogen granules.

There are two potential explanations for the relative high concentrations of PG in the PSSM horses. First, since glycogenin is present in proportion to glycogen granule number under resting conditions, the presence of smaller and more abundant glycogen molecules in the PSSM horses would imply a higher glycogenin content in the skeletal muscle of these horses. However, to date there are conflicting results for a causative role of overexpression of glycogenin in determining the extent of glycogen storage (Skurat *et al*. 1996; Skurat *et al*. 1997; Hansen *et al*. 1998). Second, increased ratio of GS to GBE activity leads to accumulation of an abnormal polysaccharide with long outer glucose chains that lack α-1.6 branch points (Massa *et al*. 1996), similar to the abnormal polysaccharide observed in PSSM horses. The increased ratio of GS to GBE activity can be a result of abnormally high intracellular concentrations of G-6-P (Massa *et al*. 1996) or of overexpression of GS (Pederson *et al*. 2003).

Horses with PSSM have a high PG/MG ratio, variably high G-6-P concentrations (Valberg *et al*. 1999), accumulation of abnormal polysaccharide with fewer branch points (Annandale *et al*. 2004) and enhanced glucose uptake in the skeletal muscle cells (De La Corte *et al*. 1999; Annandale *et al*. 2004). Therefore, it seems reasonable to hypothesize that an increased ratio of GS to GBE activity accounts for both the high PG concentrations and the abnormal polysaccharide formed in these horses. It might be argued that G-6-P does not play a causative role in the accumulation of an abnormal polysaccharide and glycogen with fewer branching points in the PSSM horses since no statistical difference has been found in G-6-P concentrations between PSSM horses and controls in the present and previous studies (Valberg *et al*. 1999; Annandale *et al*. 2004; Annandale *et al*. 2005). However, whole muscle sample concentrations of metabolites are mean values of all fibers within the sample and therefore do not express the metabolic changes within individual fibers. It is likely that PSSM is a disorder that causes metabolic abnormalities within some but not all muscle fibers since abnormal polysaccharide is found in only 1 – 25 % of fast twitch muscle fibers (Valberg *et al*. 1992). This variability thus makes even rather large changes difficult to detect in muscle homogenates unless a critical mass of fibers are affected. For example, in a recent study on PSSM horses marked variations in IMP were observed within single fibers and within pools of single muscle fibers after exercise that was not apparent in whole muscle samples (Annandale *et al*. 2005).

The exercise test did not cause any significant changes in total glycogen, PG, MG, G-6-P and lactate in muscle tissue in either the control group of horses or the PSSM horses. It is likely that the intensity and duration of the exercise test was too short to be able to demonstrate any use of glycogen as fuel for energy production in the muscle. It is well known that during submaximal exercise both glucose and fatty acids in blood are important fuel sources for aerobic ATP production (Essén-

Gustavsson & Jensen-Waern 2002). Of interest was, however, the increased concentration of free muscle glucose after light exercise in the PSSM horses but not in the control horses. The values for free glucose in the present study are based on glucose assay of muscle homogenates and are not extrapolated values for intracellular glucose. Some of the free glucose is therefore of extracellular origin in both PSSM and control horses. The free glucose concentration in the cytoplasm determines the intracellular component of the glucose transport gradient. Thus, the ability to metabolize transported glucose is critical in determining glucose flux across the cell membrane. The first step in glucose metabolism, the phosphorylation of G-6-P by the enzyme hexokinase, is crucial because it maintains a low intracellular glucose concentration. It is generally assumed that most of the glucose taken up by the muscle during exercise is metabolized at least during light and moderately intense exercise (Wasserman & Halseth 1998). The accumulation of glucose after light submaximal exercise in the PSSM horses suggest a limited or overwhelmed capacity to phosphorylate intracellular glucose. This could have several explanations. High intracellular levels of G-6-P apparently inhibit hexokinase but this is usually considered to occur as a consequence of increased glycogenolysis during intense exercise (Wasserman & Halseth 1998). This explanation is not applicable in the PSSM horses in the present study as the exercise was very light and glycogenolysis limited. It is possible that increased baseline levels of G-6-P could account for this inhibition since there was a high variability of G-6-P both pre- and post exercise. A more likely explanation is that glucose transport is stimulated to a high degree. Horses with PSSM have an enhanced glucose transport over the muscle cell membrane at rest (De La Corte *et al*. 1999; Annandale *et al*. 2004) and it is likely that this faster rate of glucose uptake is more prominent during exercise when there is an increase in plasma membrane glucose transporter content via translocation of GLUT4.

General Summary and Conclusions

- An accurate and reproducible method for determination of PG and MG in freeze dried muscle biopsy specimens from horses was established. The two fractions of glycogen were very stable with extraction time and PCA concentration during the separation procedure.

- There was good agreement between the traditional method (AC) for analysis of glycogen in muscle biopsy specimens and the PG plus MG determination technique.

- Depth at which muscle biopsy specimens were collected in the *gluteus medius* muscle affected the G_t and PG concentrations. Muscle biopsy specimens obtained more superficially had higher PG and G_t concentrations compared with specimens obtained deeper in the muscle.

- The degradation of PG and MG is affected by duration and type of exercise. PG and MG contribute equally to glycogenolysis during intense exercise in Standarbred trotters performing an incremental exercise test on a treadmill. During repeated bouts of exercise on a slope MG contributed twice as much as PG to glycogenolysis in Standarbred trotters.

- The resynthesis of G_t after repeated bouts of exercise on a slope was a slow process and required 48 hours to be completed. The rate of glycogen resynthesis during 1 to 24 hours was higher for MG than for PG. The rate of muscle glycogen resynthesis thereafter was slower and did not differ between MG and PG.

- Resting muscle glycogen concentrations were 1.5, 2.2 and 1.7 times higher for PG, MG and Gt respectively in PSSM horses than controls. Significant changes in G_t, PG, MG, G-6-P and lactate did not occur with light exercise, however, free muscle glucose increased significantly in PSSM horses after exercise.

References

Adamo, K.B. & Graham, T.E. 1998. Comparison of traditional measurements with macroglycogen and proglycogen analysis of muscle glycogen. *J Appl Physiol* 84, 908-913.

Adamo, K.B., Tarnopolsky, M.A. & Graham, T.E. 1998. Dietary carbohydrate and postexercise synthesis of proglycogen and macroglycogen in human skeletal muscle. *Am J Physiol* 275, E229-334.

Alonso, M., Lagzdins, E., Lomako, J., Lomako, W. & Whelan, W. 1995a. New and specific nucleoside diphosphate glucose substrates for glycogenin. *FEBS Lett* 359, 110-112.

Alonso, M.D., Lomako, J., Lomako, W.M. & Whelan, W.J. 1995b. A new look at the biogenesis of glycogen. *FASEB J* 9, 1126-1137.

Annandale, E.J., Valberg, S.J., Essen-Gustavsson, B. 2005. Effects of submaximal exercise on adenine nucleotide concentrations in skeletal muscle fibers of horses with polysaccharide storage myopathy. *Am J Vet Res* 66, 839-845.

Annandale, E.J., Valberg, S.J., Mickelson, J.R. & Seaquist, E.R. 2004. Insulin sensitivity and skeletal muscle glucose transport in horses with equine polysaccharide storage myopathy. *Neuromuscul Disord* 14, 666-674.

Asp, S., Daugaard, J.R., Rohde, T., Adamo, K. & Graham, T.E. 1999. Muscle glycogen accumulation after a marathon: roles of fiber type and pro- and macroglycogen. *J Appl Physiol* 86, 474-478.

Battram, D.S., Shearer, J., Robinson, D. & Graham, T.E. 2004. Caffeine ingestion does not impede the resynthesis of proglycogen and macroglycogen after prolonged exercise and carbohydrate supplementation in humans. *J Appl Physiol* 96, 943-950.

Bergström, J. 1962. Muscle electrolytes in man. Determination by neutron activation analysis on needle biopsy specimens. A study on normal subjects, kidney patients and patients with chronic diarrhoea. *Scand J Clin Lab Invest Suppl* 68, 1-110.

Bergström, J., Hermansen, L., Hultman, E. & Saltin, B. 1967. Diet, muscle glycogen and physical performance. *Acta Physiol Scand* 71, 140-150.

Bland, J.M. & Altman, D.G. 1986. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1, 307–310.

Bland, J.M. & Altman, D.G. 1990. A note on the use of intraclass correlation coefficient in the evaluation of agreement between two methods of measurement. *Comput Biol Med* 20, 337-340.

Calder, P.C. 1991. Glycogen structure and biogenesis. *Int J Biochem* 23, 1335-1352.

Campbell, D.G. & Cohen, P. 1989. The amino acid sequence of rabbit skeletal muscle glycogenin. *Eur J Biochem* 185, 119-125.

Campbell, M.J. & Machin, D. 1993. *Common pitfalls in medical statistics.* In: Medical Statistics a Commonsense Approach, 2nd edn. John Wiley & Sons, Chichester. pp 126-136.

Comline, R.S., Hall, L.W., Hickson, J.C.D., Murillo, A. & Walker, R.G. 1969. Pancreatic secretion in the horse. *J of Physiol* 204, 10P-11P.

Davie, A.J., Evans, D.J., Hodgson, D.R. & Rose, R.J. 1994. The effects of an oral glucose polymer on muscle glycogen resynthesis in Standardbred horses. *J Nutr* 124, 2740S-2741S.

Davie, A.J., Evans, D.J., Hodgson, D.R. & Rose, R.J. 1995. Effects of intravenous dextrose infusion on muscle glycogen resynthesis after intense exercise. *Equine vet J Suppl* 18, 195-198.

De La Corte, F.D., Valberg, S.J., Mickelson, J.R. & Hower-Moritz, M. 1999. Blood glucose clearance after feeding and exercise in polysaccharide storage myopathy. *Equine vet J Suppl* 30, 324-328.

Derave, W., Gao, S. & Richter, E.A. 2000. Pro- and macroglycogenolysis in contracting rat skeletal muscle. *Acta Physiol Scand* 169, 291-296.

Dunnett, M., Harris, R.C. & Orme, C.E. 1991. Reverse-phase ion-pairing high-performance liquid chromatography of phosphocreatine, creatine and creatinine in equine muscle. *Scan J clin Lab Invest* 51, 137-141.

Elsner, P., Quistorff, B., Hansen, G. & Grunnet, N. 2002. Partly ordered synthesis and degradation of glycogen in cultured rat myotubes. *J Biol Chem* 277, 4831-4838.

Essen, B. 1978. Glycogen depletion of different fibre types in human skeletal muscle during intermittent and continuous exercise. *Acta Physiol Scand* 103, 446-455.

Essen, B. & Henriksson, J. 1974. Glycogen content of individual muscle fibres in man. *Acta Physiol Scand* 90, 645-647.

Essén-Gustavsson, B. & Jensen-Waern, M. 2002. Effect of an endurance race on muscle amino acids, pro- and macroglycogen and triglycerides. *Equine vet J Suppl* 34, 209-213.

Essén, B. & Kaijser, L. 1978. Regulation of glycolysis in intermittent exercise in man. *J Physiol* 281, 499-511.

Essén-Gustavsson, B., Karlstrom, K. & Lindholm, A. 1984. Fiber type, enzyme activities and substrate utilization in skeletal muscle of horses competing in endurance rides. *Equine Vet J* 14, 197.

Essén-Gustavsson, B., McMiken, D., Karlström, K., Lindholm, A., Persson, S. & Thornton, J. 1989. Muscular adaption of horses during intensive training and detraining. *Equine vet J* 21, 27-33.

Farris, J.W., Hinchcliff, K.W., McKeever, K.H. & Lamb, D.R. 1995. Glucose infusion increases maximal duration of prolonged treadmill exercise in Standardbred horses. *Equine Vet J Suppl* 18, 357-361.

Friden, J., Seger, J. & Ekblom, B. 1985. Implementation of periodic acid-thiosemicarbazide-silver proteinate staining for ultrastructural assessment of muscle glycogen utilization during exercise. *Cell and Tissue Res* 242, 229-232.

Friden, J., Seger, J. & Ekblom, B. 1989. Topographical localization of muscle glycogen: an ultrahistochemical study in the human vastus lateralis. *Acta Physiol Scand* 135, 381-391.

Gansen, S., Lindner, A., Marx, S., Mosen, H. & Sallmann, H.P. 1999. Effects of conditioning horses with lactate-guided exercise on muscle glycogen content. *Equine vet J Suppl* 30, 329-331.

Goldsmith, E.S., Sprang, R.J. & Fletterick, R.J. 1982. Structure of maltoheptaose by difference fourier methods and a model for glycogen. *J Mol Biol* 156, 411-427.

Gottlieb, M. 1989. Muscle glycogen depletion patterns during draught work in Standardbred horses. *Equine Vet J* 21, 110-115.

Graham, T.E., Adamo, K.B., Shearer, J., Marchand, I. & Saltin, B. 2001. Pro- and macroglycogenolysis: relationship with exercise intensity and duration. *J Appl Physiol* 90, 873-879.

Hansen, B.F., Derave, W., Jensen, P. & Richter, E.A. 2000. No limiting role for glycogenin in determining maximal attainable glycogen levels in rat skeletal muscle. *Am J Physiol Endocrinol Metab* 278, E398-404.

Hansen BF, Jensen P, Barbetti F. 1998. Glycogen storage in L6 cells are increased by overexpression of glycogenin. *Diabetologia*, 41:A201.

Harris, P. 1997. Energy sources and requirements of the exercising horse. *Annu Rev Nutr* 17, 185-210.

Harris, R.C., Hultman, E. & Nordesjo, L.O. 1974. Glycogen, glycolytic intermediates and high-energy phosphates determined in biopsy samples of musculus quadriceps femoris of man at rest. Methods and variance of values. *Scand J Clin Lab Invest* 33, 109-120.

Harris, R.C., Marlin, D.J. & Snow, D.H. 1987. Metabolic response to maximal exercise of 800 and 2000 m in the Thoroughbred horse. *J Appl Physiol* 63, 12-19.

Hodgson, D.R., Kelso, T.B., Bayly, W.M. & Gollnick, P.D. 1987. *Response to repeated high-intensity exercise: influence on muscle metabolism.* In: Gillespie, J.R. & Robinson, N.E. (eds.) Equine exercise physiology 2. Davis, California, ICEEP Publications, pp 302-311.

Hodgson, D.R., Rose, R.J., DiMauro, J. & Allen, J.R. 1985. Effects of submaximal treadmill training programme on histochemical properties, enzyme activities and glycogen utilisation of skeletal muscle in the horse. *Equine vet J* 17, 300-305.

Hultman, E. 1967. Muscle glycogen in man determined in needle biopsy specimens: method and normal values. *Scand J Clin Lab Invest* 19, 209-217.

Hyyppä, S., Räsänen, L. & Pösö, R. 1997. Resynthesis of glycogen in skeletal muscle from Standardbred trotters after repeated bouts of exercise. *Am J Vet Res* 58, 162-166.

Jansson, E. 1981. Acid soluble and insoluble glycogen in human skeletal muscle. *Acta Physiol Scand* 113, 337-340.

Jensen-Waern, M., Lindberg, Å., Johannisson, A., Gröndahl, G., Lindgren, J.Å. & Essén-Gustavsson, B. 1999. The effect of an endurance ride on metabolism and neutrophils function. *Equine vet J Suppl* 30, 605-609.

Jose-Cunilleras, E. & Hinchcliff, K.W. 2004. Carbohydrate metabolism in exercising horses. *Equine and comparative exercise physiology* 1, 23-32.

Karlström, K., Essén-Gustavsson, B. & Lindholm, A. 1994. Fibre type distribution, capillarization and enzymatic profile of locomotor and nonlocomotor muscles of horses and steers. *Acta Anatomica* 151, 97–106.

Kiens, B. & Richter, E. 1998. Utilization of skeletal muscle triacylglycerol during postexercise recovery in humans. *Am J Physiol* 275, E332-337.

Kits Van Heijningen, A.J.M. & Kemp, A. 1955. Free and fixed glycogen in rat muscle. *Biochem J* 59, 487-491.

Krisman, C. & Barengo, R. 1975. A precursor of glycogen biosynthesis: alpha-1,4 glucan protein. *Eur J Biochem* 52, 117-123.

Lacombe, V.A., Hinchcliff, K.W., Geor, R.J. & Baskin, C.R. 2001. Muscle glycogen depletion and subsequent replenishment affect anaerobic capacity of horses. *J Appl Physiol* 91, 1782-1790.

Lacombe, V.A., Hinchcliff, K.W., Geor, R.J. & Lauderdale, M.A. 1999. Exercise that induced substantial muscle glycogen depletion impairs subsequent anaerobic capacity. *Equine vet J Suppl* 30, 293-297.

Lacombe, V., Hinchcliff, K.W., Kohn, C.W., Devor, S.T. & Taylor, L.E. 2004. Effects of feeding meals with various soluble-carbohydrate content on muscle glycogen synthesis after exercise in horses. *Am J Vet Res* 65, 916-923.

Lacombe, V.A., Hinchcliff, K.W. & Taylor, L.E. 2003. Interactions of substrate availability, exercise performance, and nutrition with muscle glycogen metabolism in horses. *J Am Vet Med Assoc* 223, 1576-1585.

Lehninger, A.L. 1982. *Principles of biochemistry*. Worth Publishers, Inc., New York. pp 287-289.

Lindholm, A. & Saltin, B. 1974. The physiological and biochemical response of Standardbred horses to exercise of varying speed and duration. *Acta Physiol Scand* 15, 310-324.

Lindholm, A. & Piehl, K. 1974. Fibre composition, enzyme activity and concentrations of metabolites and electrolytes in muscles of Standardbred horses. *Acta Vet Scand* 15, 287-309.

Lomako, J., Lomako, W. & Whelan, W. 1988. A self-glucosylating protein is the primer for rabbit muscle glycogen biosynthesis. *FASEB J* 2, 3097-3103.

Lomako, J., Lomako, W.M. & Whelan, W.J. 1990. The nature of the primer for glycogen synthesis in muscle. *FEBS Lett* 268, 8-12.

Lomako, J., Lomako, W.M. & Whelan, W.J. 1991. Proglycogen: a low-molecular-weight form of muscle glycogen. *FEBS Lett* 279, 223-228.

Lomako, J., Lomako, W.M., Whelan, W.J., Dombro, R.S., Neary, J.T. & Norenberg, M.D. 1993. Glycogen synthesis in the astrocyte: from glycogenin to proglycogen to glycogen. *FASEB J* 7, 1386-1393.

Lowry, O.H. & Passonneau, J.V. 1973. *A flexible system of enzymatic analysis.* Academic Press, New York. pp 1-291.

Marchand I. 2001. A quantitative qnalysis of subcellular distribution of human skeletal muscle glycogen, PhD thesis. *University of Guelph,* Guelph, ON, Canada.

Marchand, I., Chorneyko, K., Tarnopolsky, M., Hamilton, S., Shearer, J., Potvin, J. & Graham, T.E. 2002. Quantification of subcellular glycogen in resting human muscle: granule size, number, and location. *J Appl Physiol* 93, 1598-1607.

Massa, R., Lodi, R., Barbiroli, B., Servidei, S., Sancesario, G., Manfredi, G., Zaniol, P. & Bernardi, G. 1996. Partial block of glycolysis in late-onset phosphofructokinase deficiency myopathy. *Acta Neuropathol* 91, 322-329.

Nimmon, M.A. & Snow, D.H. 1983. *Changes in muscle glycogen, lactate and pyruvate concentrations in the Thoroughbred horse following maximal exercise*. In: Snow, D.H., Persson, S.G.B. & Rose, R.J. (eds.) Equine exercise physiology. Cambridge, UK, Granta Editions. pp 701-706.

Passonneau, J.V. & Lauderdale, V.R. 1974. A comparison of three methods of glycogen measurement in tissues. *Anal Biochem* 60, 405-412.

Pederson, B.A., Csitkovits, A.G., Simon, R., Schroeder, J.M., Wang, W., Skurat, A.V. & Roach, P.J. 2003. Overexpression of glycogen synthase in mouse muscle results in less branched glycogen. *Biochem Biophys Res Commun* 305, 826-830.

Pitcher, J., Smythe, C., Campbell, D.G. & Cohen, P. 1987. Identification of the 38-kDa subunit of rabbit skeletal muscle glycogen synthase as glycogenin. *Eur J Biochem* 169, 497-502.

Pitcher, J., Smythe, C. & Cohen, P. 1988. Glycogenin is the priming glucosyltransferase required for the initiation of glycogen biogenesis in rabbit skeletal muscle. *Eur J Biochem* 176, 391-395.

Roach, P.J. 2002. Glycogen and its metabolism. *Curr Mol Med* 2, 101-120.

Roach, P.J. & Skurat, A.V. 1997. Self-glucosylating initiator proteins and their role in glycogen biosynthesis. *Prog Nucleic Acid Res Mol Biol* 57, 289-316.

Roach, P.J., Cheng, C., Huang, D., Lin, A., Mu, J., Skurat, A.V., Wilson, W. & Zhai, L. 1998. Novel aspects of the regulation of glycogen storage. *J Basic Clin Physiol Pharmacol* 9, 139-151.

Rodriquez, I.R. & Whelan, W.J. 1985. A novel glycosyl-amino acid linkage: rabbit muscle glycogen is covalently linked to a protein via tyrosine. *Biochem Biophys Res Commun* 132, 829-836.

Rose, R.J. & Hodgson, D.R. 1994. *An overview of performance and sports medicine.* In: Hodgson, D.R. & Rose, R.J. (eds.) The athletic horse: Principles and practice of equine sports medicine. W. B. Saunders Company, Philadelphia. pp 3-11.

Schuback, K. & Essén-Gustavsson, B. 1998. Muscle anaerobic response to a maximal treadmill exercise test in Standardbred trotters. *Equine vet J* 30, 504-510.

Sellevold, O.F.M., Jynge, P. & Aarstad, K. 1986. High performance liquid chromatography: a rapid isocratic method for determination of creatine compounds and adenine nucleotides in myocardial tissue. *J mol Cell Cardiol* 18, 517-527.

Serrano, A.L., Petrie, J.L., Rivero, J.L. & Hermanson, J.W. 1996. Myosin isoforms and muscle fiber characteristics in equine gluteus medius muscle. *Anat Rec* 244, 444-451.

Shearer, J. & Graham, T.E. 2002. New perspectives on the storage and organization of muscle glycogen. *Can J Appl Physiol* 27, 179-203.

Shearer, J. & Graham, T.E. 2004. Novel aspects of skeletal muscle glycogen and its regulation during rest and exercise. *Exerc Sport Sci Rev* 32, 120-126.

Shearer, J., Marchand, I, Sathasivam, P., Tarnopolsky, M.A. & Graham, T.E. 2000. Glycogenin activity in human skeletal muscle is proportional to muscle glycogen concentration. *Am J Physiol Endocrinol Metab* 278, E177-188.

Shearer, J., Marchand, I., Tarnopolsky, M.A., Dyck, D.J. & Graham, T.E. 2001. Pro- and macroglycogenolysis during repeated exercise: roles of glycogen content and phosphorylase activation. *J Appl Physiol* 90, 880-888.

Shearer, J., Wilson, R.J., Battram, D.S., Richter, E.A., Robinson, D.L., Bakovic, M. & Graham, T.E. 2005. Increase in glycogenin and glycogenin mRNA accompany glycogen resynthesis in human skeletal muscle. *Am J Physiol Endocrinol Metab* 289, E508-E514.

Sherman, W.M., Costill, D.L., Fink, W.J., Hagerman, F.C., Armstrong, L.E. & Murray, T.F. 1983. Effect of a 42.2 km footrace and subsequent rest or exercise on muscle glycogen and enzymes. *J Appl Physiol* 55, 1219-1224.

Skurat, A.V., Lim, S.S. & Roach, P.J. 1997. Glycogenin biogenesis in rat-1 fibroblasts expressing rabbit muscle glycogenin. *Eur J Biochem* 245, 147-155.

Skurat, A.V., Peng, H., Chang, H.Y., Cannon, J.F. & Roach, P.J. 1996. Rate determining steps in the biosynthesis of glycogen in COS cells. *Arch Biochem Biophys* 328, 283-288.

Smythe, C., Watt, P. & Cohen, P. 1990. Further studies on the role of glycogenin in glycogen biosynthesis. *Eur J Biochem* 189, 199-204.

Snow, D.H., Baxter, P. & Rose, R.J. 1981. Muscle fibre composition and glycogen depletion in horses competing in an endurance ride. *Vet Rec* 108, 374-378.

Snow, D.H., Harris, R.C., Harman, J.C. & Marlin, D.J. 1987 *Glycogen repletion following different diets.* In: Gillespie, J.R. & Robinsson, N.E. (eds.). Equine Exercise Physiology 2. Davis CA, ICEEP Publications. pp 701-706.

Snow, D.H. and Harris, R.C. 1991. *Effects of daily exercise on muscle glycogen in the Thoroughbred horse.* In: Persson, S.G.B., Lindholm, A. & Davis, L.B.J. (eds.) Equine Exercise Physiology 3: Proceedings of the Third International Conference on Exercise Physiology, Uppsala, Sweden 15-19 July 1990. Davis CA, ICEEP Publications. pp 299-304.

Snow, D.H. and Valberg, S.J. 1994. *Muscle anatomy, physiology, and adaptions to exercise and training.* In: Hodgson, D.R. & Rose, R.J. (eds.) The athletic horse: Principles and practice of equine medicine, W. B. Saunders Company, Philadelphia. pp 145-179.

Stetten, M., Katzen, H. & Stetton, D. 1958. A comparison of the glycogen isolated by acid and alkaline procedure. *J Biol Chem* 232, 475-488.

Stryer, L. 1988. *Biochemistry* 3rd edn. W. H. Freeman and Company, New York. pp 449-468.

Valberg, S. 1986. Glycogen depletion patterns in the muscle of Standardbred trotters after exercise of varying intensities and durations. *Equine Vet J* 18, 479-484.

Valberg, S., Cardinett III, G.H., Carlson, G.P. & DiMauro, S. 1992 Polysaccharide storage myopathy associated with exertional rhabdomyolysis in the horse. *Neuromuscul Disord* 2, 351-359.

Valberg, S.J., Townsend, D. & Mickelson, J.R. 1998. Skeletal muscle glycolytic capacity and phosphofructokinase regulation in horses with polysaccharide storage myopathy. *Am J Vet Res* 59, 782-785.

Valberg, S.J., MacLeay, J.M., Billstrom, J.A., Hower-Moritz, M.A. & Mickelson, J.R. 1999. Skeletal muscle metabolic response to exercise in horses with 'tying-up' due to polysaccharide storage myopathy. *Equine vet J* 31,43-47.

Wasserman, D.H. and Halseth, A.E. 1998. *An overwiew of muscle glucose uptake during exercise. Sites of regulation.* In: Richter, E.A., Kiens, B., Galbo, H., *et al*. (eds). Skeletal muscle metabolism in exercise and diabetes. New York, Plenium Press. pp 1-16.

Willstaetter, R. & Rhodewald, M. 1934. Protein binding of physiologically important substances. The condition of glycogen in liver, muscle and leukocytes. *Z Physiol Chem* 225, 103-124.

Acknowledgements

The present study was carried out at the Department of Clinical Sciences, Faculty of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences, Uppsala, Sweden in collaboration with the Department of Clinical Studies, Large Animal Medicine, Ontario Veterinary College, University of Guelph, Canada, and the Department of Veterinary Population Medicine, College of Veterinary Medicine, University of Minnesota, St Paul, Minnesota, USA.

The studies were financed by EP Taylor Equine Trust Fund (Canada), ATG (the Swedish Horserace Totalizator Board, Sweden), the Morris Animal Foundation (USA) and the University of Minnesota Equine Research Centre through funds provided by Minnesota Racing Commission, Minnesota Agricultural Experiment Station and private donors (USA).

Many people have supported and helped me during the years I have been working with the present thesis and I am grateful to all of you. In particular, I would like to express my sincere gratitude to:

Birgitta Essén-Gustavsson, my main supervisor and Associate Professor at the Department of Clinical Sciences, for the privilege to having you as my coach through all these research projects. Your knowledge about muscle metabolism and muscle fibers is enormous. Thank you for your support, guidance, enthusiasm and for all interesting scientific discussions. You are the only person I know of who is able to work with 200 % energy and 300 % enthusiasm at 3 o'clock in the morning and at that time also start brainstorming about a complicated metabolic problem and get every person around the table completely involved. And finally - deadlines could always be pushed forward…

Stephanie Valberg, my assistant main supervisor and Professor at the Department of Veterinary Population Medicine, University of Minnesota for your friendly attitude, constructive criticism, absolutely brilliant intellect, great knowledge about muscle physiology and especially your guidance in the mystery of polysaccharide storage myopathy. Thanks a lot for letting me have access to your interesting research horses with polysaccharide storage myopathy!

Henry Stämpfli, Professor at the Department of Clinical Studies, Ontario Veterinary College and co-author for introducing me to the interesting field of muscle physiology and for stimulating my interest in large animal internal medicine. You have been my clinical advisor during my residency at the Ontario Veterinary College. Thank you for your guidance, interest, your unfailing encouragement and for always keeping me cheerful during all steps in becoming a Diplomate in Large Animal Internal Medicine at the American College of Veterinary Medicine.

Terry Graham, Professor at the Department of Human Biology and Nutritional Sciences, University of Guelph and my co-author for your excellent guidance in the field of pro- and macroglycogen and for your always optimistic and friendly attitude. Your constructive criticism and comments on the manuscripts have always been appreciated. Thanks a lot for letting me have access to your research laboratory.

Robert Jonasson, graduate student at the Department of Clinical Sciences and my co-author and co-worker in the laboratory during study III and IV of this thesis. Your thorough personality has always been appreciated – it is nice to work with a person with the same philosophy.

Susanne Holm, my co-author and co-worker in the laboratory during study IV for your enthusiasm and thoroughness in the laboratory as well as during the studies at Wångens.

Ulf Hedenström, my co-author and colleague for your enthusiasm and help with every practical moment at the study at Wången.

Fia Svensson and **Ida Sandberg** for excellent technical assistance in carrying out the study at Wången.

The rest of the staff and students at the Department of Equine Studies, Trotting and Racing School, Swedish University of Agricultural Sciences, **Wången** for excellent technical assistance during all practical moments. It has been a pleasure to do research at Wången.

Kristina Karlström, the person you can't survive without in the laboratory. Thanks for your guidance in the laboratory and for always keeping an eye on everything in the laboratory – even me!

Kristi Adamo for introducing me to the analytical methods of pro- and macroglycogen.

Agneta Egenwall for statistical advices.

Kjell-Åke Ahlin, for instant and reliable help with fading and failing computers with short notice. I wouldn't be able to produce anything without your help!

All my friends and wonderful colleagues at the Division of Equine Internal Medicine, for enormous support, help and encouragement but most of all for creating a nice familiar environment in the big chaos at the University during all cutbacks and reorganisations. **Agneta**, my co-pilot in clinics (who unfortunately left us for the excitement of all those drugs on the market) for sharing triumphs and disasters in the clinics and for having that nice personality and clinical knowledge that makes you a wonderful colleague to work with (miss you in clinics!). **Susy**, wonderful co-pilot in clinics with an enormous experience in the area of internal medicine but also my most important neighbour. If it weren't for

you – I would not be able to make the last deadline with the thesis. Spontaneously suggesting that you could take my on calls and one week in clinics despite that you had started your stud farm season so I could finish the last rush with the thesis was absolutely fantastic. **Miia** (a very good friend and an excellent internist) acted the same way and suggested that she could do clinics for me in January so I could finish writing (despite her own heavy workload). **Kia** (who left clinics temporary for doing research and producing kids) for your support and concerns with everything from clinical work to my family. Our residents **Anna** and **Sara** for your thoroughness, enthusiasm, encouragement and support in every moment of my time at work and for your interest in internal medicine – it makes my work really fun. **Pia**, who left us in clinics and now will start working at ATG, for your enthusiasm and support throughout the years – it is impossible to be in a bad mood when you are around! **John**, Professor in equine internal medicine, for your suggestion to do a residency in North America and your inspiring way of teaching students – I have learned so much from you.

The technical staff from equine internal medicine for taking care of my patients (and me!) with encouragement and for all fun moments, friendship and laughs throughout the years despite cutbacks and reorganisations; **Anders** and **Kenneth** (would I survive at work or on my farm without your support and care?), **Jennie** (in alphabetic order), **Malin**, **Ylva**, **Ulrika** and those who have left; **Cattis**, **Eva**, **Ingela**, **Lina** and **Stina**.

.

Lars Nordenberg, my colleague, very good friend and mentor in hunting for, together with your wonderful wife **Ansi,** helping me out with somewhere to live when I once started my internship in internal medicine (I did not know at the time but that was the initiating phase that started my interest in research despite my convincing statements that "I will never do research"). I really miss our discussions about veterinary medicine, hunting, politics and episodes in our lives that usually, together with a wonderful and expensive glass of brandy, accompanied our nice philosophic moments during the nights at Skediga gård. I admire both of ours wives who are able to overlook our unbelievable "time optimism" (Thanks Ansi and Caro!).

Claes Friberg, my very skilled mentor in farming and very good friend throughout the years for your support and help with everything that matters Eric-Andersgården. You are one of the persons that knows that the stories about breaking in to Läckö Castle "by mistake" and other really crazy stories where you and I have been involved actually are true!

Inger and **Gudmar**, my parents, for your support in all periods of my life and for always stimulating academic studies. **Anders** and **Anna** for being my brother and sister.

Eric-Andersgården in Arnebo village for letting my family being part of your history. It is fascinating to know by name every resident on the farm since 1626.

Last, but most important, my family. **Caroline**, my wife and very best friend for being the most important person in my life together with our kids. Thank you for always believing in me and for your love, help, support, encouragement but most of all for being my wife. **Rebecka** and **Kristoffer** for giving me the most wonderful moments in life and for always reminding me what the most important things in life are. Nothing would have been possible without you – nothing at all!