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SUBSTRATES FOR MUSCLE GLYCONEOGENESIS DURING PROLONGED SWIMMING AND RECOVERY IN RATS

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Thesis submitted to the School of Graduate Studies and
Research in partial fulfillment of the requirements
for the degree of Master of Science

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ABSTRACT

The goal of this work was to initiate a systematic investigation of the possible substrates for muscle glyconeogenesis under physiological circumstances - during recovery from prolonged submaximal exercise. The hypothesis was that amino acids mobilized from possibly increased body protein breakdown (in particular muscle protein degradation), or glycerol liberated from catabolism of body fat (especially intramuscular fat) during and after exercise might serve as substrates for muscle glyconeogenesis after exercise.

In order to assess whole body protein breakdown, muscle myofibrillar protein degradation during and after exercise as well as to determine the incorporation of putative substrates into muscle glycogen during postexercise recovery, twelve-hour-fasted rats were infused intravenously with: a) ^{14}C-U-urea, b) ^{14}C-U-threonine (a representative of gluconeogenic amino acids) and ^{3}H-6-glucose, and c) ^{14}C-U-glycerol and ^{3}H-6-glucose throughout two-hour basal, four-hour swim (or rest) and three-hour postexercise recovery periods. Arterial blood samples were taken every hour. Soleus, white and red gastrocnemius muscles were excised and assessed for 3-methylhistidine (3-MH) and tyrosine contents as well as for total and radiolabelled glycogen contents at the end of the postexercise recovery.

The results indicate that: 1) whole body protein breakdown and muscle myofibrillar protein degradation increase significantly during and after prolonged swimming in white and red gastrocnemii as evidenced by an increased rate of urea production and increased 3-MH level in blood and in these muscles, respectively; 2) glyconeogenic amino acids released from increased body proteolysis appear to serve as

substrates for glyconeogenesis after exercise in at least red gastrocnemius muscle, as demonstrated by the fact that nearly 11% of the label arising from ^{14}C-U-threonine and incorporated into muscle glycogen could be accounted for by muscle glyconeogenesis; and 3) circulating glycerol does not play a role in muscle glyconeogenesis.

Increased protein breakdown was demonstrated during the recovery from prolonged submaximal exercise, precisely in those muscle groups where glyconeogenesis was demonstrated. These therefore suggests a linkage between these two processes. The linkage is strengthened by the suggestion that a labelled amino acid (threonine) can be incorporated into muscle glycogen without a gluconeogenic hepatic conversion to glucose.

DEDICATION

This thesis is dedicated to

my husband Jingming Li and my parents.

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Finally, I like to thank all of my colleagues in this laboratory for their help and friendship.

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CHAPTER ONE

INTRODUCTION

1.1. MUSCLE GLYCONEOGENESIS AND ITS PRECURSORS

1.1.1. Introduction

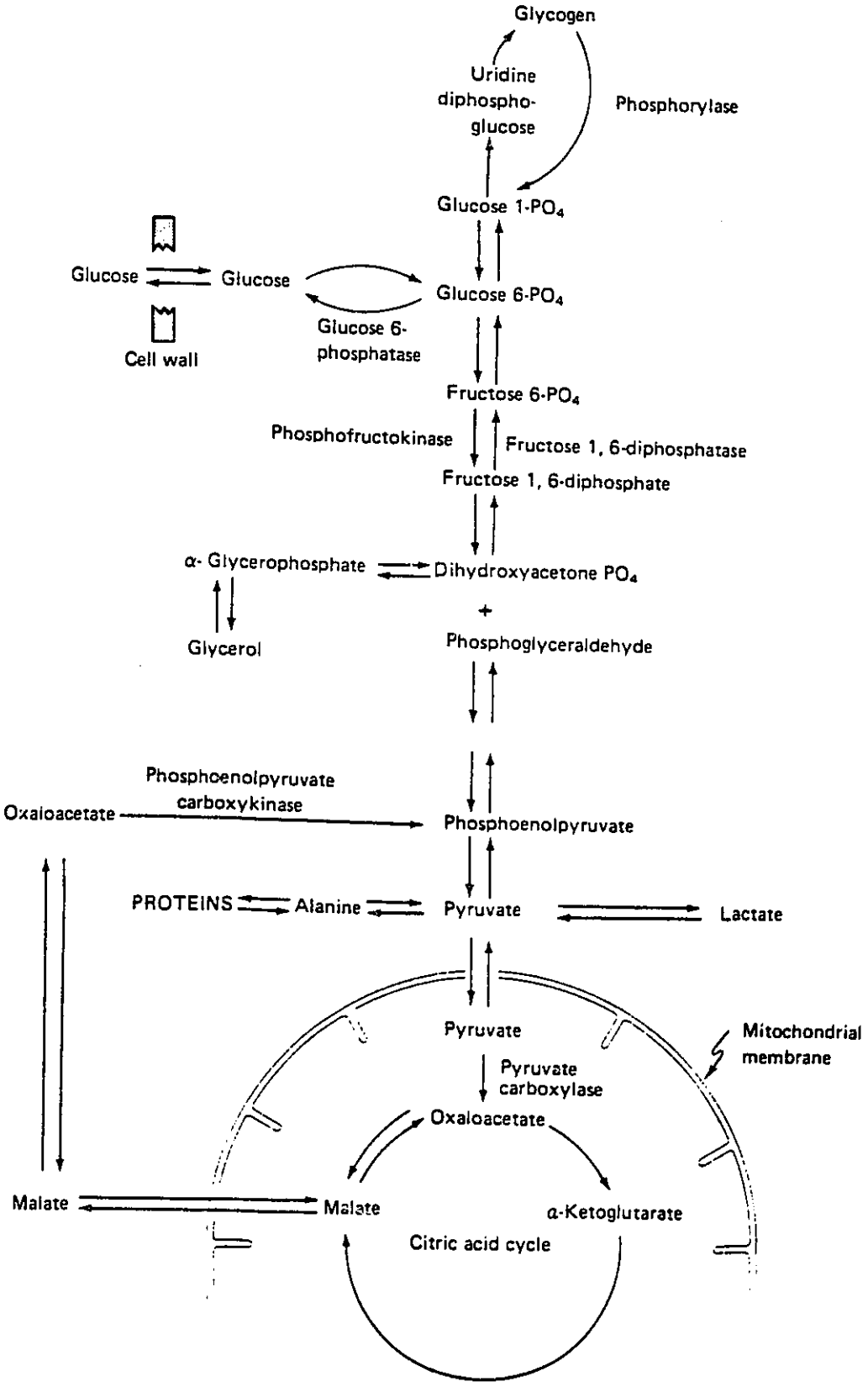
Glycogen is a storage form of glucose linked by α-1,4- and α-1,6-glycosidic bonds. It is present in most body tissues, but the major storage sites of glycogen are the liver and skeletal muscle. Since skeletal muscle contributes nearly 45% of the body weight, it contains much more glycogen than liver although the concentration of glycogen in liver is much higher than that in skeletal muscle. The physiological importance of glycogen is that it is a crucial source of fuel for energy required during strenuous muscle activity. Liver glycogen is a source of blood glucose, and it may be gradually depleted during starvation or rapidly mobilized in response to exercise or hypoglycaemia (Fell et al., 1980; Dohm et al., 1983). Muscle glycogen cannot contribute directly to glucose, since muscle lacks the enzyme, glucose-6-phosphatase which liberates glucose into blood circulation (Opie and Newsholme, 1967). Muscle glycogen is the primary and immediate energy source for muscle contraction and work performance (Hultman, 1967; Hultman and Bergstrom, 1973). Work performance and fatigue endurance have been shown to be directly related to muscle glycogen contents (Ahlborg et al., 1967; Bergstrom et al., 1967; Hermansen et al., 1967; Saltin, 1981; Johnson and Bagby, 1988). The importance of resolving metabolic pathways, substrates and the regulation of muscle glycogen synthesis is not only that muscle glycogen provides a reservoir of energy, but also because muscle

glycogen synthesis may be intimately related to the metabolism of protein, amino acids and fat.

Glycogen is synthesized and degraded by different pathways. The breakdown of glycogen is *glycogenolysis* in which glycogen is cleaved by the action of the enzyme, glycogen phosphorylase (upon stimulation by hormones such as epinephrine and glucagon) to glucose-1-phosphate which is then metabolized through glycolysis (the conversion of glucose to pyruvate or lactate with the production of ATP) and the citric acid cycle to release energy in the form of ATP which is required for muscle contraction. Significant muscle glycogenolysis occurs during exercise, the performance of work and starvation (Fell et al., 1980; Favier et al., 1987). In opposition to the glycogenolysis, *glycogenesis* refers to the formation of glycogen. It takes place during the post-absorptive period and during postexercise recovery. Glycogenesis consists of two different processes - direct glycogen synthesis in which glycogen is synthesized from the direct uptake of plasma glucose and *glyconeogenesis* which is defined as the synthesis of tissue *glycogen* from non-carbohydrate precursors, such as lactate, glycerol and gluconeogenic amino acids (alanine, glutamine, threonine, etc), via the glyconeogenic pathway (non-carbohydrate substrates → 3-carbon substrate pool → glucose-6-phosphate → glycogen). In comparison with glyconeogenesis, *gluconeogenesis* is defined as the formation of *glucose* from non-carbohydrate precursors (non-carbohydrate substrates → 3-carbon substrate pool → glucose-6-phosphate → glucose). It only takes place in liver and kidney cortex, but not in muscle itself since this tissue is not generally considered to be a gluconeogenic organ. Except in prolonged starvation during which the kidney produces

a small amount of glucose (Dohm et al., 1969), the liver is assumed to be the sole site of glucose production. The role of gluconeogensis is to maintain the plasma glucose level to meet the glucose requirement of the brain and erythrocytes, etc during the postabsorptive period and to provide additional fuel during exercise. It has been recently shown that gluconeogensis may play a role in the resynthesis of muscle glycogen during fasting postexercise recovery (Fell et al., 1980; Ryan and Radziuk, 1994). In liver, it is well documented that hepatic glycogen can be formed either by the direct pathway from glucose or by glyconeogenesis from gluconeogenic precursors (Newgard et al., 1983; Radziuk, 1979, 1982, 1989). Hepatic glyconeogenesis continues to take place even during glucose infusion and carbohydrate feeding when there is adequate glucose available to replenish glycogen via the direct pathway (Radziuk, 1982; Newgard et al., 1984). In muscle, however, the existence of glyconeogenesis is less clear. Whether muscle has the capacity to synthesize glycogen from precursors other than glucose remained incompletely resolved for many years. The possibility of muscle glyconeogenesis has been long established, especially during exogenous lactate supplementation (eg, Johnson and Bagby, 1988). The most recent evidence indicates that muscle glycogen is synthesized not only from glucose but also partially from non-carbohydrate precursors under physiological conditions during recovery from prolonged submaximal exercise in rats (Ryan and Radziuk, 1994). However, the substrate(s) involved in this muscle glyconeogenesis are not completely identified. Thus, it is the goal of this work to investigate substrates which may contribute to muscle glycogen formation via the process of glyconeogenesis *in vivo* during postexercise recovery.

Figure 1.1. Schematic of glyconeogenic and gluconeogenic pathways in liver, and the entry of the gluconeogenic precursors into these pathways.


Glycogen
Uridine diphospho-glucose
Phosphorylase
Glucose 1-PO4
Glucose
Glucose
Glucose 6-PO4
Glucose 6-phosphatase
Cell wall
Fructose 6-PO4
Phosphofructokinase
Fructose 1, 6-diphosphatase
Fructose 1, 6-diphosphate
α- Glycerophosphate
Dihydroxyacetone PO4
Glycerol
Phosphoglyceraldehyde
Phosphoenolpyruvate carboxykinase
Oxaloacetate
Phosphoenolpyruvate
PROTEINS
Alanine
Pyruvate
Lactate
Pyruvate
Mitochondrial membrane
Pyruvate carboxylase
Oxaloacetate
Malate
Malate
α-Ketoglutarate
Citric acid cycle

The pathways of glyconeogenesis and gluconeogenesis, as well as the entry points of substrates into these pathways in liver are shown in Figure 1.1, Pathways of glyconeogenesis and gluconeogenesis in liver. Key enzymes are included. The pathway(s) of muscle glyconeogenesis, however, remains more obscure.

1.1.2. Muscle Glyconeogenesis

Muscle glyconeogenesis was first reported by Meyerhof and co-workers who demonstrated the significant conversion of lactate into muscle glycogen in perfused frog hindlimbs in the early 1900's (Meyerhof, 1920; Meyerhof et al., 1922). He claimed that a major fraction (80%) of lactate formed in muscle was converted directly into muscle glycogen within muscle and only 20% of lactate underwent oxidation generating CO_2. Thereafter, two separate lines of evidences had led to conflicting conclusions about muscle glycogen. Hill et al. (1924) and Cheosakul et al. (1926) supported Meyerhof's view by showing that glycogen was formed from lactate in frog hindlimbs and hepatectomized rats. In the meantime, however, reports which did not suggest muscle glyconeogenesis were made by a number of investigators who had failed to detect any glycogen formation from lactate in various animal muscles (eg. Eggleton and Evants, 1930; Sacks and Sacks, 1935). Based on *in vivo* studies, Cori et al. (1929) and Himwich et al. (1930) concluded that skeletal muscle had no capacity of glyconeogenesis and that lactate must first escape from muscle into blood circulation, be carried to liver, and be converted to glucose. Then this glucose is transferred to muscle and is ultimately converted to glycogen. Later studies further dismissed muscle glyconeogenesis based on

the observations that three key enzymes (pyruvate carboxylase, phosphoenolpyruvate carboxykinase and fructose 1,6-diphosphatase) required for generating glycogen from lactate were never found together in the same muscle (Krebs, 1964; Krebs and Woodford, 1965; Opie and Newsholme, 1967; Crabtree et al., 1972). Due to these conflicting results, the controversy around muscle glyconeogenesis remained an unsettled issue for over fifty years. At that time, it was still generally accepted that the conversion of glucose into glycogen is the only route of glycogen synthesis in muscle.

In 1970, however, Bendall and Taylor (1970) reinvestigated this problem and showed an increased glycogen formation from lactate in muscle. This led to many subsequent investigations on muscle glyconeogenesis. In 1977, the first such human study was done by Hermansen and Vaage (1977) using A-V flow techniques across the leg (Hermansen and Vaage, 1977). Their results showed that a rapid disappearance of accumulated muscle lactate coincided with a rapid glycogen resynthesis in muscle during postexercise recovery. Only 10% of this lactate effluxed from muscle to the blood circulation. At the same time, glucose uptake by the leg during recovery could account for less than 5% of the glycogen synthesized. Moreover, although 90% of the lactate had been metabolized within the muscle itself, only 15% of the lactate metabolized in muscle was oxidized to CO_2. Thus, the remaining 75% of lactate must have been converted into glycogen within muscle. However, at this point, all evidence that support muscle glyconeogenesis was indirect. In 1979, the first direct evidence of muscle glyconeogenesis was obtained by Mclane and Holloszy (Mclane et al., 1979) *in vitro* by using an isotope tracer study. They had demonstrated the significant increase of glycogen formation in

perfused fast-twitch muscle of rats when lactate was the only perfusing substrate and also detected the direct incorporation of ^{14}C-lactate into muscle glycogen. Their results also indicated that glyconeogenic capacity was correlated with the presence of the appropriate enzymes. Subsequently, several studies confirmed that muscle does indeed have a capacity to synthesize glycogen from lactate *in vitro* by using labelled lactate experiments (eg. Shiota et al., 1984; Stevenson et al., 1987; Talmadge et al., 1989; Bonen et al., 1990).

Although these studies demonstrated that muscle glyconeogenesis occurred in muscle, all these results were obtained from *in vitro* experiments in which muscle glycogen was depleted by pre-exercise or pre-stimulation. However, whether this muscle glyconeogenesis could take place *in vivo* remained unknown. Hermansen and Vaage (1977) had provided strong evidence of muscle glyconeogenesis *in vivo* during postexercise recovery, and Hultman et al. (1986) reported that only 10% of lactate formed during exercise was taken up by liver and maximally 40% was oxidized. A further 7% remained as part of the lactate pool. The remainder (40%) of lactate must therefore be converted into glycogen within muscle itself. All this evidence however was indirect. In contrast to above conclusions, Gaesser and Brooks (1980) reported that the incorporation of label from ^{14}C-U-lactate into muscle glycogen was stimulated by exercise but accounted for less than 20% of lactate removal during the postexercise recovery. Most of the infused ^{14}C-lactate was collected as $^{14}CO_2$. Thus, they concluded that oxidation is the major fate of lactate removal after exercise. Futre et al. (1987) confirmed Brooks' findings. Their results however did not entirely rule out the possibility of direct conversion of lactate into muscle glycogen.

In 1988, Johnson and Bagby (1988) provided the first direct evidence of incorporation of ^{14}C-lactate into muscle glycogen via muscle glyconeogenesis (as distinct from that of ^{14}C-glucose arising from hepatic gluconeogensis) *in vivo* during postexercise recovery in white muscle of rats. However, this muscle glyconeogenesis only occurred when the plasma lactate level was maintained high by exogenous infusion of lactate or glucose. Thus, they concluded that participation of the glyconeogenic pathway in glycogen synthesis within muscle *in vivo* is dependent on a sustained period of high lactate delivery. In their study, infusions of double isotope tracers, ^{3}H-6-glucose and ^{14}C-U-lactate, into exhausted rats from a treadmill running along with a load: 1) saline; 2) glucose; 3) lactate, were used. Since ^{3}H label originating in ^{3}H-6-glucose is lost to water when ^{3}H-labelled C-3 precursors are incorporated into glycogen through the glyconeogenic pathway, the contributions of lactate carbon into muscle glycogen via glyconeogenic pathway were assessed qualitatively by comparing the ^{3}H/^{14}C ratio in plasma glucose to the same ratio in muscle glycogen. If the only source of muscle glycogen is plasma glucose, this ratio in glycogen will be the same as that in plasma glucose. However, significant incorporation of ^{14}C-lactate into muscle glycogen via muscle glyconeogenic pathway will result in an enhancement of ^{14}C relative ^{3}H in muscle glycogen. A lower ^{3}H/^{14}C ratio in muscle glycogen relative to that found in plasma glucose would therefore result. Indeed, their results showed a significant lowering of ^{3}H/^{14}C ratios in glycogen from white gastrocnemius muscle from rats which were loaded with lactate or glucose during recovery from exercise, but not in those which were saline loaded (as compared with the ^{3}H/^{14}C ratio in plasma glucose). The requirement of high lactate concentration

for muscle glyconeogenesis is consistent with that observed in *in vitro* studies in which muscle glyconeogenesis was found to occur (Mclane et al., 1979; Shiota et al., 1984; Stevenson et al., 1987; Talmadge et al., 1989). Wasserman et al. (1991) claimed that only when circulating lactate concentration was high, intramuscular lactate level would be retained and be available for muscle glyconeogenesis. If extracelluar lactate concentration is low, intracellular lactate would be able to diffuse out. These investigations suggested that high substrate availability is necessary for eliciting muscle glyconeogenesis.

The existence of muscle glyconeogenesis was thus demonstrated *in vitro* and *in vivo* under conditions of high lactate, a non-physiological circumstance. Whether it can take place *in vivo* under physiological circumstances remained an open question. The most recent work done by Ryan and Radziuk (1994) has for the first time demonstrated that significant muscle glyconeogenesis indeed occurs in white and red gastrocnemius muscles *in vivo*, under a particular set of physiological circumstance - during fasting recovery in rats from prolonged submaximal exercise which is characterized as low intensity exercise with minimal lactate accumulation in plasma and muscle (Favier et al., 1987; Ryan et al., 1993). In this study, a double isotope tracer method was used. Since lactate formed in muscle during exercise may not equilibrate well with that in circulation, ^{14}C-lactate infusion into the circulation may only track glyconeogenesis in muscle from systemic lactate. Thus, in addition to ^{14}C-lactate, a ^{14}C-1-glucose infusion was also used to efficiently label the intramuscular lactate pool and to trace any glycogen formation from intramuscular glyconeogenic precursors. The tracer strategy included: 1) infusion of ^{14}C-lactate throughout entire study in which rats swim for four hours and then recover for

three hours to determine if circulating lactate enters muscle glycogen via the muscle glyconeogenic pathway; and 2) the infusion of ^{14}C-1-glucose throughout the study to label the muscle 3-carbon substrate pool and to examine any contribution of substrates in this pool to muscle glyconeogenesis. ^{3}H-6-glucose was infused concurrently with ^{14}C-label and used to correct for the ^{14}C-glucose (from ^{14}C-lactate or recycled) which is directly taken up into muscle from the circulation. The glyconeogenic formation of glycogen in muscle from circulating lactate and from any precursors in muscle 3-carbon pool were effectively estimated by comparing ^{3}H/^{14}C ratio in plasma glucose to that in tissue glycogen and by comparing ^{3}H/^{14}C-6 ratio in plasma glucose to that in muscle glycogen, respectively. Their results showed that ^{14}C-lactate in the circulation was not incorporated into muscle glycogen, whereas the ^{14}C-6-glucose-P (arising locally from ^{14}C-1-glucose via muscle glyconeogenic pathway) was incorporated into glycogen in white and red gastrocnemius muscles and up to 25% of new glycogen was synthesized by muscle glyconeogenesis. Moreover, it was suggested that lactate produced in muscle may be used locally without equilibrating with circulating lactate.

With the demonstration of glyconeogenesis in muscle under physiological conditions, the emphasis shifted from its existence to its substrates. The questions as to what are the substrates involved in muscle glyconeogenesis under this particular set of physiological circumstances and to what extent individual substrate contributes to muscle glycogen via muscle glyconeogenic route have not been investigated.

1.1.3. Possible Substrates For Muscle Glyconeogenesis

I. Introduction

It is well known that in liver, the gluconeogenic and glyconeogenic pathway involve the process: gluconeogenic precursors → 3-carbon substrate pool → G-6-phosphate → glucose or glycogen. Thus, the 3-carbon pool is the most important substrate pool for hepatic glyconeogenesis. It has also been demonstrated that muscle glycogen can also arise from a 3-carbon pool endogenous and exogenous to muscle *in vivo* (eg. Hermansen and Vaage, 1977; Hultman, 1986; Favier et al., 1987; Johnson and Bagby, 1988; Ryan and Radziuk, 1994). In particular, Ryan has shown an incorporation of ^{14}C into muscle glycogen from labelled muscle 3-carbon substrate pool by using label recycled from ^{14}C-1-glucose. This evidence indicates that the muscle 3-carbon pool is involved in muscle glyconeogenesis. Therefore, substrates which can contribute to the 3-carbon substrate pool are expected to be the possible muscle glyconeogenic substrates. They are most likely to be lactate, glycerol and gluconeogenic amino acids since prolonged exercise results in not only an accumulation of lactate, but also the utilization of body fat and protein. Lactate and gluconeogenic amino acids are known to contribute to the 3-carbon pool through pyruvate, and glycerol contributes to the 3-carbon pool via triose-phosphates.

Muscle glyconeogenesis has only been shown to occur during recovery from exercise (Ryan and Radziuk, 1994). This suggests that exercise must in some way affect or elicit glyconeogenesis in muscle. However, various studies also indicated that high substrate availability induces glyconeogenesis in muscle (Stevenson et al., 1984; Johnson and Bagby, 1988; Wasserman et al., 1991). Therefore, most likely, the effects of exercise

and additional substrate(s) on muscle glyconeogenesis are synergistic. Based on these observations, to qualify as a potential substrate for muscle glyconeogenesis during recovery from prolonged submaximal exercise, metabolites might be characterized by: firstly, an increased availability in the circulation and/or especially in muscle; secondly, an ability to contribute to the muscle 3-carbon pool.

II. Lactate As A Possible Substrate For Muscle Glyconeogenesis

The history of the existence of muscle glyconeogenesis is, in fact, a history of lactate as a substrate contributing to muscle glycogen via glyconeogenic pathway. Since Meyerhof 's first demonstration of conversion of lactate into muscle glycogen in perfused muscle, lactate has been implicated as a major substrate for muscle glyconeogenesis *in vitro* (eg. Mclane et al., 1979; Bonen et al., 1991), and *in vivo* (eg. Hultman, 1986; Johnson and Baage, 1988) when circulating lactate was maintained at a high concentration. Ryan and Radziuk have recently demonstrated that significant muscle glyconeogenesis takes place in white and red gastrocnemius muscle during a fasting recovery from prolonged submaximal exercise without maintaining high circulating lactate concentration by exogenous administration (Ryan and Radziuk, 1994). Whether lactate, itself is a major glyconeogenic substrate for muscle glyconeogenesis in this case is still not proven. In their study, there was no direct evidence of incorporation of lactate into muscle glycogen when ^{14}C-lactate was infused intravenously. In fact, it appears that lactate in circulation may not equilibrate well with the intramuscular 3-carbon pool which may be available for muscle glyconeogenesis.

It has been well documented that prolonged submaximal exercise only causes a minimal lactate accumulation in both plasma and muscle (Brooks et al., 1973; Fell et al., 1980; Favier et al., 1987). Consistent with this, Ryan has shown a maximally two-fold rise in plasma lactate concentration during the first hour of swimming and thereafter a gradually return to pre-exercise level after three hours of recovery (Ryan et al., 1993). This seems to negate the possibility of lactate serving as a significant glyconeogenic precursor in muscle after prolonged submaximal exercise. However, in their study, Ryan and Radziuk (1994) did provide evidence which implicates a 3-carbon pool equilibrating with intracellular lactate as a potential substrate for muscle glyconeogenesis under their experimental condition. Firstly, this demonstration was based on the recycling of ^{14}C label from ^{14}C-1-glucose to the C-6 position of the glucosyl unit of glycogen in muscle. Brooks et al. (1980) has reported that there is some exchange of label between lactate and the amino acid pool in muscle after exercise, but the majority of the label should remain in lactate and then undergo muscle glyconeogenesis. Secondly, with a lactate load infusion during recovery, glycogen synthesis was found to be dramatically increased (Ryan, thesis). In addition, labelled glycogen made by muscle glyconeogenesis was increased from 25% to 50% of total new labelled glycogen synthesis when a lactate load was administered during recovery. These data strongly suggest that lactate is a substrate involved in muscle glyconeogenesis after prolonged submaximal exercise. However, in light of the minimal lactate accumulation in muscle during prolonged submaximal exercise and recovery, the potential importance of other substrates for muscle glyconeogenesis becomes an issue.

III. Glycerol As A Possible Substrate For Muscle Glyconeogenesis

- Is additional glycerol available during and after prolonged low intensity exercise?

It is well known that oxidation of body fat in adipose tissue and muscle provides the primary energy for muscular contraction during prolonged exercise (Holloszy et al., 1978; Newsholme, 1983). Studies employing either normal prolonged exercise (Carlson et al., 1971; Froberg, 1971; Reiman et al., 1973; Baldwin et al., 1973; Spriet et al., 1985) or electrical stimulation of muscle in situ (Barclay and Stainsby, 1972) have shown that endogenous muscle triglyceride stores can be decreased or depleted by contractile activity to provide energy. Indirect evidence suggested that intramuscular triglycerides may provide approximately 50% to 75% of the total free fatty acids (FFA) oxidized during prolonged exercise (Havel et al., 1967; Issekutz and Paul, 1968). The hydrolysis of stored triglycerides during prolonged exercise liberates not only FFA for beta-oxidation and aerobic energy metabolism to supply energy for muscle activity, but also release glycerol, a highly gluconeogenic substrate. The increased rate of hydrolysis of triglycerides by exercise can be further enhanced by starvation (Terblanche et al., 1981; Fell et al., 1983). In his study, Terblanche showed that overnight fasting resulted in an approximately two-fold increase in glycerol concentration in plasma, muscle and liver. In response to prolonged swimming, glycerol concentration increased further by a factor of two. After exercise, plasma glycerol level gradually returned to a resting fasting range which was still significantly elevated as compared with that in fed rats after three hours of recovery. Furthermore, he concluded that muscle and liver had the capacity to actively take up glycerol from the circulation. This was based on the observation that muscle and liver

glycerol concentrations were two- to three-fold higher than that in the blood after a glycerol feeding following prolonged swimming. Nevertheless, Havel et al. (1967) reported a large utilization of intramuscular triglycerides during prolonged exercise and an insignificant release of glycerol from muscle following exercise. Therefore, there is undoubtedly a substantial increase in glycerol concentration in plasma and especially muscle during prolonged submaximal exercise and recovery.

-Can glycerol serve as a substrate for muscle glyconeogenesis after exercise?

Glycerol is a highly gluconeogenic substrate and has been reported to be the second to lactate in importance as a gluconeogenic precursor during prolonged exercise (Ahlborg et al., 1974). Exercise results in a significant accumulation of glycerol in plasma and muscle. The possibility that glycerol as a carbon source contributes to muscle glycogen via the muscle glyconeogenic pathway, therefore, exists. Two studies have considered the contribution of glycerol to muscle glycogen resynthesis after exercise. Neither found a significant contribution (Terblanche et al., 1981; Favier et al., 1987). In Terblanche's study, muscle glycogen was depleted by prolonged swimming, the animals were then maintained on a low carbohydrate diet overnight. The experiments examining direct incorporation of ^{14}C-glycerol into muscle glycogen were performed in perfused hindquarter on the next day. Since there was no significant incorporation of ^{14}C into muscle glycogen under either physiological or very high concentration of glycerol, they concluded that muscle has no capacity to synthesize glycogen from glycerol. These studies were done *in vitro* and after a significant delay post-exercise. The potential of

muscle glyconeogenesis from glycerol *in vitro* during the recovery from exercise was not therefore completely eliminated. In another study, Favier and co-workers examined muscle glycogen resynthesis during a fasting recovery immediately after exhaustive swimming. Muscle glycogen resynthesis after exercise was not inhibited by pharmacological blockade of lipolysis (propranolol or nicotinic acid given immediately after exercise), but was significantly decreased by dichloroacetate (DCA) which reduce pyruvate concentrations by stimulating pyruvate oxidation (activating pyruvate dehydrogenase), they concluded that glycerol does not play a major role in muscle glycogen repletion during postexercise recovery. These studies however do not focus specifically on muscle glyconeogenesis since they inhibit the formation of substrates for both hepatic glucose and muscle glycogen. Whether the substrate at the level of the muscle is glucose or gluconeogenic substrate was not investigated. Glycerol levels, moreover, remain elevated in spite of the blockade of lipolysis. The specific role of this molecule is therefore difficult to evaluate.

The entry points of glycerol into the glyconeogenic pathway would be either by phosphorylation to glycerol-3-phosphate, and subsequent dehydrogenation to dihydroxyacetone-phosphate or by oxidation to lactate. The former pathway is energetically more favourable. Both pathways require glycerol kinase activity for the initial phosphorylation step. As is well known, muscle cannot metabolize glycerol because of the lack of significant glycerol kinase. However, it has been reported that the activity of glycerol kinase in muscle is significantly enhanced after prolonged exhaustive exercise (Newsholme and Taylor, 1969). This again suggests that glycerol mobilized from the hydrolysis of triglycerides during exercise could potentially be able to contribute to

muscle glycogen via the glyconeogenic pathway within muscle.

IV. Gluconeogenic Amino Acids As Putative Substrate For Muscle Glyconeogenesis

-Are additional free amino acids available during and after prolonged low-intensity exercise?

A. Whole-body Protein Metabolism During and After Exercise.

The issue whether or not exercise stimulates whole-body net protein breakdown has been investigated for over a century, yet it has not been completely resolved. Possible reasons for the widely varying conclusions concerning protein metabolism in response to exercise may include the consideration of the age, sex, evaluation model, experimental conditions, nutrition status, training status and the type, duration and intensity of the exercise, as well as the time at which metabolism was evaluated (Dohm, 1986; Lemon, 1987).

Protein and amino acid metabolism during exercise has not received much attention since the traditional view holds that protein does not provide a significant portion of the energy during exercise. This was based on many early investigations which failed to observe significant change in urinary nitrogen excretion during exercise (see ref cited in Cathcart, 1925). More recently, Astrand et al. (1970) has supported this traditional view by the finding that urinary nitrogen excretion was not increased during muscular work in human subjects. Since 1976, however, a number of studies have suggested that whole-body protein catabolism, in fact, is increased in strenuous exercise. The evidence has been derived from three lines of investigation. Most early studies have focused on the

effects of exercise on urea excretion since most of the nitrogen released as a result of protein catabolism is incorporated into the ornithine cycle to form non-toxic urea, which is considered to be the final product of the nitrogen disposal pathways. The rate of urea synthesis (production), therefore, is considered to be a true indicator of net protein catabolism. Moreover, under most circumstances, the majority of urea is excreted in urine (Cerny et al., 1975), hence its urinary excretion rate closely reflects its rate of production (Jahoor and Wolfe, 1987). In contrast with the traditional view, Haralambie and Berg, 1976) showed a dramatic increase in plasma urea concentration during prolonged intensive running in human subjects, beginning after 60 to 70 minutes of exercise. This increase was much greater than that could be accounted for by reduced urea removal due to any decrease in kidney function. Moreover, they found plasma tyrosine, an essential amino acid that is not metabolized by muscle tissue, was elevated during exercise and its concentration increased progressively with the duration of exercise. Therefore, they concluded that whole-body protein breakdown increased in prolonged exercise. Rennie et al. (1981) examined protein metabolism in humans during moderate, long-lasting treadmill running. Their results indicated a gradual rise in plasma urea concentration throughout the exercise period and this urea concentration remained close to the value obtained at the end of exercise for at least five hours after exercise. Subsequently, there have been a substantial number of reports that showed urea excretion was increased in humans after extended skiing, running and cycling (Refsum et al., 1979; Haralambie and Senser, 1980; Decombaz et al., 1979; Calles et al., 1984), and in rats after exhaustive running at various intensities (Dohm et al., 1982b; Dohm, 1983, Dohm et al., 1985).

Meanwhile, several studies showed that urea excretion did not change during a number of exercise protocols in trained subjects (Consolazio et al., 1975; Marable et al., 1979; Butterfield and Calloway, 1984). From these reports, Dohm (1986) have concluded that there are transient increases in urea excretion during and after a single strenuous bout of exercise (acute exercise or a competitive event), whereas nitrogen excretion over a prolonged period of training is not increased. This is consistent with the observation that athletes in training do not continue to lose muscle mass (Gantzea et al., 1975). In addition, urea excretion was found to increase with exercise intensity and duration (Lemon et al., 1984).

Net protein breakdown reflected by urea production is a function of whole-body protein synthesis and protein catabolism. Also, urea production was estimated by urea excretion which may have been reduced by decreased kidney clearance of urea, irrespective of urea production during and after exercise (Refsum et al., 1974; Rennie et al., 1981; Poortmans, 1984). Furthermore, urea synthesis may even be inhibited by exercise (Metz et al., 1968; Wolfe et al., 1982). These observations suggest that actual protein catabolism in response to exercise may be underestimated by measuring urea excretion. Therefore, a number of studies have used isotope tracer methods (1-^{13}C-Leucine infusion) to investigate the effects of endurance exercise on amino acids and protein metabolism (White and Brooks, 1981; Wolfe et al., 1982, Hagg et al., 1982; Evans et al., 1983; Booth and Watson, 1985; Dohm et al., 1982a; Laurent and Milward, 1980; Meredith et al., 1989, Wolfe, 1984, Fielding et al., 1991). In such studies, whole-body protein catabolism and synthesis during exercise were estimated from the oxidation and

the fluxes of leucine, an essential amino acid that can only arise from body protein breakdown in the fasting state and was reported to be taken up by exercising muscle (Ahlborg et al., 1974). All these studies demonstrated striking increases in the rate of oxidation of leucine. Although leucine oxidation was significantly increased, leucine concentration was not changed (Rennie et al., 1981; Dohm et al., 1985; Wolfe, 1884; Hagg et al., 1982). The amount of leucine oxidation was greater than the total free leucine in the body (Dohm et al., 1985) and thus the only source for the leucine that was oxidized was from net breakdown of body protein. Several of the studies (Booth and Watson, 1985; Hagg et al., 1982; White and Brooks, 1981; Rennie et al., 1981) have demonstrated a reduction in whole-body protein synthesis for some time after exercise and this depression of protein synthesis was influenced by both intensity and duration of the exercise (Dohm et al., 1985).

The other line of evidence for an increased protein breakdown was obtained from studies in which total and essential amino acids were found to be increased during exercise in plasma, muscle and liver (Dohm et al., 1981; Dohm et al., 1985, Rennie et al., 1981). Maclean et al. (1991) demonstrated that total amino acids and total essential amino acids in plasma were not significantly altered during a prolonged submaximal cycling in human, whereas the contents of these amino acids in muscle was significantly increased at exhaustion.

It seems clear, therefore, that exercise may cause a net breakdown of whole-body protein and this is accomplished by a decrease in the rate of protein synthesis and an increase in protein catabolism. However, not all data are in agreement. Wolfe et al. (1982)

has pointed out that in N excretion studies, urea production has never been directly measured, and the indirect estimate of urea production from urea excretion has pitfalls. Therefore, they used a stable isotope tracer $^{15}N_2$-urea infusion to directly measure the urea production during prolonged mild exercise in human, and showed that plasma urea concentration and urea production did not change. Also Stein et al. (1987) reported that there was no increase in urea production in the human subjects who cycled for five hours and then ran for three hours when the same tracer method was used. Therefore, they have concluded that exercise does not stimulate a net breakdown of whole-body protein.

Although the latter studies did not find a whole-body net protein breakdown during exercise, they did observe an increased leucine oxidation with an unchanged plasma leucine concentration (Wolfe et al., 1982). In agreement with their findings, a recent study done by Carraro and co-workers showed an unchanged urea production concurrent with an apparent acceleration of muscle protein breakdown in rats in response to exercise (Carraro et al., 1990). They thus proposed that amino acids mobilized from tissue protein breakdown during exercise could be accumulated in the body and used in subsequently stimulated muscle protein synthesis during recovery. From all of the above observations, it can be surmised that some exercise may cause a net protein mobilization and free amino acid accumulation, although there is no general agreement on the extent of breakdown.

Most studies regarding protein metabolism in response to exercise have focused on examining the changes during the exercise period. Only a few have investigated the metabolic responses of protein after exercise (Rennie et al., 1981; Booth and Watson,

1985; Devlin et al., 1990; Pivarnik et al., 1989; Tarnopolsky et al., 1991). Tarnopolsky et al. (1991) reported that an increase in whole-body protein synthesis was not observed two hours after exercise. Rennie et al. (1981) and Booth et al. (1985) have suggested that the increased catabolism and the decreased synthesis of protein induced by exercise continue to occur for sometime after exercise. However, the exact time-course of these events was not determined and it may depend on several factors, such as the type, intensity and duration of the exercise, training status, as well as the experimental conditions (Lemon, 1987).

B. Muscle Protein Metabolism During and After Exercise.

As seen above, some studies suggest a net mobilization of body protein during exercise. It is of interest whether this increased protein mobilization takes place in muscle, so that mobilized amino acids would accumulate there and potentially be available for muscle glyconeogenesis after exercise. A net loss of tissue protein would occur if there is a decrease in the rate of protein synthesis and/or an increase in the rate of protein degradation within a tissue. There is some evidence that muscle protein synthesis is depressed during and after an acute bout of exercise *in vitro* (Bylund-Fellenins et al., 1984; Cook et al., 1981; Davis and Karl, 1986; Balon et al., 1990) and *in vivo* (Dohm et al., 1982; Dohm et al., 1985; Booth and Watson, 1985; Hagg et al., 1982; Rennie et al., 1981; Carraro et al., 1990). This depression was directly influenced by the intensity and the duration of the exercise (Dohm et al., 1985).

The effect of exercise on the degradation of muscle protein, however, is less clear.

In previous *in vivo* studies, muscle protein degradation during and after exercise was found to be increased (Frisch et al., 1984; Dohm et al., 1982; Dohm et al., 1985, Wolfe et al., 1982; Carraro et al., 1990), decreased (Rennie et al., 1981; Radha and Bessman, 1983; Young and Torun, 1981), or unchanged (Rennie and Davies, 1981; Wolfe, 1984; Decombaz et al., 1979; Calles et al., 1984). In most of these studies, the rate of muscle protein degradation was determined by measuring the urinary excretion of 3-methylhistidine (3-MH). As reviewed elsewhere (Young and Munro, 1978; Wolfe, 1987), 3-methylhistidine is a minor amino acid found exclusively in the contractile myofibrillar protein (actin and myosin), and after being released by degradation of actin and myosin, it is neither reincorporated into protein nor metabolized. The only fate of 3-MH is entry into the blood circulation and urinary excretion. Since most of the contractile proteins are found in skeletal muscle (Lemon, 1987), 3-MH excretion has been widely used as an estimate of skeletal muscle protein degradation. The disparities in the above studies were explained as the lack of a consistent effect of exercise on protein degradation: it is increased under one set of conditions, but not changed or even decreased under other sets of conditions. Dohm et al. (1982 and 1985) and Booth et al. (1985) have suggested that muscle protein degradation is increased with the intensity and the duration of the exercise. Performing exercise on consecutive days could enhance 3-MH excretion.

The inconsistencies in results regarding muscle protein degradation led Dohm and co-workers (Dohm and Askew, 1985) to examine the time-course of changes in the 3-MH excretion under different conditions during running. Their results showed that 3-MH excretion was depressed during exercise in all cases. During the recovery period, it

returned to control value, and in some cases exceeded the control value depending on the experimental conditions. The biphasic change in 3-MH excretion in running subjects could explain some of the variation in the previous observations.

The validity of 3-MH excretion as a specific indicator of skeletal muscle degradation has also been questioned. A portion of the excreted 3-MH was found to originate from the degradation of the small amount of actin and myosin in the gut and skin (Wassner and Li, 1982; Rennie and Millward, 1983). Even though the proportion of the non-skeletal muscle 3-MH is low, its contribution may be higher since the turnover rate of non-muscle 3-MH pool is more rapid than that of skeletal muscle. On the other hand, the continued use of the 3-MH excretion as a measure of skeletal muscle protein degradation was advocated (Ballard et al., 1983; Tomas et al., 1984). Comparable rates of protein synthesis and degradation were found when the 3-MH excretion was measured and when constant tracer infusion techniques were used, which suggested that under most conditions 3-MH excretion is a valid method for muscle protein degradation studies (Tomas et al., 1984).

A decrease in muscle myofibrillar protein degradation during exercise seems inconsistent with the exercise-induced net body protein mobilization. However, muscle protein consists not only of myofibrillar protein, but also non-myofibrillar proteins. The myofibrillar protein comprises about 50% - 60% of total muscle protein, whereas non-myofibrillar proteins contribute to the remainder 40% - 50% of total muscle proteins (Highlander, 1961). In the basal state, the degradation of the myofibrillar protein is several times slower than that of non-myofibrillar proteins (Bates, 1983). In addition, it has been

reported that degradation of the two classes of proteins are regulated independently in response to starvation and hormones (Li et al., 1984; Smith et al., 1986). In these studies, the degradation of the two classes of proteins was assessed by measuring both 3-MH excretion and tyrosine release. Tyrosine release was used as an indicator of muscle net protein degradation since it is an essential amino acid and it can not be metabolized in muscle, but it can be reincorporated into muscle protein. In fact, in response to exercise, an increased plasma tyrosine concentration was observed *in vivo* (Haralambie and Berg, 1976), and an increased release of tyrosine *in vitro* from perfused muscle or hindquarter preparations was also found after a bout of exercise *in vivo* (Dohm et al., 1980; Kasperek and Snider, 1985; Goodman and Gomez, 1987; Balon et al., 1990). Kasperek and co-worker (Kasperek and Snider, 1989) concluded that muscle protein degradation was increased during exercise, and this increase, observed immediately after exercise, was due to the breakdown of the non-myofibrillar proteins in muscle. This was based on an increased tyrosine release and an unchanged 3-MH release from exercised muscle in their studies.

Changes in net protein breakdown in the muscle during and after exercise may therefore depend on the balance between myofibrillar and non-myofibrillar protein metabolism, and the specific conditions under which exercise takes place.

- Can gluconeogenic amino acid(s) serve as substrate for muscle glyconeogenesis after exercise?

It is known that during exercise, amino acids liberated from protein catabolism

have three likely fates: they may accumulated in the free amino acid pool, be oxidized to CO_2 to supply energy, or be converted into alanine or glutamine and thus contribute to hepatic gluconeogensis (Dohm, 1986). However, after exercise, the utilization of amino acids for oxidation and hepatic gluconeogensis is decreased consistently with the decreased energy requirement, whereas the net muscle protein breakdown induced by exercise may be maintained for sometime after exercise (Bylund-Fellenius et al., 1984; Davis and Karl, 1986; Kasperek and Snider, 1985; Goodman and Gomez, 1987; Balon et al., 1990). An increased myofibrillar protein degradation after exercise may also contribute to a part of the amino acid accumulation seen in muscle (Dohm et al., 1985). Thus, there is an increased availability of free amino acids in muscle immediately after exercise. Glyconeogenesis occurs concurrently in muscle (Ryan and Radziuk, 1994). Thus it is possible that mobilized gluconeogenic amino acid(s) could be contributing substrates for this process. Favier and co-workers (Favier et al., 1987) have suggested that alanine could play an important role in muscle glycogen resynthesis during fasting recovery from prolonged exhaustive exercise. They did not distinguish however whether this contribution of alanine into muscle glycogen is via hepatic gluconeogensis or by muscle glyconeogenesis. The potential role of amino acids as local substrates for glyconeogenesis, therefore, remains to be examined.

1.2. THE PROBLEM AND THE AREAS OF INVESTIGATION

1.2.1. The Problem

The first direct evidence that muscle glyconeogenesis occurs during fasting

recovery from prolonged submaximal exercise *in vivo* has been provided by Ryan and Radziuk (1994). Although indirect evidence strongly suggested that lactate produced locally in muscle during exercise may serve as a substrate for muscle glyconeogenesis after intensive exercise, even under physiological circumstances *in vivo*, isotope tracer infusion techniques failed to detect significant conversion of ^{14}C-lactate into muscle glycogen via the process of muscle glyconeogenesis. Lactate accumulation in plasma and muscle in prolonged submaximal exercise, however, is low (Fell et al., 1980; Favier et al., 1987; Ryan et al., 1993). It is, therefore, possible that the substrate pool which is labelled by the metabolism of ^{14}C-glucose to 3-carbon precursors may include glycerol and/or precursors of amino acid metabolism. This is probably attractive as a hypothesis since there may be local mobilization of these substrates within muscle (Holloszy et al., 1978; Newsholme, 1983; Lemon, 1987). Thus the goal of this study was to begin the examination of i) the availability of substrates in muscle following exercise; and ii) their possible incorporation into glycogen after a prolonged submaximal exercise, such as swimming.

1.2.2. Areas of the Investigation.

The work described here can be subdivided as follows:

I. Amino acids as substrate for muscle glyconeogenesis after prolonged swimming.

A. Does net protein breakdown occur during and after extended submaximal exercise?

This question will be addressed by: 1) examining plasma urea concentrations during a four-hour swim as well as three-hour recovery period in rats, and 2) measuring

urea production to ensure that changes in urea levels in fact reflect changes in urea production and not (to any important extent) changes in the volume of distribution or renal clearance of urea. This will be done using tracer methods and ^{14}C-U-urea.

B. Can any protein breakdown seen be accounted for by myofibrillar protein degradation in muscle?

This question will be examined by measuring the levels of tyrosine and 3-MH both in plasma during exercise and recovery and in muscles at the end of the recovery period. Tyrosine is an essential amino acid, not considered to be metabolized in muscle and therefore serves as an index of generalized protein breakdown. 3-MH arises by methylation of histidine in myofibrillar protein and is therefore specific to this type of protein. Its only fate after mobilization from muscle is excretion by the kidney. With some caution it can be used as an index of myofibrillar protein breakdown. Since it is also present in skin and intestine (where the protein turns over more rapidly), both tyrosine and 3-MH will be measured in the muscle (soleus, white and red gastrocnemius muscles) at the end of the recovery period to ascertain whether tissue changes correspond to those seen in plasma. An elevation of muscle free 3-MH would be highly suggestive of a local breakdown of myofibrillar protein.

C. Can amino acids which may be produced in muscle be metabolized and incorporated into glycogen locally?

This problem was investigated by choosing a representative amino acid and

measuring the incorporation of its labelled counterpart into glycogen in muscle. The amino acid chosen was threonine since it is not synthesized *in vivo* and is metabolized to pyruvate thus likely labelling the same pool as ^{14}C-glucose which had been previously shown to contribute label to glycogen locally by the glyconeogenic pathway (Ryan and Radziuk, 1994). Threonine is a strongly gluconeogenic (Hetenyi et al., 1984) so that significant label incorporation into glucose and glycogen would be expected.

To distinguish between local synthesis of glycogen from 3-carbon substrates labelled by ^{14}C-threonine and hepatic gluconeogensis from same substrates, ^{3}H-glucose was infused concurrently with ^{14}C-threonine. The incorporation of circulating ^{14}C-glucose into muscle glycogen could be tracked from the ratio of ^{3}H/^{14}C in glycogen and the mean ratio during the recovery period after exercise.

II. Glycerol as substrate for muscle glyconeogenesis after prolonged swimming in rats.

The elevations of glycerol in plasma and tissue after exercise have been previously established. We therefore restricted our investigation on the possibility of the incorporation of ^{14}C-label from circulating glycerol into muscle glycogen without the formation of glucose in the liver as an intermediate step. The method of proceeding was identical to that for ^{14}C-threonine with the concurrent infusion both ^{14}C-glycerol and ^{3}H-glucose.

In summary, we are thus planning to initiate a systematic investigation of the possible substrates, other than glucose, for glycogen synthesis in muscle under

physiological circumstances - during recovery from submaximal exercise.

1.3. APPROACH TO THE PROBLEM.

To determine the substrates of muscle glyconeogenesis *in vivo* under a physiological condition, we have chosen the same exercise model as described by Ryan and Radziuk (1994), in which the rats swim for a prolonged period (four hours), and recover for three hours under fasting condition, since the direct evidence of physiological muscle glyconeogenesis has been obtained in this model. As described by Ryan and Radziuk (1993 and 1994), models of exercise to exhaustion are the most useful in examining muscle glyconeogenesis *in vivo* under physiological circumstances. They are characterized by significant muscle glycogen depletion (Gaesser and Brooks, 1980; Hermansen and Vaage, 1977; Johnson and Bagby, 1988), as well as preferential (Fell et al., 1980; Gaesser and Brooks, 1980) and rapid repletion of muscle glycogen, even in the absence of exogenous substrate (Gaesser and Brooks, 1980; Hermansen and Vaage, 1977; Johnson and Bagby, 1988). In addition, prolonged submaximal exercise is an excellent model for studying the effects of substrates other than lactate on muscle glyconeogenesis. Prolonged submaximal exercise is characterized not only by significant muscle glycogen depletion (Favier et al., 1987; Fell et al., 1980) as well as repletion, but also by minimal lactate accumulation (Favier et al., 1987), significant utilization of stored triglycerides (Newgard, 1983) and of tissue protein (Lemon, 1987).

Three specific muscles - the soleus, white gastrocnemius and red gastrocnemius were chosen to study the contributions of amino acids or glycerol to muscle glycogen by

the glyconeogenic route. Each represents one type of fibre that has a different metabolic profile and glyconeogenic capacity (Ariano et al., 1973; Armstrong and Phelps, 1984). The soleus muscle is comprised primarily of slow-twitch red fibres (85%) which are type I fibres. Type I fibres have a high respiratory capacity and are highly dependent on aerobic oxidation of both carbohydrates and free fatty acids for energy (Ariano et al., 1973; Armstrong and Phelps, 1984) and have low glycogenolytic activity (Peter et al., 1972). The white gastrocnemius muscle is formed primarily (83%) of fast-twitch white fibres which are type IIb (Armstrong and Phelps, 1984). Type IIb fibres are highly glycolytic and glycogenolytic, and have low respiratory capacities (Peter et al., 1972). The red gastrocnemius muscle is made up by approximately 62% of fast-twitch red fibres which are type IIa (Armstrong and Phelps, 1984). Type IIa fibres are both glycolytic and oxidative in nature with high respiratory and glycogenolytic capacities, as well as having higher concentrations of mitochondria and myoglobin than white fibres. A depletion of glycogen during prolonged swimming and a significant glycogen resynthesis after exercise in all these three muscles have been reported (Conlee et al., 1978; Favier et al., 1987; Terblanche et al., 1981; Terjung et al., 1974).

1.4. TRACER METHODOLOGY

1.4.1. Introduction

The complex substrate, pathway and hormonal interrelationships make the *in vivo* study of metabolism more difficult. The reason that isotopic tracer methods have been chosen for this *in vivo* study is that: 1) using tracer dilution as an indicator, the actual turnover rates of metabolites can be determined; 2) the synthesis of muscle glycogen by different pathways can be distinguished; and 3) physiological concentrations and the fluxes of the metabolites would not be altered by the use of tracers.

Isotopic tracers are molecules with one or more atoms replaced by isotopes of a different atomic mass (i.e. which contain the same number of protons, but differing numbers of neutrons). It can be stable (^{2}H, ^{13}C) or radioactive (^{3}H, ^{14}C) depending on the stability of the isotopes. The stable isotopes can be detected by Mass Spectrometry and the radioactive isotopes can be detected using Liquid Scintillation Counting. The use of isotope tracers also relies on the assumption that the labelled molecules have the same metabolic behaviour as the compound traced (tracee). This enables the labelled molecules to track the movement of unlabelled molecules without altering their metabolism. In this study, radioactive tracers and a primed-constant infusion technique was used. A primer is used since otherwise hours may elapse before isotopic equilibrium is reached. The primer minimizes this time since in conjunction with a continuous isotope infusion, it instantaneously labels the total miscible pool of metabolite to the equilibrium enrichment.

1.4.2. Application of the Tracer Method to (Glucose and) Urea Metabolism

A: Steady state kinetics

A physiological steady state consists of a dynamic equilibrium in which opposing processes are precisely balanced, and the size of the substrate metabolic pools are not changing with time. The rate of appearance (Ra) is equal to the rate of disappearance (Rd) of the substrate, and a constant plasma concentration of the substrate (C) is therefore achieved. Similarly, the rate of appearance of tracer (Ra*) which is tracer infusion rate is equal to its disappearance rate (Rd*), yielding a constant plasma tracer concentration. In steady state, we have therefore:

$$\frac{Ra^*}{Ra} = \frac{C^*}{C} \quad ; \quad \frac{Rd^*}{Rd} = \frac{C^*}{C} \qquad (1.4.1)$$

$$Ra = Ra^* * \frac{C}{C^*} \qquad (1.4.2)$$

Note also that Rd = Ra.

Since Ra^* is equal to tracer infusion rate (I), we can write

$$Ra = \frac{I}{SA} \qquad (1.4.3)$$

where SA is the specific activity of the labelled substrate in plasma (C^*/C).

The metabolic clearance rate of substrate (MCR) is defined as the volume of the plasma that is completely cleared of metabolite in one minute. It is an indicator of substrate utilization and is calculated from (eg, Ryan et al., 1993):

$$MCR = \frac{Rd}{C}$$

From equation (1.4.1) and $Rd^* = Ra^*$

$$MCR = \frac{Rd^*}{C^*} = \frac{Ra^*}{C^*} \qquad (1.4.4)$$

or

$$MCR = \frac{I}{C^*} \qquad (1.4.5)$$

B: Nonsteady-state kinetics.

A great number of *in vivo* metabolic events occur under nonsteady-state conditions (such as exercise, meals etc.) where the Ra is not equal to the Rd. This leads to changing substrate concentrations. Moreover, when a tracer is infused, the specific activity, under general conditions will also be variable. Under conditions where rapid changes in metabolite fluxes are not expected, the one-compartment model has been shown to perform well for glucose (±15%, Radziuk, 1978) as well as for urea (Wolfe, 1981). For the metabolite in question, conservation of mass gives:

$$V*\frac{dC}{dt} = Ra - Rd \qquad (1.4.6)$$

where C is plasma substrate concentration, t is the time, V is the volume of distribution of substrate in the body. Rd is the time- and concentration- dependent disappearance rate

of substrate. Since tracer is chemically identical to the molecules traced, its kinetics will also be the same. Therefore we have:

$$V*\frac{dC^*}{dt} = Ra^* - Rd* \qquad (1.4.7)$$

where C* is tracer concentration in plasma, Ra* is appearance rate of tracer which is equal to infusion rate, Rd* is the tracer disappearance rate, V is the same volume of distribution. Using equations (1.4.6) and (1.4.7) the following equation can be derived for Ra (Steele, 1959):

$$Ra = \frac{Ra^*}{SA} - \frac{VC}{SA}*\frac{dSA}{dt} \qquad (1.4.8)$$

This calculation assumed a uniform distribution of the substrate throughout the volume, V. In order to compensate for the non-uniform distribution of substrate, a pool fraction, p, is used to generate an effective volume of distribution. The final equations for the calculations of production rate and metabolic clearance rate are:

$$Ra = \frac{Ra^*}{SA} - \frac{pVC}{SA}*\frac{dSA}{dt} \qquad (1.4.9)$$

$$MCR = \frac{1}{C^*}*(p*V*\frac{dC^*}{dt} - Ra^*) \qquad (1.4.10)$$

If, for example, substrate concentrations are changing rapidly, a fixed effective volume

of distribution may not, in general, apply (e.g. Issekutz et al., 1976; Allsop et al., 1978). When concentrations change more slowly, p was optimized at 0.5 (Steele, 1959), 0.65 (Cowan and Hetenyi, 1971) and 0.75 (Radziuk, 1978) in the case of glucose and near 0.5 (pV=100 ml/kg) for urea (Wolfe, 1981).

1.4.3. Application of Tracer Method to Muscle Glycogen Metabolism

A. A double tracer method for glycogen synthesis

It has recently been demonstrated that under physiological circumstances glycogen in muscle is synthesized from more than one source and via two different pathways - direct and glyconeogenic routes (Ryan and Radziuk, 1994). In order to distinguish between the two processes and to calculate the contributions of different precursors to new glycogen synthesis, a double tracer method is utilized. ^{3}H-6-glucose was chosen to trace the direct glycogen synthesis since the tritium label on the sixth carbon in glucose is lost in the glyconeogenic pathway (in the equilibration of oxaloacetate and fumarate) (Dunn et al., 1967; Newgard et al., 1983; Shiota et al., 1984; Wolfe, 1984). The ^{3}H-glycogen in muscle is therefore considered to have arisen by direct incorporation of ^{3}H-6-glucose. ^{14}C-labelled glyconeogenic precursors are then used to trace potential glyconeogenic contributions to the muscle glycogen. Since these potential substrates for muscle glyconeogenesis are not yet known, several putative muscle glyconeogenic substrates were used with ^{14}C-label to identify possible sources of such glyconeogenic substrate in muscle. If the substrate traced is indeed a muscle glyconeogenic precursor, significant ^{14}C-glycogen made from this labelled precursor (over and above that made

from direct incorporation of such ^{14}C-label from the circulation) via glyconeogenesis should be detected in muscle. We thus predict whether a substrate could be a glyconeogenic precursor in muscle, based on a significant incorporation of ^{14}C from its labelled counterpart into glycogen when the ^{14}C-labelled substrate is infused systemically as a tracer.

B. ^{14}C-U-threonine as the glyconeogenic tracer in muscle.

In order to test whether amino acids serve as substrates for muscle glyconeogenesis, tracer techniques were utilized. A labelled amino acid, ^{14}C-U-threonine was chosen as the tracer and used to assess the presence of glyconeogenesis in muscle and, if this was detected, to estimate the extent of the glyconeogenesis from this amino acid. The choice of threonine as a representative amino acid was made for a number of reasons. Firstly, threonine is a known precursor for glucose and it is metabolized through the common 3-carbon pool. Therefore, it may also be a glyconeogenic precursor in muscle. Secondly, threonine is an essential amino acid and therefore cannot be synthesized from any other sources *in vivo*. In estimations of glyconeogenesis, its specific activity (particularly intracellular) would not be diluted by new synthesis of threonine. In addition, the additional threonine should be available if an increased muscle proteolysis occurs during exercise and/or postexercise recovery.

The tracer approach designed to determine whether threonine is a muscle glyconeogenic substrate in the recovery period following exercise (see Figure 1.2) is

Figure 1.2: Tracer strategy: pathways labelled by ^{3}H-6-glucose and ^{14}C-U-threonine. The gluconeogenic formation of glycogen in muscle from systemic threonine is traced by ^{14}C-U-threonine infusion into the blood circulation. ^{3}H-6-glucose is infused to track the direct glycogen synthesis from plasma glucose.

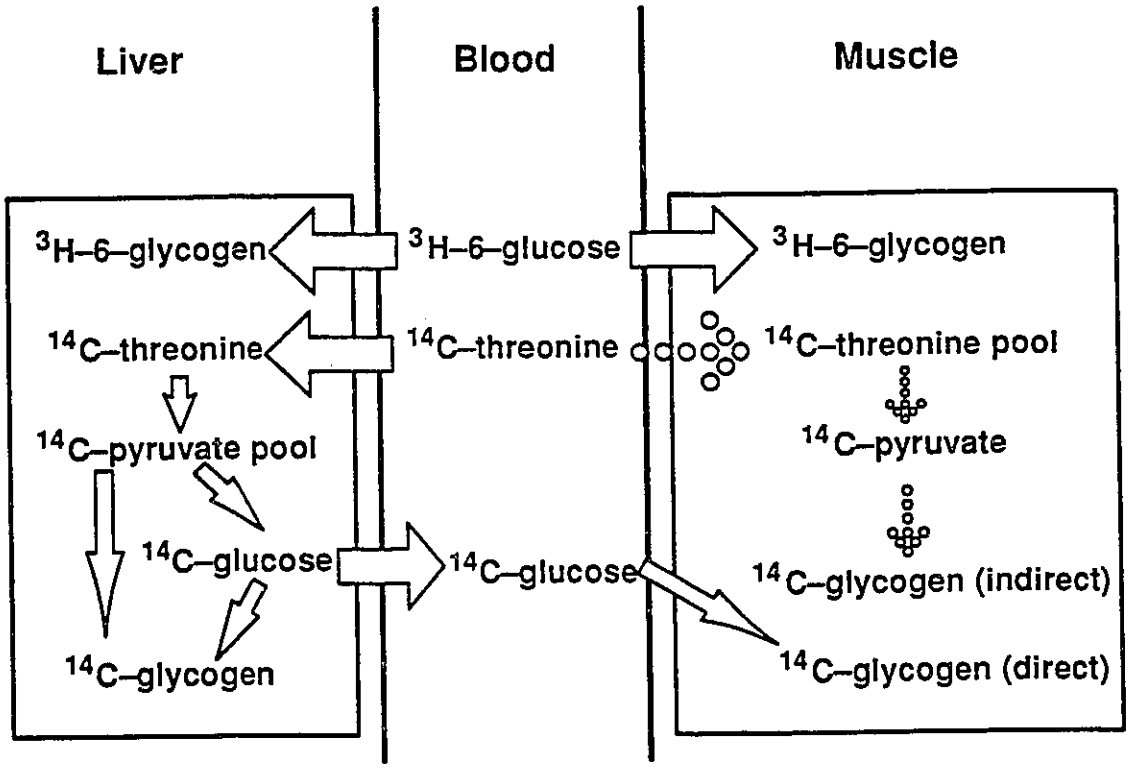


Figure 1.2: Tracer strategy and metabolic pathways labelled by various tracers

based on the method used in previous studies (Ryan and Radziuk, 1994; Johnson and Bagby, 1988). Following the infusion of ^{3}H-6-glucose and ^{14}C-U-threonine, ^{3}H-label traces only the direct incorporation of glucose into muscle glycogen (since this label will be lost in the glyconeogenic pathway). Circulating ^{14}C-glucose (made by gluconeogenesis from ^{14}C- threonine) may be taken up by muscle and converted to glycogen (direct ^{14}C-glycogen). This will, however, occur in parallel with ^{3}H-6-glucose. The incorporation of the latter into glycogen will therefore track that of ^{14}C-glucose. Putatively, ^{14}C-U-threonine would also be taken up by muscle and might follow the glyconeogenic pathway. Any ^{14}C-glycogen which cannot be accounted for by the direct route is deemed to be synthesized by glyconeogenic pathways. The calculations are made as follows. The ratio of [^{3}H]/direct [^{14}C] in glycogen equals the mean ratio of [^{3}H]/ [^{14}C] in plasma glucose:

$$\frac{[^{3}H]\text{-}glucose}{[^{14}C]\text{-}glucose} = \frac{[^{3}H]\text{-}glycogen}{[^{14}C]\text{-}glycogen(direct)}$$

The ^{14}C-glycogen synthesized by the direct uptake of ^{14}C-glucose from plasma is thus calculated as:

$$Direct\ [^{14}C]\text{-}glycogen = \frac{tissue\ [^{3}H]\text{-}glycogen\ d.p.m.}{plasma\ \frac{[^{3}H]\text{-}glucose\ d.p.m.}{[^{14}C]\text{-}glucose\ d.p.m.}} \qquad (1.4.11)$$

where the ratio of [^{3}H]/[^{14}C] in d.p.m. of plasma glucose is the average ratio during three-hour recovery period. The total ^{14}C-glycogen in muscle would be equal to the sum of the ^{14}C-glycogen arising from plasma ^{14}C-glucose via the direct route (direct ^{14}C) and the ^{14}C-

glycogen newly synthesized from ^{14}C-threonine via muscle glyconeogenesis (indirect ^{14}C). The difference between the total amount of ^{14}C-glycogen present and the direct ^{14}C-glycogen calculated would represent the glyconeogenic contribution of threonine to muscle glycogen.

Tissue glycogen synthesized from the direct uptake of plasma glucose is calculated by

$$Direct\ Synthesis = \frac{[^3H]\text{-}glycogen\ d.p.m.\ in\ tissue}{SA\ (plasma[^3H]\text{-}glucose)} \qquad (1.4.12)$$

where SA is the average specific activity of ^{3}H-glucose in plasma (C*/C) during the three-hour recovery period.

C. ^{14}C-U-glycerol as glyconeogenic tracer in muscle

Although glycerol is not generally thought to be phosphorylated in muscle, the possibility of its incorporation into muscle glycogen during recovery from exercise was tested. This was done using a similar tracer approach to the above (see Figure 1.3). Followed infusion of ^{3}H-6-glucose and ^{14}C-U-glycerol, muscle uptake recycled ^{14}C-glucose generating direct ^{14}C-glycogen. At the same time, ^{14}C-U-glycerol might enter the muscle triose-phosphate pool and yield indirect ^{14}C-glycogen via glyconeogenesis. The direct [^{14}C]-glycogen and the direct synthesis of glycogen were calculated using equations (1.4.11) and (1.4.12), respectively. Should the results be negative (i.e. no incorporation),

Figure 1.3: Tracer strategy: pathways labelled by ^{3}H-6-glucose and ^{14}C-U-glycerol. ^{3}H-6-glucose was infused to track the direct glycogen synthesis from plasma glucose. ^{14}C-U-glycerol was infused to trace the glyconeogenesis in muscle from systemic glycerol.

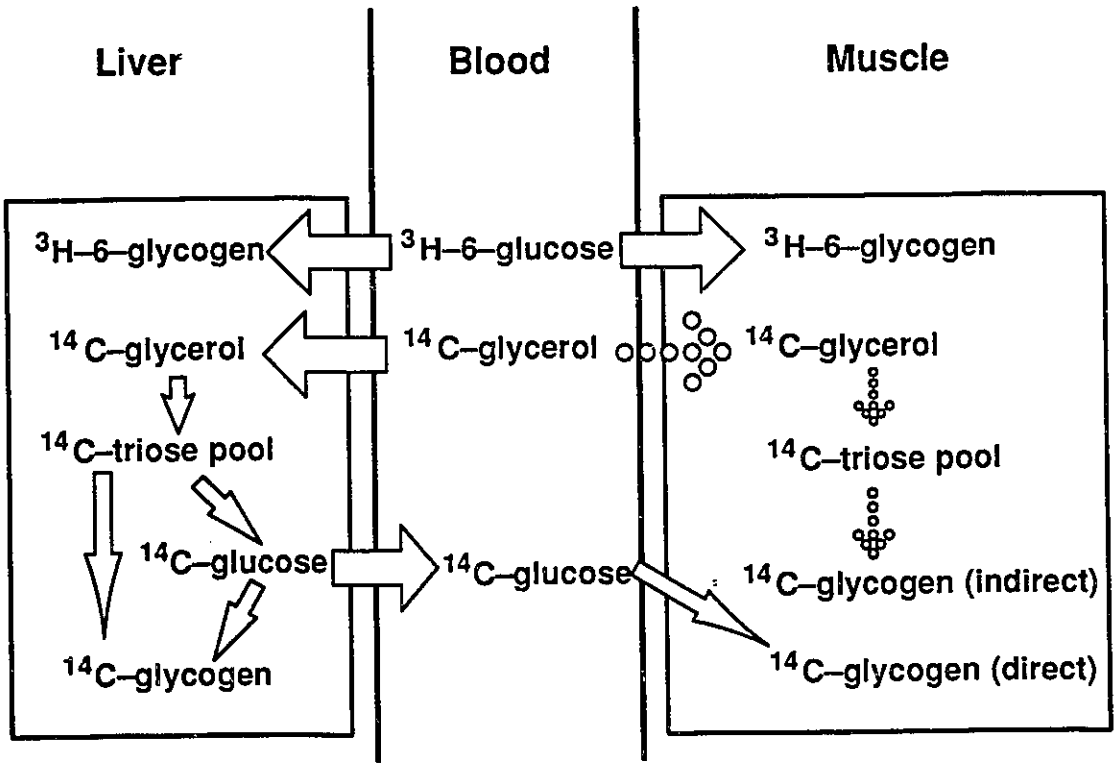


Figure 1.3: Tracer strategy and metabolic pathways labelled by various tracers

they would be useful in verifying the consistency of the methodology since previous data from this laboratory suggested no incorporation of label from the triose-phosphate level into glycogen in muscle (Ryan and Radziuk, 1994).

1.4.4. The Limitations and Problems of Tracer Method

A . Application of One-compartment kinetics

There are a number of limitations when using a single compartment to describe the nonsteady-state kinetics of glucose or urea. These limitations are primarily derived from the assumptions described previously by Steele (1959). These are that there is a single well-mixed substrate pool in the body and there is uniform instantaneous mixing of the infused labelled substrate pool with the unlabelled pool. It is also required that once having left the substrate pool, no molecule reenters this pool. The use of a one-compartment model in deriving an equation for the measurement of glucose or urea production (the "Steele" equation) includes all of the above potential problems. The assumption of uniform and instantaneous mixing of existing, and newly arrived, labelled and unlabelled molecules within the volume of distribution may not always be the case for either glucose or urea. This could lead, for both glucose and urea, to situations where the appropriate volume of distribution may appear variable (Allsop et al., 1978; Wolfe, 1981). In addition, in the case of urea, a suggested selective reincorporation of carbon from urea back into newly synthesized urea (Long et al., 1978) could lead to errors in the calculations. In the case of glucose, ^{3}H-6-glucose is an almost irreversible tracer and can therefore be used to circumvent the potential errors of calculation which could be induced

by glucose recycling (Wolfe et al., 1979).

B. The problems of estimating glyconeogenesis in muscle from labelled precursors.

A number of potential problems can arise from the label dilution and label exchange prior to its incorporation into glycogen when glyconeogenesis in muscle is estimated from labelled precursors. Firstly, the true precursors for glyconeogenesis are intramuscular precursors which may not be in equilibrium with plasma precursors. Thus, the specific activity of the label in muscle may be different compared with that in plasma. Secondly, there are metabolic exchanges of the label between different metabolic pathways in common metabolite pools. These have been well defined in the liver. For example, in the Krebs cycle, there exists an exchange between ^{14}C atoms from labelled precursors and ^{12}C atoms from acetyl CoA in the oxaloacetate (OAA) pool. This leads to the "loss" of the label and thus the underestimation of glyconeogenesis from labelled precursors. Hetenyi has proposed a method to correct for the metabolic exchange (Hetenyi, 1982; Hetenyi et al., 1982). This particular exchange may be to a lesser degree for labelled glycerol since glycerol does not pass through the OAA pool on its way to glycogen even if glycerol is a true glyconeogenic precursor in muscle. Due to these shortcomings, the assessment of muscle glyconeogenesis from labelled precursors is only an estimate.

CHAPTER TWO

MATERIAL AND METHODS

2.1. ANIMALS

2.1.1. Animals and Housing

Male Sprague-Dawley rats, weighing approximately 180-200 grams, were obtained from the Charles River, Ottawa, Ontario. The animals were housed in a controlled lighting 12:12 hour light/dark cycle environment (light on at 0700 hours), and had free access to food (Purina rat chow) and water. Normally rats were kept in groups of two or three at 26.5°C in plastic cases for at least a week before pre-experimental surgery.

2.1.2 Pre-Experimental Animal Preparation

A week after arrival, the rats underwent catheter implant surgery. They were anesthetized with Ketamine (120 mg/kg) and Xylazine (8.3 mg/kg). The catheters, made of silastic medical grade tubing connected with polyethylene tubing by blunted needles, were implanted into the superior vena cava via the right jugular vein and into the aortic arch via the left carotid artery for intravenous isotope tracer infusions and arterial blood sampling, respectively. The catheters were fixed to the skin at the back of rat's neck. Thereafter, the animals were allowed to recover from surgery for four days in individual cages with free access to water and food. On the third and the fourth days of recovery, rats were swum for 30 minutes each day in a rectangular tank (55.5 cm width, 1.4 m

length, 42 cm depth) to allow them to become familiar with swimming in water (36.0°C). Prior to the day of experiment, rats were fasted for 14 hours overnight in order to minimize glucose absorption from the gut. Fasting was begun after a one hour feeding period during the dark cycle. At the same time, the rats were transferred to the experimental room in individual cages with only a water supply.

2.2. UREA KINETICS, PROTEIN CATABOLISM AND GLUCOSE TURNOVER DURING AND FOLLOWING PROLONGED SWIMMING IN RATS

2.2.1. Experimental Protocol

14-hour fasted rats were weighed. The indwelling jugular catheters were connected with tracer infusion syringes and carotid arterial catheters were connected with sampling syringes through a harness-swivel system. A primed constant double radioisotope tracer infusion was given intravenously to all of the rats. D-[^{3}H-6]-glucose (HPLC purified using Bio-Rad HPX-87P column) and [^{14}C-U]-urea were infused continuously for 9 hours by using a Harvard compact infusion pump (Model 975) to assess glucose turnover and urea kinetics, respectively. The priming dose of D-[^{3}H-6]-glucose was approximately 4.5 μCi/min. The rats were divided into two groups - rest control group (n=8) and exercise group (swimmer, n=8). Rats in both groups underwent a 2-hour basal period in which rats stayed at rest to allow a tracer steady-state to be achieved. Thereafter, the swimmer would swim for four hours in the tank, whereas the control rats rested in their cages. After the four-hour exercise period, swimmers were taken out of the water, dried with towels, and allowed to recover for three hours under heating lamps. Meanwhile, the

control rats rested for three further hours during the recovery period. During the study, 1 ml of arterial blood samples were taken at 90, 120, 180, 240, 300, 360, 420, 480, and 540 minutes. Samples were mixed with one drop of heparin (1000 U/ml) and spun in a microcentrifuge (Jouan M 14.11) at 1000 r.p.m. for 5 minutes to separate red blood cells and plasma. Plasma (approximately 0.5 ml) was saved for various analyses, while the red blood cells were resuspended in approximately 0.5 ml of plasma which was obtained from fasted non-experimental rats before the experiments, and re-injected into corresponding experimental rats. At the end of the experiment (after the 540 minutes), rats were sacrificed with an intravenous sodium pentobarbitol (65 mg) injection.

2.2.2. Chemical Analysis

Preparation of the tracer standards.

Tracer standards were prepared by diluting the infusate (1 ml) with glucose (100 mg) and urea (10 mg) to 100 ml with double distilled water. The purpose of making standard solutions was to measure the total amount of radioactivity infused and to check the recoveries of label from the assays.

Chemicals: Unless otherwise stated, all chemicals were purchased from BDH, Poole, UK.

Deproteinization of plasma samples.

To plasma sample (200 μl), $Ba(OH)_2$ (0.4 ml, 0.3 N) was added followed by the addition of $ZnSO_4$ (0.4 ml, 0.5% w/v) and water (10 ml) (Somogyi, 1952). The samples

were mixed well and left to react for 20 minutes (room temperature). The precipitate was removed by centrifugation (20 minutes, 2000 rpm, Beckman GPR, Palo Alto, CA). The supernatant was decanted and frozen until required for further chemical assays. Standards and blanks (200 µl distilled water) were prepared in the same manner as the samples.

Measurement of plasma glucose.

Total glucose concentration: Total glucose concentration was measured using the glucose oxidase method (2300 Stat Glucose/L-l acetate Analyzer, YSI, Yellow Springs, OH).

Determination of plasma ^{3} H-glucose: The deproteinized plasma samples were used to determine ^{3}H-glucose concentrations. To separate labelled glucose from labelled organic acids and amphoteric compounds, the deproteinized plasma samples were passed through ion exchange resin columns (Dowex 1 X 8 and Dowex 50 W X 8, 200-400 mesh, Biorad, Richmond CA) as described previously (Issekutz, 1976). The eluate (containing labelled glucose and other labelled uncharged compounds) was collected. An aliquot (2 ml) was placed in the counting vials and freeze dried (to remove tritiated water). To the dried samples, water (0.6 ml) and 15 ml of the scintillation cocktail (Formula 989, New England Nuclear, Boston MA) were added. The level of radioactivity was determined using a liquid scintillation analyzer (2200 CA TriCarb Canberra Packard). Recovery was determined by running standard solutions in parallel.

Plasma Lactate Concentration.

Total plasma lactate concentration was measured by using a Yellow Springs

Instruments 2300 Stat Glucose/L-lactate Analyzer.

Measurement of plasma urea.

Determination of plasma urea concentration: Plasma urea concentration was measured by using a coupled enzymatic UV-method (Test-Combination Urea Kit, Boehringer Mannheim) which was described previously by Gutmann & Bergmeyer (1974). The assay was based on the following reactions:

$$urea + H_2O \xrightarrow{urease} NH_3 + CO_2$$

$$2\,\alpha\text{-}oxoglutarate + 2NH_4 + 2NADH \xrightarrow{GLDH} 2L\text{-}glutamate + 2NAD^+ + 2H_2O$$

In the process, NADH is used to form NAD. The decrease in absorbance at 340 nm measured by spectrophotometry (Spectronic 1001 Bausch and Lomb) is directly proportional to the amount of urea in the sample. The standard curve was prepared by serial dilution of a known concentration of urea in water. Plasma (5 µl) was used directly in the assay. A blank was prepared from distilled water (5 µl). This was used to eliminate the system error.

Determination of ^{14}C-urea: ^{14}C-urea was determined using the modifications of the method described previously (Huskisson and Ward, 1978). Briefly, plasma (100 µl) was placed directly into counting vials. H_3PO_4 (0.5 ml, 1N) was added. This was left to stand (4°C, 18 hour). The reaction vials were sealed with rubber caps fitted with centre wells containing filter paper (Whatman no.1) and hyamine hydroxide (0.2 ml, ICN) and NaOH

(0.75 ml 1N) and urease (0.3 ml, 10 mg/3 ml H_2O) were injected into the vials. Twenty minutes later, 1 N HCl (1 ml) was injected into the vials. $^{14}CO_2$ released from the hydrolysis of ^{14}C-urea was trapped in centre wells. After an overnight refrigeration, the centre wells were cut into clean scintillation vials. Then double distilled water (0.8 ml) and scintillation fluid (15 ml, Formula 989) were added. Samples were counted on Scintillation Analyzer. Recovery of ^{14}C-urea was determined using standards which had gone through the same assay.

2.2.3. Calculations

As discussed in section 1.4.2., the rates of appearance (Ra) and metabolic clearance (MCR) of urea or glucose were calculated by using the equations (de Bodo et al., 1963; Ryan et al., 1993):

$$Ra = \frac{Ra^*}{SA} - \frac{pVC}{SA} * \frac{dSA}{dt} \qquad (2.2.1)$$

and

$$MCR = \frac{1}{C^*}[pV\frac{dC^*}{dt} - Ra^*] \qquad (2.2.2)$$

Where C^* is plasma concentration of ^{14}C-urea or ^{3}H-6-glucose. C is total plasma concentration of urea or glucose. V is the volume of distribution of urea (pV=100 ml/kg) or glucose (V=20% of body weight) , and p is the pool fraction of urea (p = 0.5) or glucose (p=0.65). Ra* is the rate of infusion of [^{14}C-U]-urea or [^{3}H-6]-glucose. SA is the

specific activity of plasma labelled urea or glucose (C^*/C).

Statistical analyses on plasma glucose and urea data were done using Dunn's procedure for *a priori* planned multiple comparisons. Plasma lactate data was compared with one-way ANOVA treating time as the repeated measure. The level of significance for each test was set at $p < 0.05$.

2.3. MUSCULAR PROTEIN BREAKDOWN DURING AND FOLLOWING PROLONGED SWIMMING IN RATS

2.3.1. Experimental Protocol

The experimental design used here was similar to that described in section 2.2.1, this was, however, done without tracers. There were four rats in the exercise group and six rats in the control group. Three muscles of both hindlimbs - soleus, white gastrocnemius and red gastrocnemius muscle which are composed of slow-twitch oxidative fibres, fast-twitch glycolytic and fast-twitch oxidative/glycolytic fibres, respectively, were excised and frozen clamped between aluminium tongs pre-cooled in liquid nitrogen at the end of the study (after 540 minutes). The plasma samples as well as the muscle biopsies were analyzed for 3-methylhistidine and tyrosine. Tissues were kept at -85°C before chemical assays.

2.3.2. Chemical Analysis

3-Methylhistidine and tyrosine concentrations in plasma and tissues obtained at the end of the study were measured by electrochemical detection of their o-phthaldialdehyde

(OPA) derivatives following reverse phase separation.

Extraction of amino acids from plasma samples for HPLC analysis

Plasma samples were deproteinized using a modification of the method previously described by Joseph and Davies (1983). To plasma (200 µl), methanol (800 µl) was added. After mixing, the samples were centrifuged (at 8,000 Xg, 5 minutes, room temperature) and an aliquot (0.9 ml) was evaporated to dryness under vacuum. The residue was dissolved in double distilled water (1 ml).

The samples were further purified by passing an aliquot (0.9 ml) of deproteinized samples down a Dowex 50W x 8 (OH^- form, Biorad California) mini column (0.3 x 1 cm). The anionic and neutral compounds were removed by washing the columns with degassed distilled water (3 ml). The amino acids were eluted using sodium borate (0.1 N, 4 ml). The elute was used in preparing the amino acid OPA derivatives.

Recovery was determined by running standards through the above procedure.

Extraction of amino acids from tissue samples for HPLC analysis

The method of Hogan et al. (1982) was used to extract amino acids from three skeletal muscles. Briefly, frozen tissues were weighed, cut into pieces and ground in 5-sulfosalicylic acid (4% w/v, 1 ml). The homogenized tissues were centrifuged (IEC Clinic Centrifuge at 8,000 Xg, 15 minutes, room temperature). An aliquot (0.9 ml) of the supernatant was neutralized with sodium hydroxide (0.1 N). The volume of the neutralized extract was brought up to 2.5 ml with double distilled water. An aliquot (2

ml) was applied to a Dowex 50W X 8 column as above.

Derivatization of amino acids in plasma and tissue samples

Amino acids can be separated by HPLC by using either 1) ion exchange columns with post-column derivatization or 2) reverse phase columns with pre-column derivatization. The latter was the method used here. The o-phthaldialdehyde (OPA, Sigma) derivatives were used. The OPA reacts with the amino acids to form an isoindole group which is electrochemically active. These derivatives are well retained and separated by reverse phase chromatography (Lindroth et al., 1979). The OPA-amino acid derivatization method described here is a modification of that used by Joseph and Davies (1983).

Preparation of OPA-2-mercaptoethanol reagent: The reagent was prepared by dissolving o-phthaldialdehyde (27 mg) (Sigma Chemical Co., St. Louis, Mo.) in absolute ethanol (0.5 ml) then adding sodium borate (5 ml, 0.1 M) and 2-mercaptoethanol (20 µl) (Sigma Chemical Co.). This solution was flushed with nitrogen and allowed to sit (room temperature, 24 hour) prior to use. 2-Mercaptoethanol (10 µl) was added every two days to maintain the maximal yield. Fresh reagent was prepared every two weeks.

Derivatization of the samples: To an aliquot (150 µl) of the plasma or tissue extract, the OPA-reagent (5 µl) was added. The solution was mixed well and allowed to stand (room temperature, one minute) to complete the reaction (Hodgin et al., 1979).

Quantitative analysis of amino acids by HPLC

The HPLC system used consisted of two constant flow liquid chromatography pumps (LCGA, Shimadzu, Kyoto, Japan); a gradient controller (SCL-6B, Shimadzu); an electrochemical detector, fitted with a glossy carbon electrode, applied potential 0.5 V and 100 namperes full scale deflection (Dionex, Sunnyvale, CA) and a recorder (Model 4270, Dionex, attenuation 1024, chart speed 0.25 cm/min). The system was fitted with an inline filter (5 μ, Waters Millipore, Milford MA) and a fixed volume injector (100 μl, Rheodyne).

Separation of the amino acids was achieved using a Licrosorb C-18, 5 μ column (4.5 x 250 mm, Phenomenex, Torrance, CA), fitted with a guard column of the same packing. The solvents used were: Solvent A, 0.02 M sodium acetate and 1% tetrahydrofuran (THF) in chromatography grade water and Solvent B, 1% THF in chromatography grade methanol. The column was equilibrated with 100% Solvent A prior to injecting the sample. The elution of the amino acids was achieved using a 2-step linear gradient; step 1, 0 to 15% solvent B over 5 minutes and step 2, 15 to 40% Solvent B over 45 minutes at a flow rate of 1.0 ml/min. After each injection, the column was washed (75% Solvent B, 25% Solvent A) and re-equilibrated with Solvent A.

Standard curves for 3-methylhistidine and tyrosine were prepared from serial dilutions of solutions containing known quantities of these amino acids. The standards went through the same derivatization and column separation process as the samples.

The location of the 3-MH and tyrosine in the sample chromatography was determined by: 1) comparing the retention times of the samples with those of the

standards and 2) comparing the chromatography of selected samples with and without the addition of standards.

The standard error of the assay was determined by repeated injections of the same standards or samples.

The STD errors calculated from repeated run of the same standard solution (n=6) through the assay are 4.57% and 2.26% for 3-MH and tyrosine, respectively. The STD errors calculated from repeated run of the same sample (n=6) through the assay are 5.26% and 5.30% for 3-MH and tyrosine, respectively.

2.3.3. Calculations

The calculation of 3-MH and tyrosine concentrations in plasma and tissues is based on the proportional relationship between the areas under the 3-MH and tyrosine peaks on the chromatogram and the areas and known concentrations of the standards. Plasma 3-MH and tyrosine concentrations are calculated by:

$$C_{p\text{-}3MH}\ (mg/ml) = \frac{24.2 * area_{p\text{-}3MH}}{slope_{p\text{-}3MH}} \qquad (2.3.1)$$

$$C_{p\text{-}tyr}\ (mg/ml) = \frac{24.2 * area_{p\text{-}tyr}}{slope_{p\text{-}tyr}} \qquad (2.3.2)$$

where: $C_{p\text{-}3MH}$ and $C_{p\text{-}tyr}$ are plasma 3-MH and tyrosine concentrations, $area_{p\text{-}3MH}$ and $area_{p\text{-}tyr}$ are the areas under 3-MH and tyrosine peaks of plasma sample chromatograms, respectively. 24.2 is the sample dilution. $Slope_{p\text{-}3MH}$ and $Slope_{p\text{-}tyr}$ are the slopes calculated from 3-MH and tyrosine standard curves, respectively.

Tissue 3-MH and tyrosine concentration were calculated as:

$$C_{T\text{-}3MH}\ (mg/g\ tissue) = \frac{5.56 * area_{T\text{-}3MH}}{slope_{T\text{-}3MH} * wt} \qquad (2.3.3)$$

$$C_{T\text{-}tyr}\ (mg/g\ tissue) = \frac{5.56 * area_{T\text{-}tyr}}{slope_{T\text{-}tyr} * wt} \qquad (2.3.4)$$

Where $C_{T\text{-}3MH}$ and $C_{T\text{-}tyr}$ are 3-MH and tyrosine concentrations in tissue sample solution. $area_{T\text{-}3MH}$ and $area_{T\text{-}tyr}$ are the areas under 3-MH and tyrosine peaks of tissue sample chromatograms, respectively. 5.56 is the dilution of tissue samples. $Slope_{T\text{-}3MH}$ and $Slope_{T\text{-}tyr}$ are the slopes calculated from 3-MH and tyrosine standard curves, respectively. wt, is the weight of tissues.

Statistical analyses on plasma 3-MH and tyrosine data were done using one-way ANOVA treating time as the repeated measure. Comparisons on muscle 3MH and tyrosine contents were done using one-way ANOVA of the means. The level of significance was set at $p < 0.05$.

2.4. AMINO ACIDS AS THE SOURCE OF SUBSTRATES FOR MUSCLE GLYCONEOGENESIS AFTER PROLONGED SWIMMING IN RATS

2.4.1. Experimental Protocol

The experimental design used in this study was identical with the one described previously in section 2.2.1. with the following exceptions. Instead of ^{14}C-urea, the radioisotope tracer [^{14}C-U]-threonine was infused together with [^{3}H-6]-glucose throughout the entire study. The priming dose for [^{14}C-U]-threonine was approximately 3.94 μCi which was followed by a constant intravenous infusion of approximately 0.03 μCi/min. At the end of the study, soleus, white gastrocnemius and red gastrocnemius muscles were taken and freeze-clamped for the assessment of the direct glycogen synthesis, total glycogen and radio-labelled glycogen contents. Tissues were kept at -85°C until assays.

2.4.2. Chemical Analysis

Measurement of plasma glucose.

The preparation of tracer standards, deproteinization of plasma, as well as the analyses of plasma total glucose and ^{3}H-glucose levels were the same as described in section 2.2.2.

Determination of plasma ^{14}C-glucose: Together with ^{3}H-glucose, ^{14}C-glucose was separated from labelled organic acids and amphoteric compounds by using ion exchange columns. To isolate the ^{14}C-glucose from other similarly labelled compounds in the water elute from the ion exchange column, the potassium gluconate was prepared using the method described by Jones and Stoodley (1963). An aliquot (2 ml) of the column elute

was mixed with carrier glucose (60 mg) and freeze-dried. The dried samples were dissolved in anhydrous methanol in a boiling water bath. Iodine (1 ml, 170 mg/ml methanol) was added to each sample. Potassium hydroxide solution (4% in anhydrous methanol) was then added dropwise over a period of 40 minutes until the mixture became colourless. The potassium gluconate was collected by filtration (Whatman No. 5). The wafer was dried and weighed into a tared scintillation counting vial. Double distilled water (1 ml) and scintillation fluid (15 ml Formula 989) were added. The samples were counted by Liquid Scintillation Analyzer. The recovery of the assay was determined by comparing the tritiated counts of column elute fraction from the ^{3}H-glucose assay and that obtained in the gluconate assay. Plasma ^{3}H-glucose and ^{14}C-glucose concentrations were calculated as:

$$d.p.m.[^{3}H]\text{-},[^{14}C]\text{-}glucose\ /\ ml = \frac{\frac{d.p.m.(^{3}H,^{14}C)*T.W.}{A.W.}*dilution}{recovery} \qquad (2.4.1)$$

$$recovery = \frac{wafer\ [^{3}H]\ d.p.m./ml\ plasma}{column\ eluate\ [^{3}H]\ d.p.m./ml\ plasma} \qquad (2.4.2)$$

Where T.W. is the theoretical weight (78 mg), and A.W. is the actual weight of the potassium gluconate wafers. The dilution is equal to the dilution of deproteinized sample (11/0.2) divided by the volume of column elute used in the gluconate assay (2 ml).

Measurement of tissue glycogen

Determination of total glycogen content in muscle: The method used was the modification of Hassid et al. (1957). Frozen tissue samples were weighed and digested in boiling KOH (2 ml, 30%) for 30 minutes. Glycogen was precipitated using ethanol (2.3 ml, 95%). After an overnight refrigeration, the samples were centrifuged (2000 r.p.m, 20 minutes) and pellets were washed with 60% ethanol (to remove any remaining free glucose or other impurities) and recentrifuged. The ethanol was decanted off and the final traces expelled by heating the tubes in boiling water. The glycogen was then suspended in HCl (2ml 1N) and heated in boiling water (2 hour) to hydrolyse the glycogen. The samples were cooled and made up to a final volume (2.0 ml) with double distilled water. Total glycogen (in the form of hydrolysed glucosyl units) was quantified by using a spectrophotometric coupled hexokinase method (Glucose SR Kits, MSA). The principal of the glucose assay is based on the following reactions:

$$D\text{-}glucose + ATP \xrightleftharpoons{HK} G\text{-}6\text{-}p + ADP$$

$$G\text{-}6\text{-}p + ATP + NAD^{+} \xrightarrow{G\text{-}6\text{-}PD} 6\text{-phosphogluconate} + NADH$$

The increase of absorbance at 340 nm is directly proportional to the amount of NADH produced, thus, proportional to glucose content in samples. The standard curve was prepared by serial dilutions of a standard glucose solution in HCl (1N).

Determination of [^{3}H]-, and [^{14}C]-glycogen (glucose): An aliquot (1ml) of the tissue extract was mixed with scintillation fluid (15 ml, Universal, ICN Biomedical Inc. U.S.A.). The level of radioactivity was determined.

2.4.3. Calculations

Tissue glycogen synthesized from the direct uptake of plasma glucose was calculated as:

$$Direct\ Synthesis = \frac{[^3H]\text{-}glycogen\ d.p.m.\ in\ tissue}{SA\ (plasma[^3H]\text{-}glucose)} \qquad (2.4.3)$$

Where SA is the average specific activity of ^{3}H-glucose in plasma (C^*/C) during the three hour recovery period.

Tissue ^{14}C-glycogen synthesized from the direct uptake of ^{14}C-glucose from plasma was calculated by:

$$Direct\ [^{14}C]\text{-}glycogen = \frac{tissue\ [^3H]\text{-}glycogen\ d.p.m.}{plasma\ \frac{[^3H]\text{-}glucose\ d.p.m.}{[^{14}C]\text{-}glucose\ d.p.m.}} \qquad (2.4.4)$$

Where the ratio of [^{3}H]/[^{14}C]-glucose in plasma is the average of those during the three-hour recovery. The gluconeogenic formation of glycogen (indirect glycogen) was assessed by calculating the differences between the total amount of ^{14}C-glycogen *versus* the amount of the direct synthesis of ^{14}C-glycogen during the recovery period.

Statistical comparisons on muscle glycogen data were performed with one-way ANOVA of the means (using the statistical package, SAS). The level of significance for all statistical tests was set at $P < 0.05$.

2.5. ROLE OF FAT: GLYCEROL AS A PUTATIVE SUBSTRATE FOR MUSCLE GLYCONEOGENESIS AFTER PROLONGED SWIMMING IN RATS

2.5.1. Experimental Protocol

The experimental design used here is identical with that described in section 2.4.1. with the following exception. Instead of a [^{14}C-U]-threonine infusion, [^{14}C-U]-glycerol was infused along with ^{3}H-6-glucose into the rats throughout the entire study. The prime dose for [^{14}C-U]-glycerol was 3.94 μCi, this followed by a constant intravenous infusion (approximately 0.03 μCi/min).

2.5.2. Chemical Analysis

The preparation of tracer standards, deproteinization of plasma samples, and the analyses of plasma total glucose and ^{3}H-glucose levels were the same as those described in section 2.2.2; plasma ^{14}C-glucose was determined as described in section 2.4.2; tissue total glycogen and labelled glycogen contents were determined as described in section 2.4.2.

2.5.3. Calculations

The tissue glycogen synthesized from the direct uptake of plasma glucose was calculated by the equation 2.4.3 (section 2.4.3). SA is the average specific activity of ^{3}H-glucose in plasma during recovery period. Tissue ^{14}C-glycogen arising from the direct uptake of ^{14}C-glucose from plasma was calculated by equation 2.4.4 (section 2.4.3), where the ratio of [^{3}H]/[^{14}C] in glucose is the average ratio during the recovery period. The

glyconeogenesis in muscle from systemic glycerol could be assessed by the difference between the total and direct ^{14}C-glycogen counts.

Statistical comparisons on muscle glycogen data were done with one-way ANOVA of the means. The level of significance for all tests was set at $P < 0.05$.

CHAPTER THREE

RESULTS

3.1. GLUCOSE TURNOVER

3.1.1. Rat Weight

1-2 day after catheter implant surgery, the rats normally lost 10-15 g of body weight. On the fourth day, the rats had recovered from surgery and weight had returned to presurgical levels. The pre-experiment overnight fast resulted in an average of 15 g weight loss. Therefore, the fasted pre-experimental weight of the exercise group was 206 ± 4 g (n=15), and that of the resting control group was 198 ± 5 g (n=15).

3.1.2. Plasma Glucose Concentration (Figure 3.1.A).

In the resting control group, there were no significant changes in plasma glucose levels during the entire experimental period. Within the two-hour basal period, plasma glucose levels were not different between resting control and exercise groups (1.04 ± 0.017 mg/ml vs. 1.04 ± 0.035 mg/ml, respectively, n=8). However, plasma glucose concentrations of exercising rats increased to 1.15 ± 0.085 mg/ml and 1.12 ± 0.09 mg/ml during the first two hours of the swimming period as compared with its basal levels, although this was not statistically significantly. In the last two hours of the exercise period, plasma glucose concentrations in the exercising group remained similar to those in the resting control group. Plasma glucose levels of the exercising rats increased again during the first hour of the post-exercise recovery (1.20 ± 0.077mg/ml), but the increases

Figure 3.1: Plasma glucose and lactate concentrations during rest, exercise and postexercise recovery in rats.

Plasma glucose (upper graph) and lactate concentrations (lower graph) were determined in rats during the two-hour basal, four-hour exercise (or four-hour rest) and the three-hour recovery periods. Rest control group is represented by the dashed lines, exercise group is represented by the solid lines. The data are expressed as means ± SEM. * significantly different from the resting controls ($P < 0.05$). $n = 8$ for both the resting control and the exercise groups.

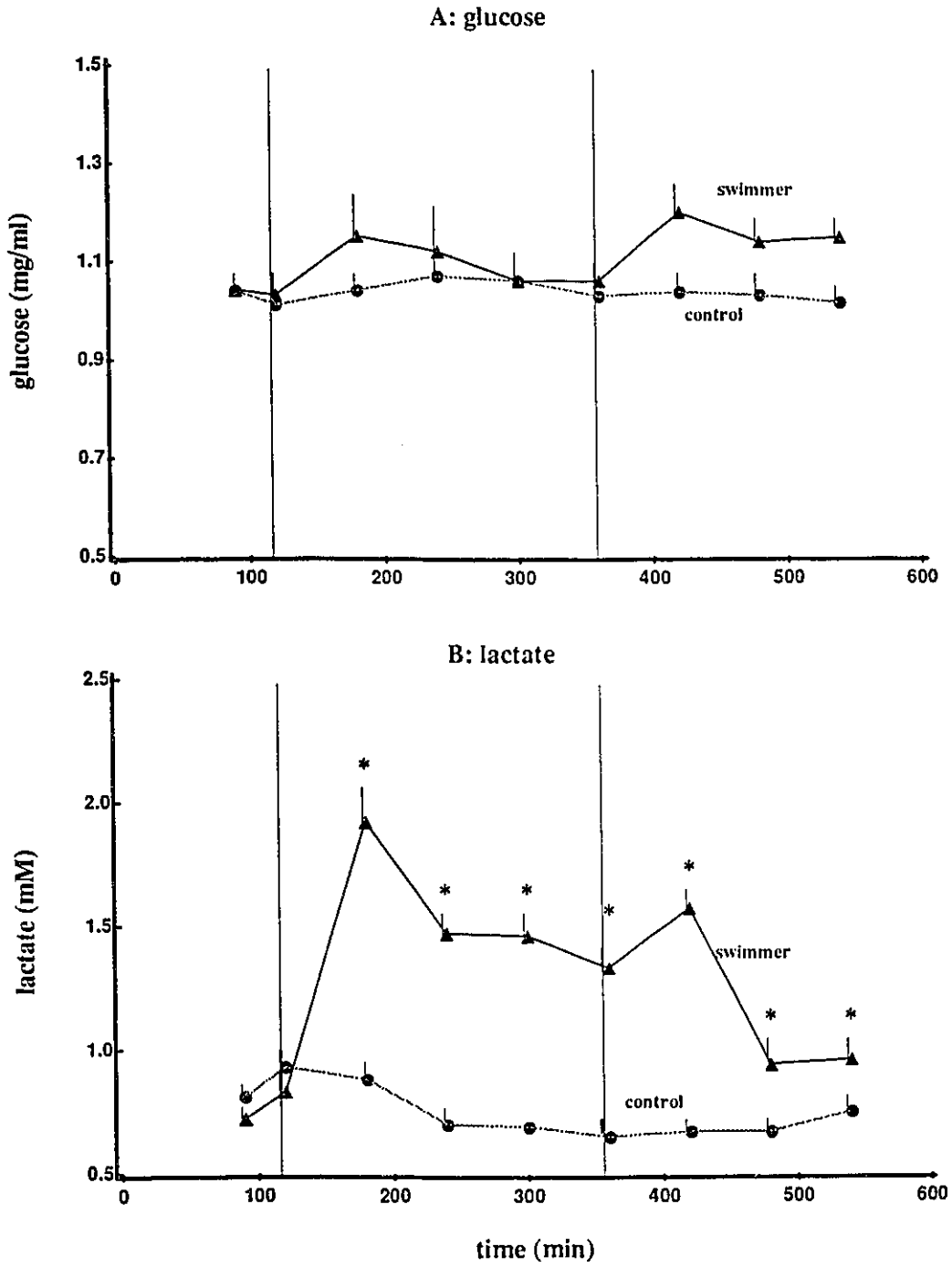


Figure 3.1: Plasma glucose and lactate concentrations during rest, exercise and recovery in rats

were not statistically significant. This level then gradually returned to the resting control level during the next two hours of recovery.

3.1.3. Plasma Lactate Concentration (Figure 3.1. B).

There were no significant changes in the resting controls throughout the experimental periods, and also there were no differences in plasma lactate levels between the resting control and exercise groups during the two-hour basal period (0.93 ± 0.05 mM vs. 0.83 ± 0.006 mM). However, exercise resulted in a doubling of the plasma lactate levels (up to 1.92 ± 0.06 mM) after the first hour of the exercise period. The plasma lactate concentrations declined gradually toward the basal levels, but remained significantly elevated during the next two hours of exercise and the three hours of the post-exercise recovery, as compared with those in the resting control group. After three hours of the recovery, the plasma lactate concentration reached 0.94 ± 0.06 mM.

3.1.4. Metabolic Clearance and Production of Glucose (Figure 3.2).

There were no significant changes in glucose fluxes in the resting control group over the experimental period. Ra in exercising rats doubled from the basal level (3.95 ± 0.036 mg/min v.s 1.612 ± 0.10 mg/min) after 80 minute swimming and remained elevated for the next three hours of exercise. There was a simultaneous doubling of glucose clearance during 80 minutes of the exercise (1.56 ± 0.10 ml/min to 3.40 ± 0.27 ml/min). MCR of glucose remained significantly elevated in the subsequent three hours of exercise. The Ra and MCR of glucose decreased after the initial peak and returned to the basal level after

Figure 3.2: Rates of glucose production and metabolic clearance during rest, exercise and postexercise recovery in rats.

The rates of glucose production (lower graph) and clearance (upper graph) were determined in rats during two-hour basal, four-hour exercise (or four-hour rest) and three-hour postexercise recovery with a constant ^{3}H-6-glucose infusion. Resting control group is represented by the dashed lines, and the exercise group is represented by the solid lines. The data are expressed as means ± SEM. *significantly different from the resting control group ($P < 0.05$). $n = 8$ for both the resting and the exercise groups.

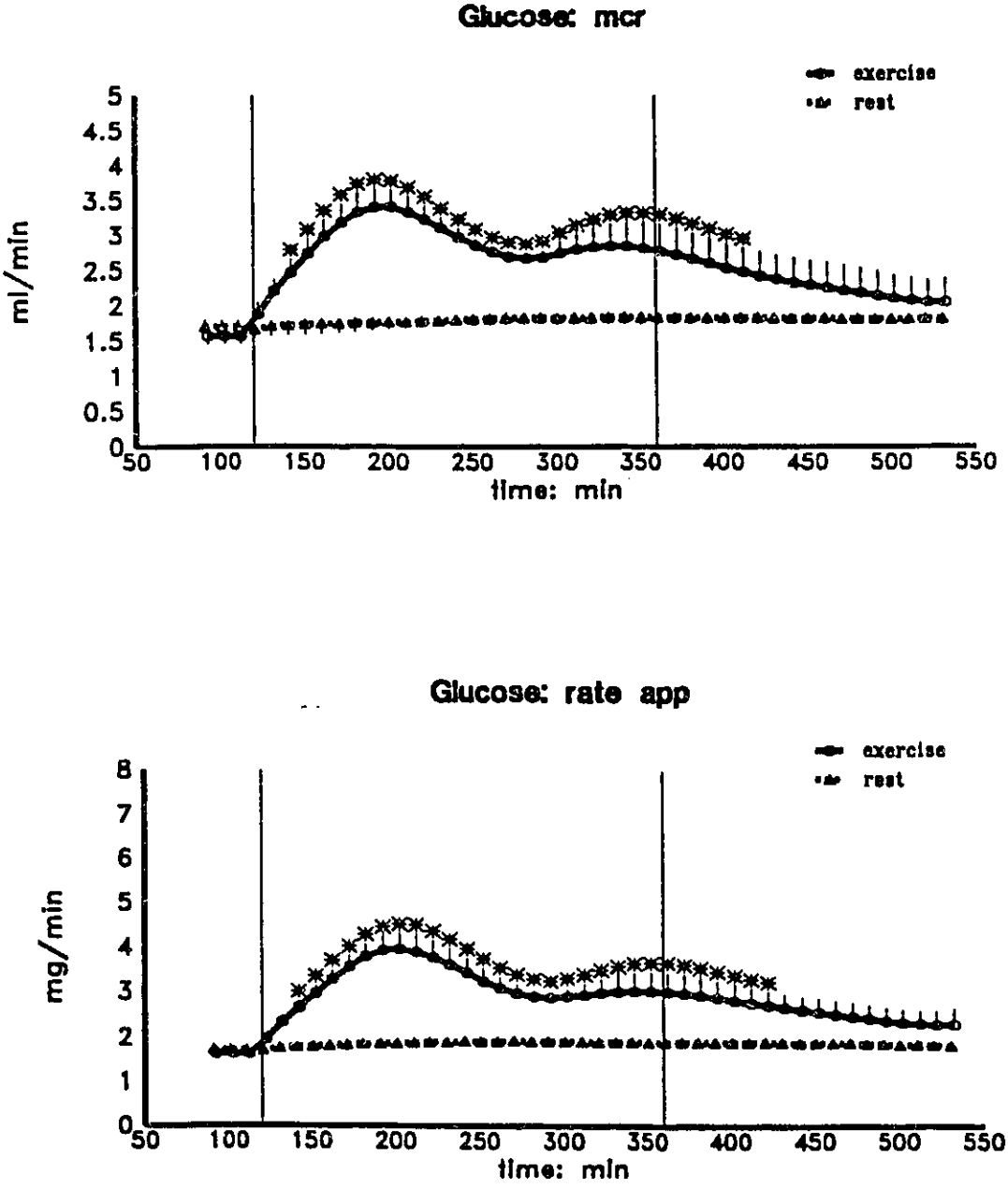


Figure 3.2: Rates of glucose production and metabolic clearance during rest, exercise and postexercise recovery in rats.

the three hours of recovery. A nearly matched increase in MCR and Ra of glucose during the exercising period led to a maintenance of plasma glucose concentrations during this period (Fig., 3.1,A).

3.2. UREA TURNOVER AND NET BODY PROTEIN CATABOLISM

3.2.1. Plasma Urea Concentration (Figure 3.3).

There was a slight, gradual decrease in plasma urea levels in the resting control group over the experimental period (from 2.00 ± 0.16 to 1.25 ± 0.12 mM). During the two-hour basal period, urea levels were not significantly different between the control and exercise groups (1.88 ± 0.18 mM vs. 1.61 ± 0.12 mM). However, the swim induced a significant increase in plasma urea levels during the first hour of exercise (up to 2.21 ± 0.12 mM). This level increased gradually, well into the subsequent three hours of the exercise period (up to 3.36 ± 0.27 mM) and the three hours of postexercise recovery, finally reaching a level nearly four-fold that of controls (4.26 ± 0.31 mM vs. 1.25 ± 0.12 mM).

3.2.2. Metabolic Clearance and Production of Urea (Figure 3.4).

There were no significant changes in the rate of urea appearance (Ra) nor in the rate of metabolic clearance (MCR) of urea in the resting control group over the experimental period. MCR of urea in the exercise group was maintained unchanged and was not significantly different from that of the control group during the entire experimental protocol. The Ra of urea in the exercising group increased gradually and reached a level which was significantly higher (from 8.01 ± 0.699 mg/min to 11.15 ± 0.719 mg/min)

Figure 3.3: Plasma urea concentrations during rest, exercise and postexercise recovery in rats.

Plasma urea concentrations were determined in rats during the two-hour basal, four-hour exercise (or four-hour rest) and the three-hour postexercise recovery periods. The resting control group is represented by the dashed lines and the exercise group is represented by the solid lines. The data are expressed as means ± SEM. * significantly different from the resting control group ($P < 0.05$). $n = 8$ for both the resting control and the exercise groups.

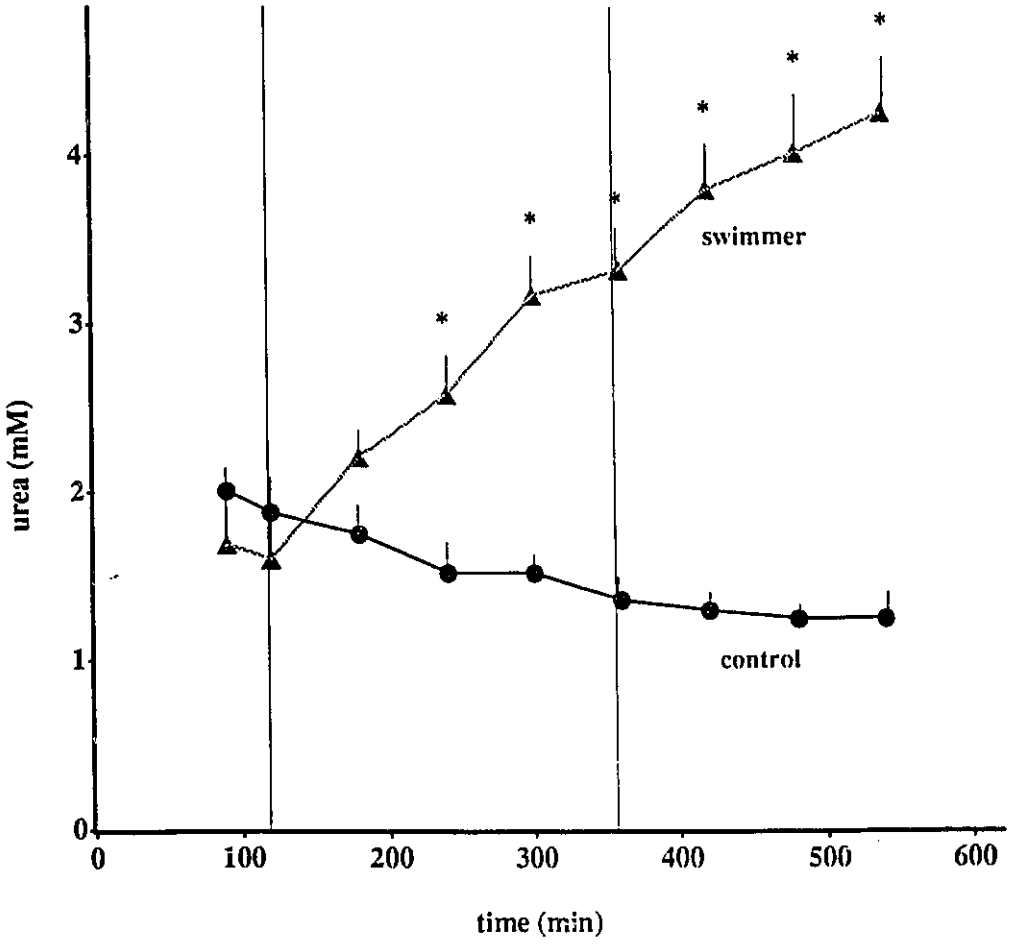


Figure 3.3: Plasma urea concentraions during rest, exercise and recovery in rats

Figure 3.4: Rates of urea production and metabolic clearance during rest, exercise and postexercise recovery in rats.

The rates of urea production (lower graph) and clearance (upper graph) were determined during the two-hour basal, four-hour exercise (or four-hour rest) and the three-hour postexercise recovery periods with a constant ^{14}C-U-urea tracer infusion into the rats. Resting control group is represented by the dashed lines and the exercise group is represented by the solid lines. The data are expressed as means ± SEM. * significantly different from the resting control group ($P < 0.05$). $n = 8$ for both the resting control and the exercise groups.

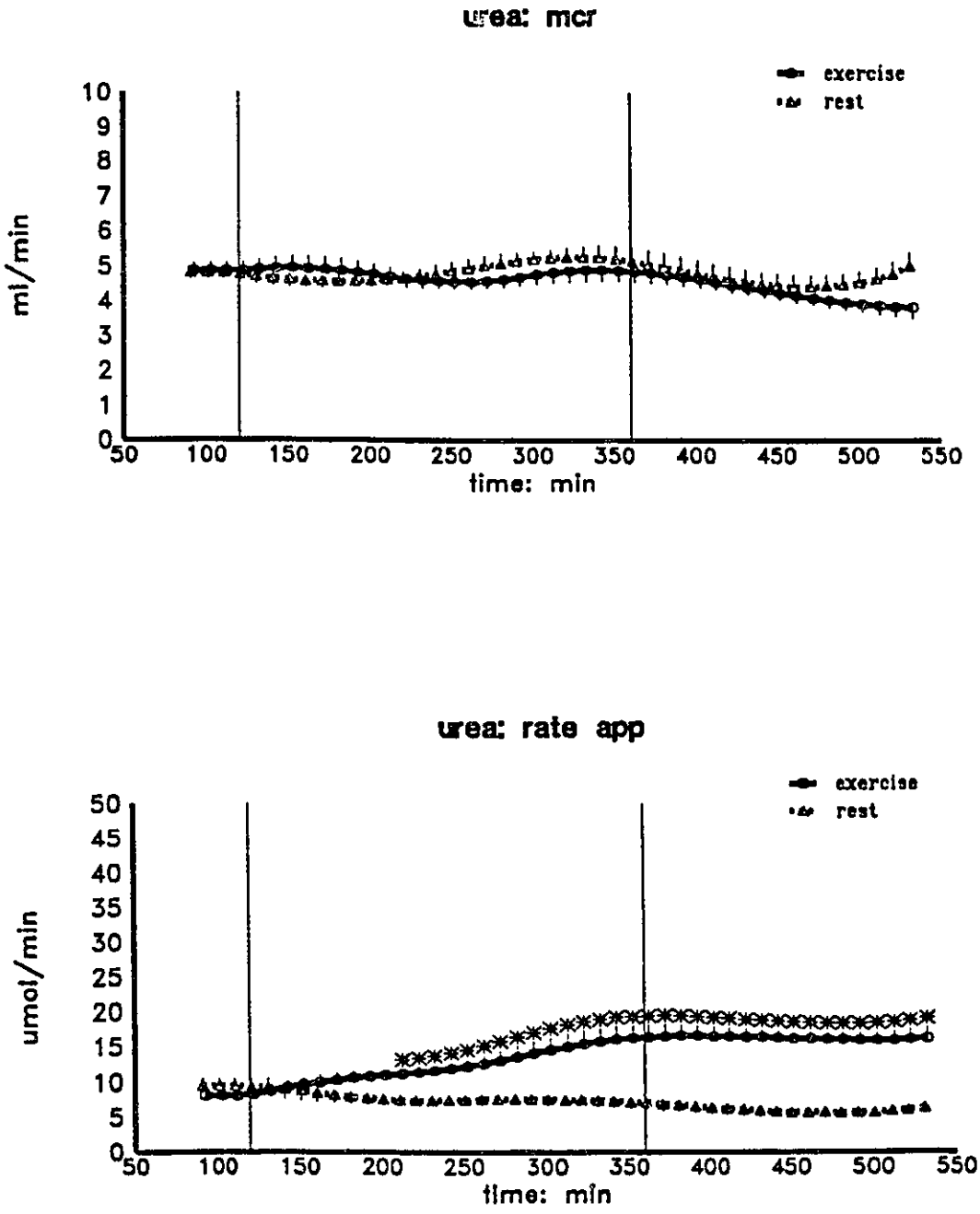


Figure 3.4: Rates of urea production and metabolic clearance during rest, exercise and postexercise recovery in rats.

during the first hour and thirty minutes of swimming. This level continued to increase during the subsequent three hours of swimming and the first thirty minutes of the recovery (up to 16.50 ± 1.517 mg/min). Thereafter it remained approximately two-times higher than its basal level for the next two hours and thirty minutes of postexercise recovery. The maintenance in MCR of urea together with the gradual increases in Ra of urea in the exercise group during the exercising and the postexercise recovery periods indicated that the increase in urea production accounted for the elevated plasma urea concentrations in exercised group (Fig. 3.3). This suggested a significant increase of body protein catabolism in the exercise group during prolonged swimming and at least three hours after exercise.

3.3. 3-METHYLHISTIDINE AND TYROSINE METABOLISM AND MUSCLE PROTEIN BREAKDOWN

3.3.1. Determination of Plasma 3-Methylhistidine and Tyrosine by HPLC (Figure 3.5, I and II).

The peaks of the chromatograms in Fig. 3.5, I and II indicate various amino acids in the standard solution of 3-MH and tyrosine (Fig. 3.5.I, upper graph), in the plasma samples (Fig. 3.5.II) and in the mixture of the plasma sample (at rest) and the standard solution (Fig. 3.5.I, lower graph). The peak of 3-MH occurred at a retention time of 25 minutes and the peak of tyrosine occurred at a retention time of 33 minutes in the standard chromatogram (Fig. 3.5.I, upper graph). For the mixture which contains a half volume of both the standard solution and plasma sample from a control rat (Fig. 3.5.I,

Figure 3.5.I: Determination of plasma 3-methylhistidine and tyrosine on High Performance Liquid Chromatography.

Upper curve is the amino acid chromatogram of the standard solution (both 3-methylhistidine and tyrosine are at the concentration of 0.000625 mg/ml). Lower curve is the amino acid chromatogram of the mixture solution which contains a half volume of the resting rat plasma sample (after seven-hour rest) and a half volume of the standard solution at concentration 0.0025 mg/ml for both of 3-MH and tyrosine.

Figure 3.5.II: HPLC separation of plasma amino acid in the rested and exercised rats.

The amino acids in plasma were determined after seven-hour rest for a resting rat (upper curve) and one hour after exercise for a exercise rat (lower curve).

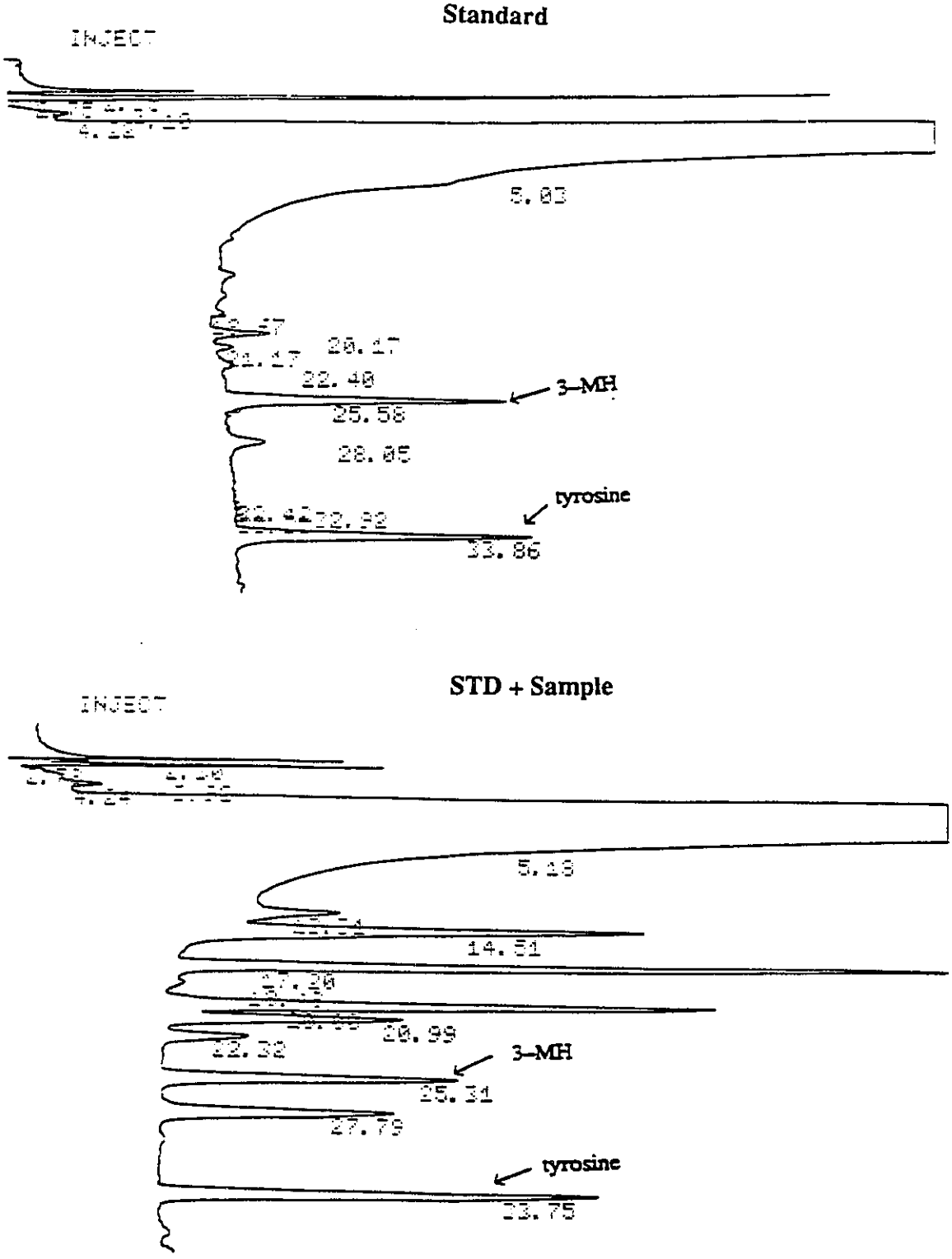


Figure 3.5.I: Determination of plasma 3-methylhistidine and tyrosine on HPLC.

Amino acids in plasma

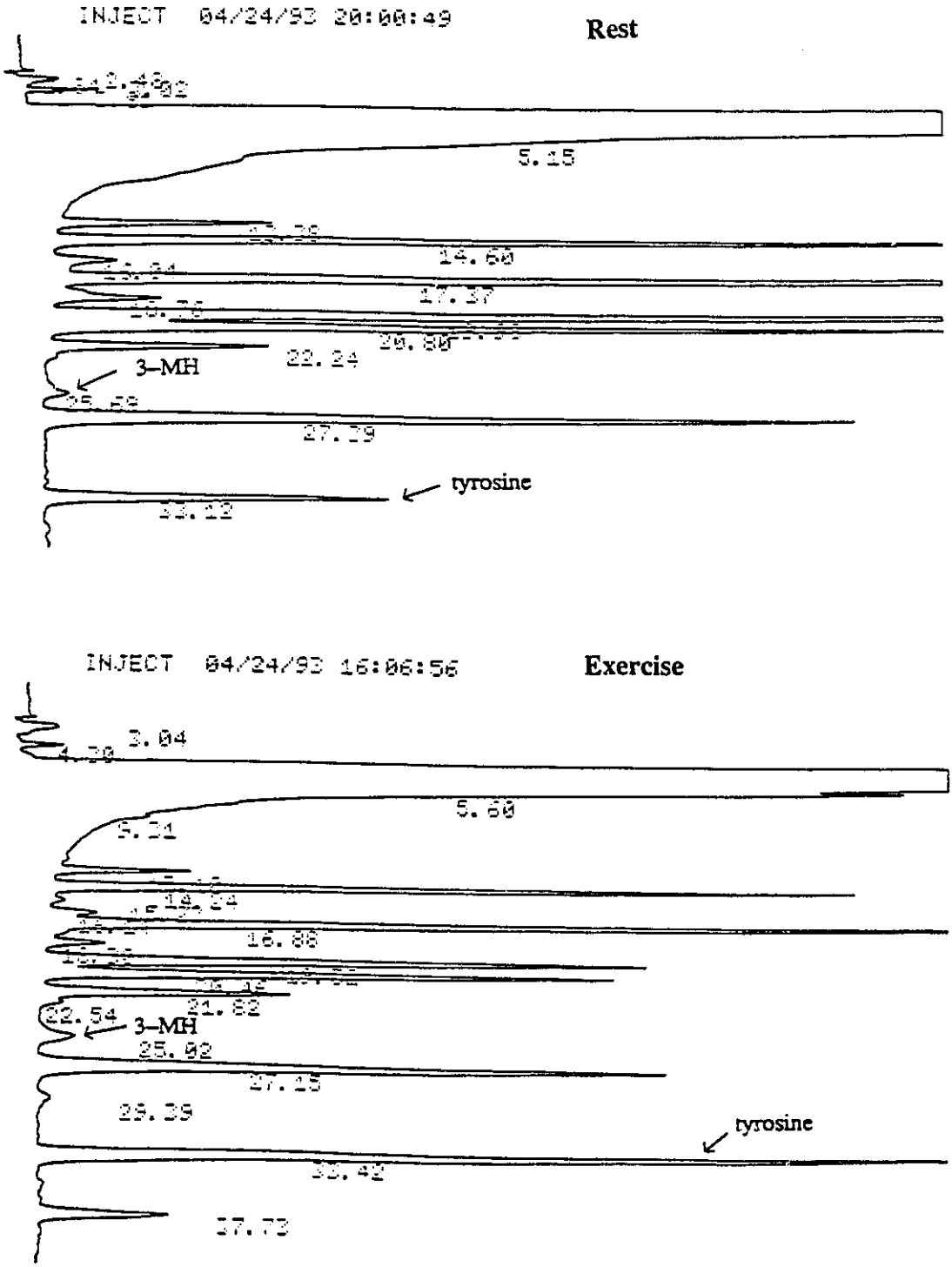


Figure 3.5.II: HPLC separation of plasma amino acids in rested and exercised rats.

lower graph), the retention times for the two amino acids were approximately the same as the corresponding retention times for the control plasma sample alone (Fig. 3.5.II, upper graph). Furthermore, only the two peaks at the retention times of 25 and 33 minutes were increased. The above observations confirmed that in the HPLC amino acid assay of the plasma sample, the peaks occurring at 25 and 33 minutes were 3-MH and tyrosine peaks respectively. In the exercising group (Fig. 3.5.II, lower graph), the areas under both 3-MH and tyrosine peaks were larger than those in resting control group (Fig. 3.5.II, upper graph). This indicates an increased plasma 3-MH and tyrosine concentrations in the exercising group.

3.3.2. Plasma 3-Methylhistidine Concentration (Figure 3.6).

There were no significant changes in plasma 3-MH concentration in resting controls over the entire experimental period (approximately 1.28 ± 0.021 μg/ml). The pre-exercise plasma 3-MH levels in the exercise group were not significantly different from those in resting control group (1.28 ± 0.021 μg/ml vs. 1.29 ± 0.050 μg/ml; 1.29 ± 0.011 μg/ml vs. 1.39 ± 0.073 μg/ml for the two samples taken during the basal period, respectively). However, plasma 3-MH concentrations increased in the exercising group starting from the first sample, one hour after the start of the exercise, reaching a peak level significantly elevated above the resting control levels (1.81 ± 0.181 μg/ml vs. 1.29 ± 0.012 μg/ml, $P < 0.05$). The 3-MH levels, then, declined gradually but still remained significantly higher than the control 3-MH levels during the next three hours of the swim. During recovery from the swim, plasma 3-MH reached a level double that seen in the basal period (2.31

Figure 3.6: Plasma 3-methylhistidine concentrations during rest, exercise and postexercise recovery in rats.

Plasma 3-MH concentrations were determined in rats during two-hour basal, four-hour exercise (or four-hour rest) and three-hour postexercise recovery periods. The resting control group is represented by the dashed lines and the exercise group is represented by the solid lines. The data are expressed as means ± SEM. * significantly different from the resting control group ($P < 0.05$). $n = 4$ for the exercise group and $n = 6$ for the resting control group.

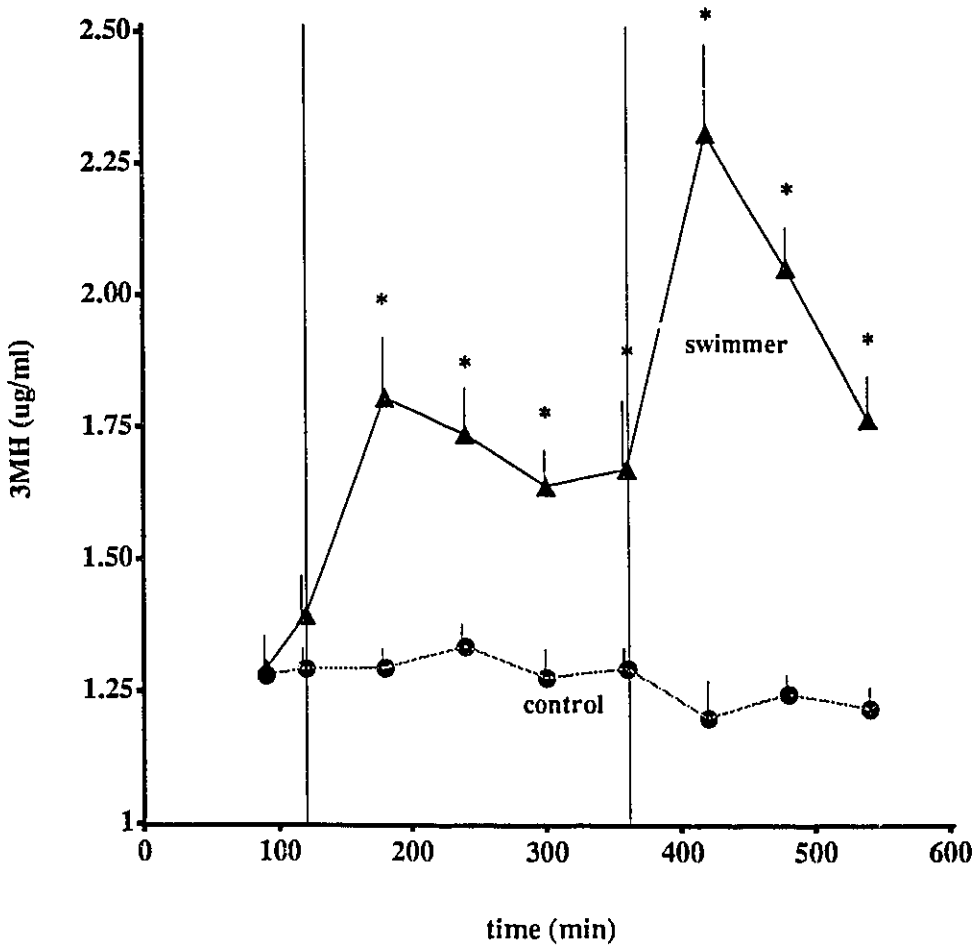


Figure 3.6: Plasma 3MH concentrations during rest, exercise and recovery in rats

± 0.127 μg/ml) after the first hour of the postexercise recovery. This level declined again during the subsequent two hour of the recovery to a level which remained significantly elevated as compared with that in resting controls (1.77 ± 0.060 μg/ml vs. 1.22 ± 0.017 μg/ml, $P < 0.05$).

3.3.3. Plasma Tyrosine Concentration (Figure 3.7).

Plasma tyrosine concentrations in the exercise group were not significantly different from those in the control group during the two hours of the pre-exercise period (9.84 ± 0.623 μg/ml vs. 7.44 ± 0.839 μg/ml). There were no significant changes in plasma tyrosine concentrations throughout the experimental period in the control group. However, the swim induced a doubling of plasma tyrosine levels to 19.34 ± 3.330 μg/ml, and 20.59 ± 5.40 μg/ml after one hour and two hours of the swim, respectively. The plasma tyrosine concentration then declined gradually to 15.85 ± 3.40 μg/ml after four hours of exercise. In a fashion similar to the plasma 3-MH concentrations, the tyrosine level increased again during the recovery period, reaching a secondary peak level (22.45 ± 5.23 μg/ml) after the first hour of recovery. During the next two hours of recovery period, plasma tyrosine level fell to 13.03 ± 3.11 μg/ml which was not significantly different from the control levels. The changes in the plasma tyrosine concentrations followed the same pattern as those of plasma 3-MH concentrations in the exercising group during the exercise and recovery periods.

Figure 3.7: Plasma tyrosine concentrations during rest, exercise and postexercise recovery in rats.

Plasma tyrosine concentrations were determined in rats during two-hour basal, four-hour exercise (or four-hour rest) and three-hour postexercise recovery periods. The resting control group is represented by the dashed lines and the exercise group is represented by the solid lines. The data are expressed as means ± SEM. * significantly different from the resting control group ($P < 0.05$). $n = 4$ for the exercise group and $n = 6$ for the resting control group.

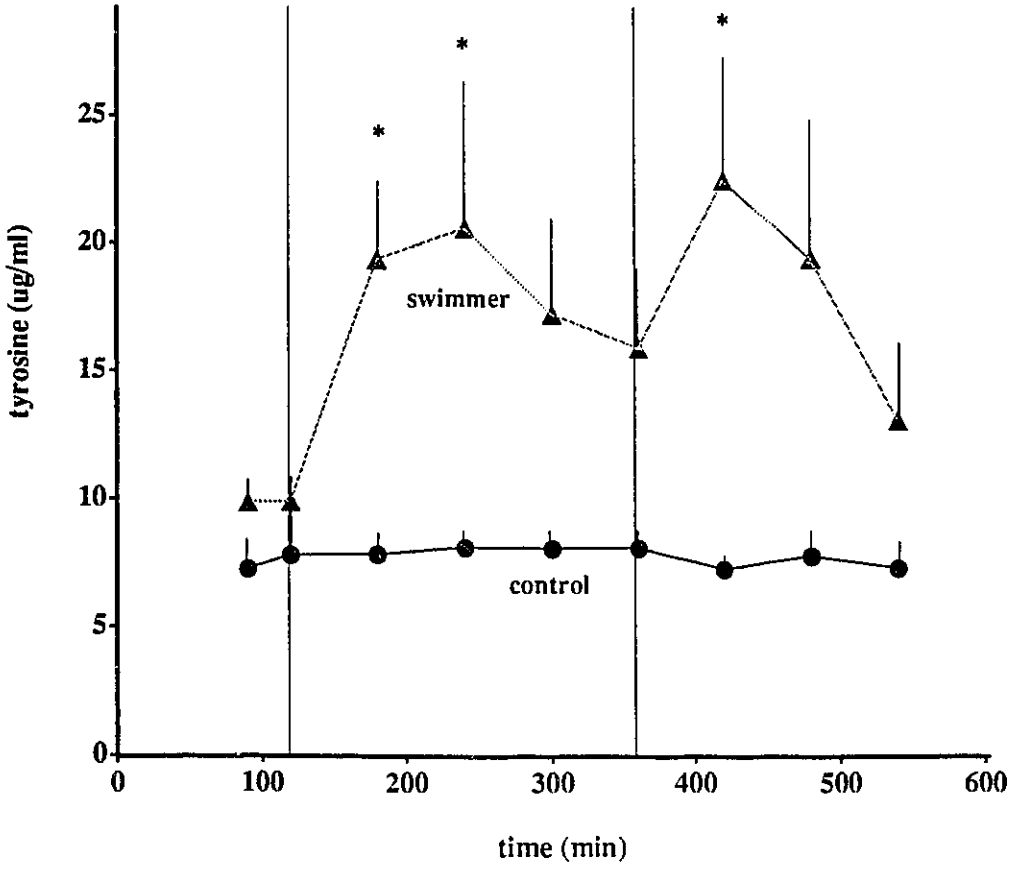


Figure 3.7: Plasma tyrosine concentrations during rest, exercise and recovery in rats

3.3.4. Determination of 3-Methylhistidine and Tyrosine contents in Muscle (Figure 3.8, I and II).

The peaks in Fig. 3.8.I and II represented various amino acids. All amino acids in tissue samples were determined by HPLC after a nine-hour rest or after three hours of the postexercise recovery. In the assay of the standard solution (Fig. 3.8.I, upper graph), 3-MH and tyrosine were eluted at the retention time near 29 min and 40 min, respectively. For the mixture which contains a half volume of tissue sample (rest) and a half volume of standard solution (Fig. 3.8.I, lower graph), the retention times for various amino acids were similar to the corresponding retention times for the rested muscle sample alone. Only the peaks at the retention times of 29 and 40 min were increased, however, the others were decreased, as compared with the peaks in chromatogram of the rest control tissue sample (Fig. 3.8.II, upper graph). Therefore, the two peaks eluted at the retention times of 29 and 40 minutes in the tissue samples indeed corresponded to 3-MH and tyrosine. In the chromatogram of the exercised red gastrocnemius muscle (Fig. 3.8. II), the areas under the 3-MH and the tyrosine peaks were greater than those of the controls. This indicated that 3-MH and tyrosine contents in the red gastrocnemius muscle increased after the three-hour postexercise recovery.

3.3.5. 3-Methylhistidine contents in muscles (Figure 3.9).

3-MH contents were determined after either a nine-hour rest for the control group or after the three-hour postexercise recovery period for the exercise group. There were no significant differences in 3-MH content between the resting control group and the exercise

Figure 3.8.I: Determination of muscle 3-methylhistidine and tyrosine on High Performance Liquid chromatography.

Upper curve is the amino acid chromatogram of the standard solution in which both 3-MH and tyrosine are at the concentration of 0.0025 mg/ml. Lower curve is the amino acid chromatogram of the mixture solution which contains a half volume of red gastrocnemius muscle sample from the resting rats and a half volume of the standard solution (at the concentration of 0.005 mg/ml for both 3-MH and tyrosine). The muscle sample was taken after nine-hour rest.

Figure 3.8.II: HPLC separation of muscle amino acids in rats after rest or postexercise recovery.

Amino acids in red gastrocnemius muscle were determined after nine-hour rest for a resting rat (upper curve) and after three-hour postexercise recovery for a exercise rat (lower curve).

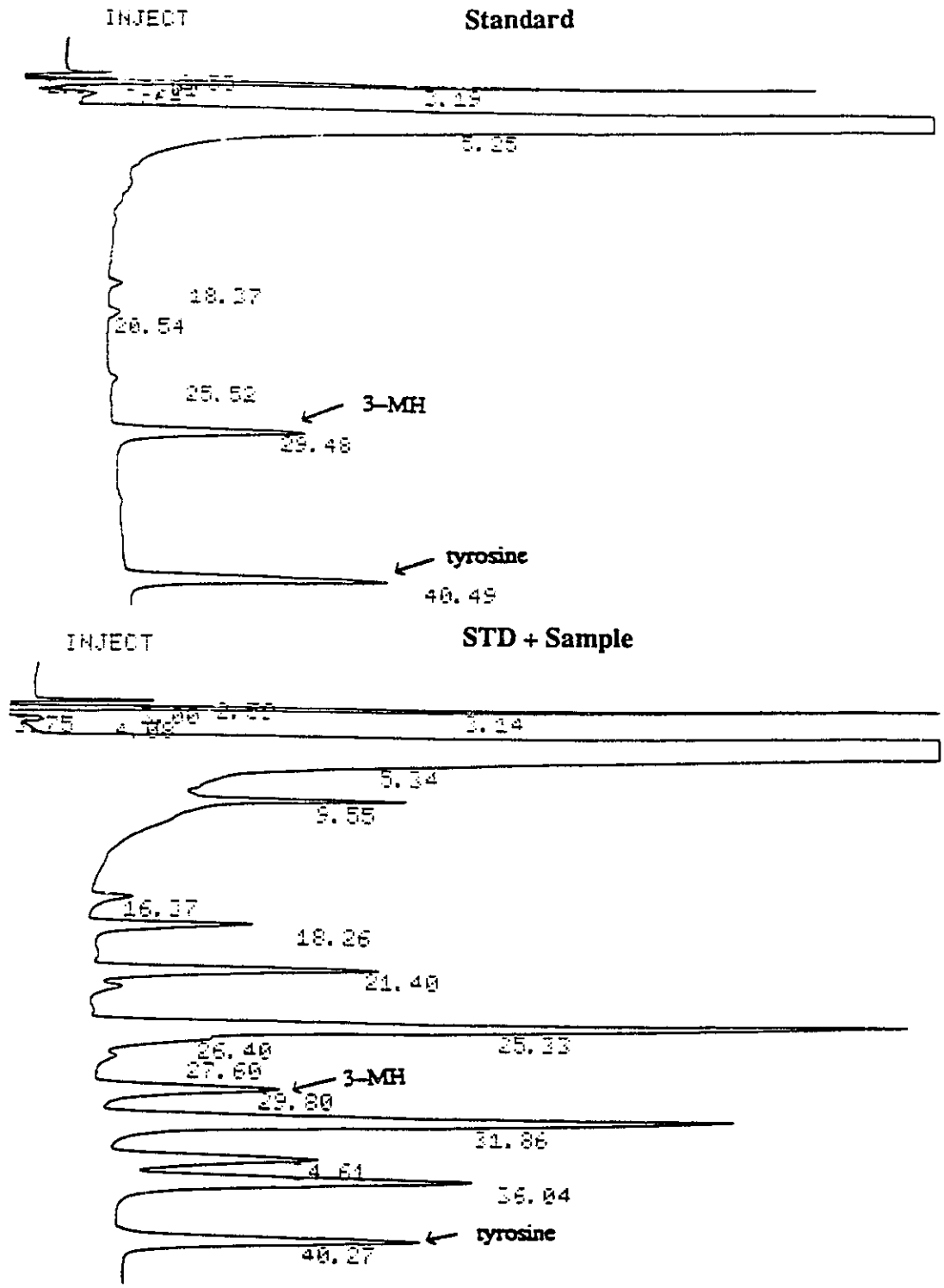


Figure 3.8.I: Determination of muslce 3-methylhistidine and tyrosine on HPLC.

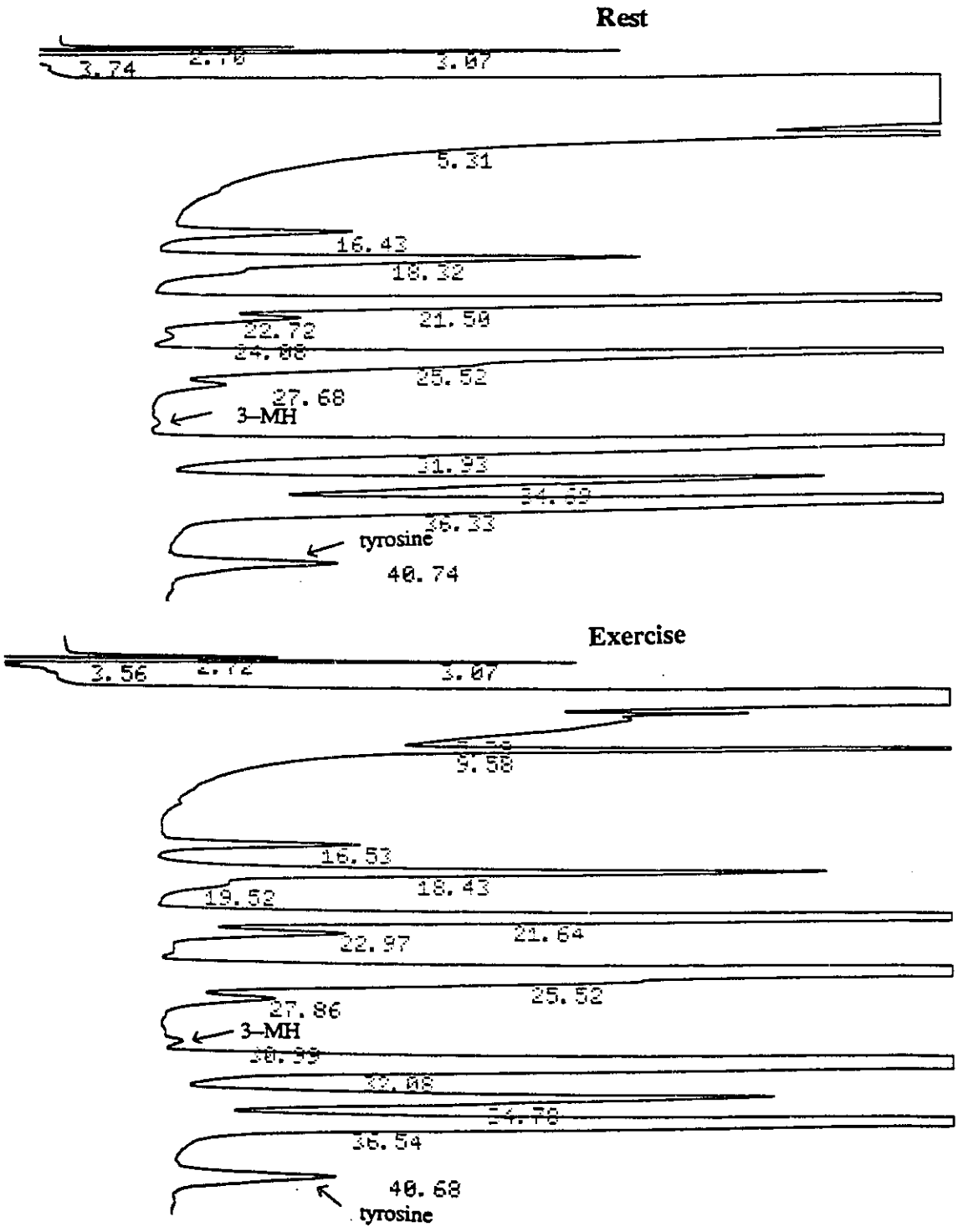


Figure 3.8.II: HPLC separation of muscle amino acids in rested and exercised rats.

Figure 3.9: 3-methylhistidine concentrations in muscles in rested and exercised rats after the recovery period.

3-methylhistidine concentrations were determined in soleus, white and red gastrocnemius muscles in the resting control group after the nine-hour rest or in the exercise group after three-hour postexercise recovery. The resting control group is represented by the open bars and the exercise group is represented by the filled bars. The data are expressed as means ± SEM. * significantly different from the resting control group ($P < 0.05$). $n = 4$ for the exercise group, and $n = 6$ for the resting control group.

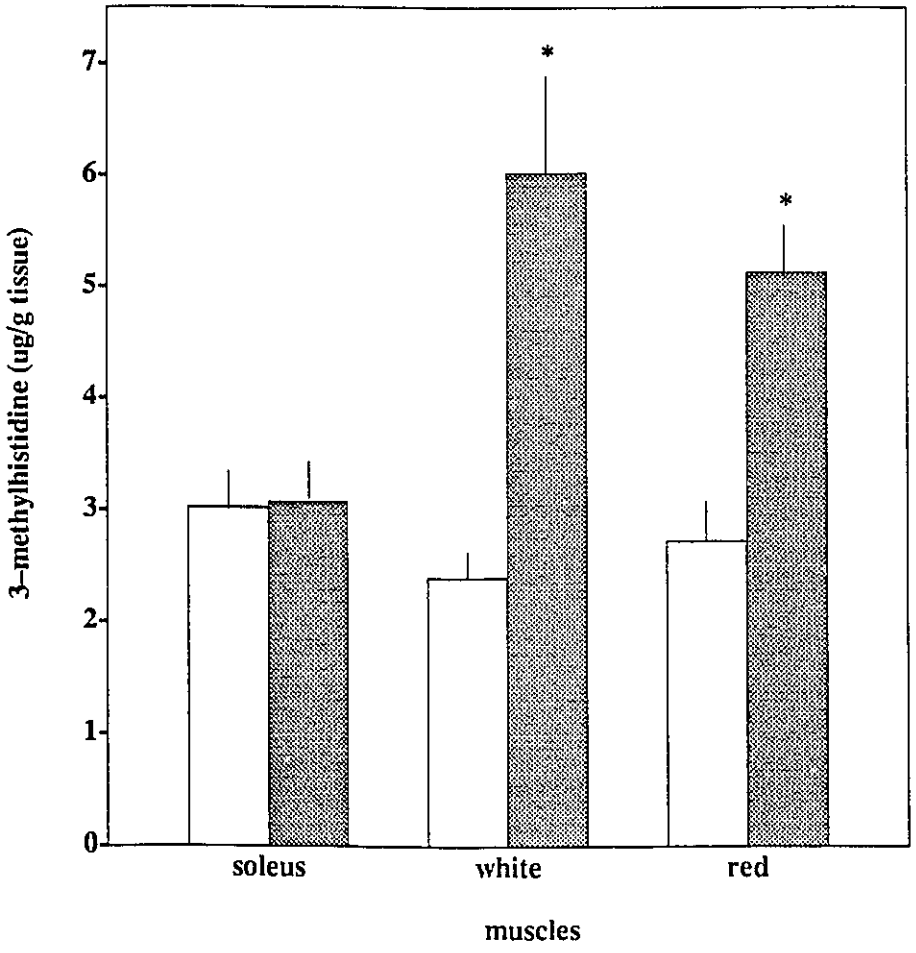


Figure 3.9: 3–MH concentrations of various tissues in rested and exercised rats after postexercise recovery period

Figure 3.10: Tyrosine concentrations in muscles in rested and exercised rats after the recovery period.

Tyrosine concentrations were determined in soleus, white and red gastrocnemius muscles in the resting control group after the nine-hour rest or in the exercise group after the three hour postexercise recovery. The resting control group is represented by the open bars and the exercise group is represented by the filled bars. The data are expressed as means ± SEM. n = 4 for the exercise group, and n = 6 for the resting control group.

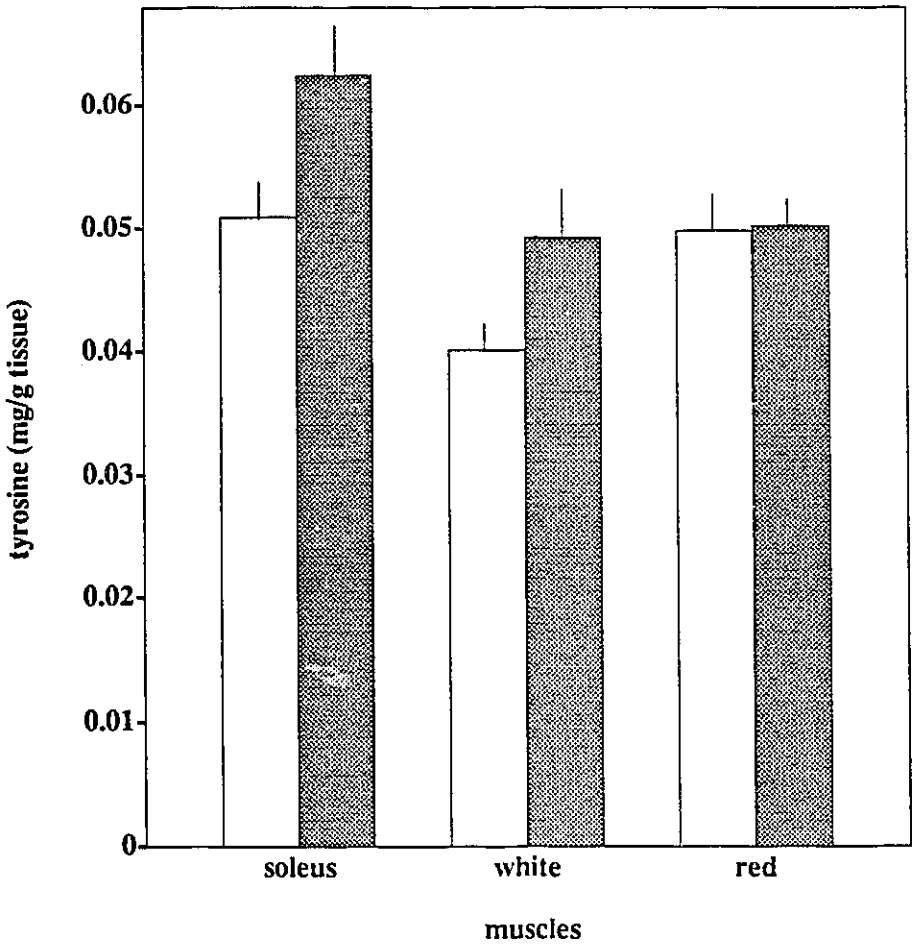


Figure 3.10: Tyrosine contents in muscles in rested and exercised rats after postexercise recovery period

group in the soleus muscle (3.02 ± 0.395 μg/g tissue vs. 3.07 ± 0.82 μg/g tissue). In white gastrocnemius muscle, 3-MH content was 2.46 ± 0.668 μg/g tissue in the rest control group and increased two and a half-fold to 6.02 ± 0.92 μg/g tissue in the exercise group. In red gastrocnemius muscle, 3-MH concentration in the rest control group was 2.65 ± 0.50 μg/g tissue. As in the white gastrocnemius muscle, the swim resulted in a doubling of 3-MH content (to 5.13 ± 0.29 μg/g tissue).

3.3.6. Tyrosine concentrations in muscles (Figure 3.10).

Tyrosine contents were determined either after a nine-hour rest for the control group or after three-hour postexercise recovery for the exercise group. Tyrosine content in the exercise group was not significantly different from that in the resting controls after the three-hour recovery in soleus muscle (62.5 ± 11.4 μg/g tissue vs. 51.3 ± 5.7 μg/g tissue); white gastrocnemius muscle (49.2 ± 8.0 μg/g tissue vs. 40.1 ± 6.3 μg/g tissue); and red gastrocnemius muscles (50.2 ± 2.6 μg/g tissue vs. 49.8 ± 6.4 μg/g tissue).

3.4. AMINO ACIDS AS SUBSTRATE FOR MUSCLE GLYCONEOGENESIS

3.4.1. Soleus Glycogen in ^{14}C-Threonine Infused Rats (Figure 3.11).

The upper panel of Fig. 3.11. shows the total d.p.m of ^{14}C-glycogen in soleus and that resulting from the direct uptake of ^{14}C-glucose from plasma (direct ^{14}C-glycogen). The lower panel shows the total soleus glycogen and the glycogen synthesized from the direct uptake of plasma glucose in either the resting control group after a nine-hour rest, or the exercise group after the three-hour postexercise recovery in rats infused with ^{3}H-

glucose and ^{14}C-threonine as the tracers.

RESTING CONTROL. There was a high glycogen turnover in the resting soleus muscle as indicated by the high counts of total ^{14}C-glycogen ($0.810 \times 10^4 \pm 0.078 \times 10^4$ dpm/g muscle) and the direct ^{14}C-glycogen ($0.830 \times 10^4 \pm 0.085 \times 10^4$ dpm/g muscle, upper panel), as well as the large amount of new glycogen synthesized via the direct route (1.16 ± 0.081 mg/g muscle, lower panel). This can be contrasted with the data in white and red gastrocnemius muscles in the resting state (follows). Specifically, the ^{14}C incorporation into glycogen in the soleus was ten times higher than that in white gastrocnemius muscle and 2.4-times higher than that in red gastrocnemius muscle (see Figure 3.13, Figure 3.14 upper panels). The ^{14}C-glycogen arising from the direct uptake of ^{14}C-glucose from plasma was not significantly different from total ^{14}C-glycogen in rested soleus muscle (upper panel). This suggested that there was no significant gluconeogenic formation of glycogen in rested soleus muscle in rats infused with ^{14}C-threonine. Total soleus glycogen content in the resting controls was 3.20 ± 0.262 mg/g muscle (lower panel). The glycogen made via the direct route was 1.10 ± 0.081 mg/g muscle.

EXERCISE GROUP. Exercise followed by recovery induced a significant increase in the incorporation of ^{14}C into glycogen. Total ^{14}C-glycogen in exercised soleus was increased by 54% relative to that in the resting controls (to $1.242 \times 10^4 \pm 0.095 \times 10^4$ dpm/g muscle, $P < 0.05$, upper panel), while the direct ^{14}C-glycogen rose 32% (to $1.094 \times 10^4 \pm 0.072 \times 10^4$ dpm/g muscle, $P > 0.05$). The ^{14}C-glycogen which could be accounted for by the direct uptake of plasma ^{14}C-glucose was lower than the total ^{14}C-glycogen in exercised soleus,

Figure 3.11: Soleus glycogen contents after rest or postexercise recovery in rats infused with ^{3}H-glucose and ^{14}C-U-threonine.

Soleus glycogen contents in ^{3}H-glucose and ^{14}C-threonine infused rested or exercised rats were determined after three hours of postexercise recovery. Upper panel: open bars are total ^{14}C-glycogen d.p.m. counts in soleus muscle, the filled bars are the ^{14}C-glycogen d.p.m. counts arising from the direct uptake of ^{14}C-glucose from plasma in soleus muscle. Lower panel: open bars are total glycogen concentrations in soleus muscle, the filled bars are the glycogen synthesized via the direct uptake of glucose from plasma in soleus muscle. All data are expressed as means ± SEM. *significantly different from the corresponding total ^{14}C-glycogen counts or total glycogen content, respectively ($P < 0.05$). +significantly different from the resting controls ($P < 0.05$). $n = 5$ for the exercise group , and $n = 6$ for the resting group.

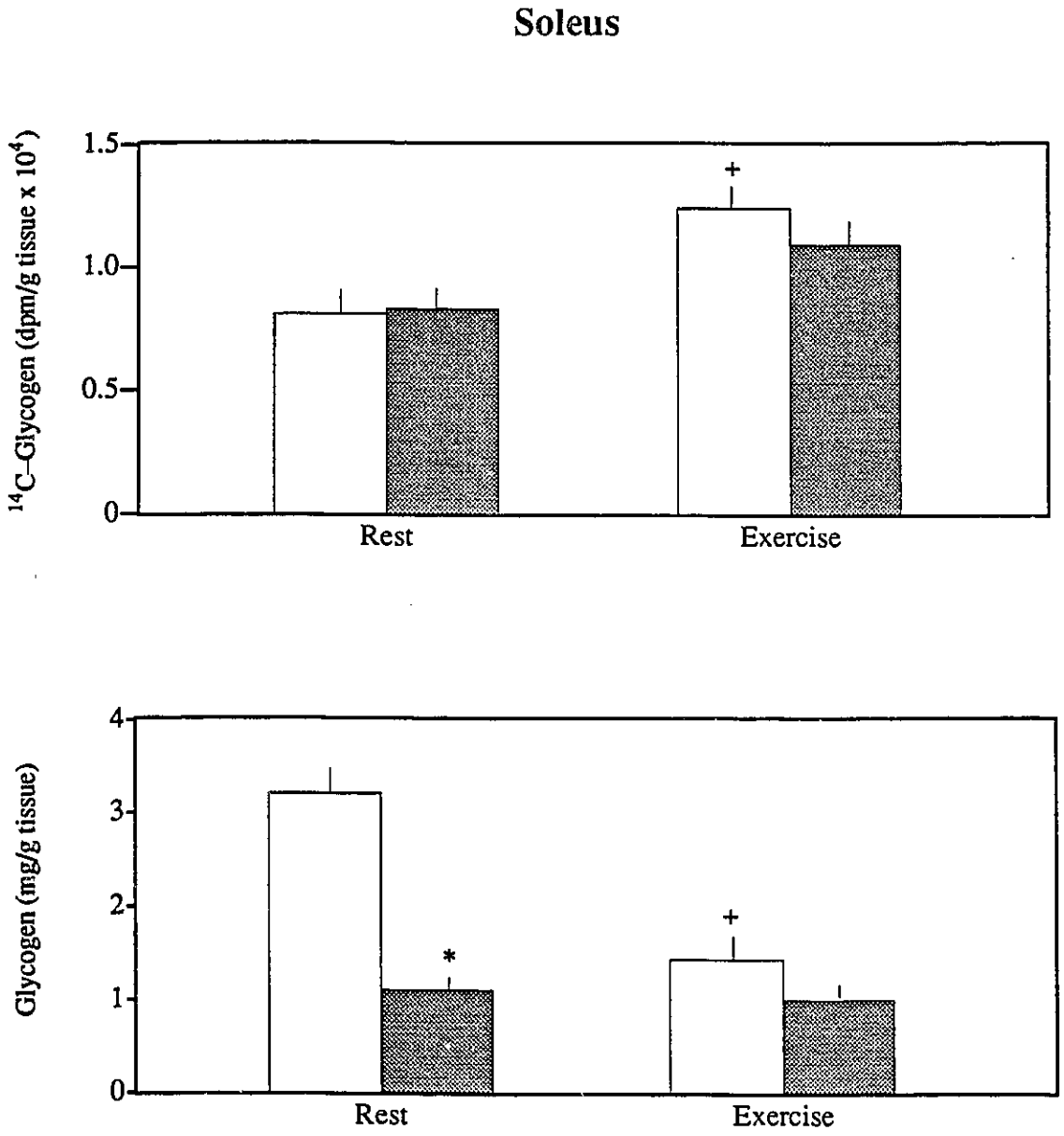


Figure 3.11 : Soleus glycogen contents after rest and postexercise recovery in rats infused with[^{3}H-6]-glucose and [^{14}C-U]-threonine

although this was not statistically significant (upper panel). Total soleus glycogen in the exercise group was 1.432 ± 0.023 mg/g muscle, significantly lower (55% lower) than that in rested soleus (lower panel, $P < 0.05$). The glycogen made via the direct route in the soleus was not significantly different between the control and exercise groups (1.11 ± 0.081 mg/g muscle *versus* 1.00 ± 0.092 mg/g muscle, lower panel). The direct glycogen synthesis in exercised soleus was 31% lower than the total glycogen.

3.4.2. White Gastrocnemius Glycogen in ^{14}C-Threonine Infused Rats (Figure 3.12).

RESTING CONTROL. In contrast to soleus muscle, there was a very low glycogen turnover in white gastrocnemius muscle in the resting state. This was evidenced by the very low incorporation of ^{14}C into glycogen ($0.081 \times 10^4 \pm 0.027 \times 10^4$ dpm/g muscle for total ^{14}C incorporation and $0.089 \times 10^4 \pm 0.032 \times 10^4$ dpm/g muscle for the direct ^{14}C incorporation, upper panel) as well as the small amount of the direct glycogen synthesis (0.14 ± 0.063 mg/g muscle, lower panel) in resting white gastrocnemius muscle, as compared with those in resting soleus and red gastrocnemius muscles. The lack of significant difference between total ^{14}C-glycogen and that resulting from the direct uptake of ^{14}C-glucose from plasma suggested that all of the new glycogen was made via the direct route in resting white gastrocnemius muscle. The total glycogen in white gastrocnemius muscle in the control group was 4.28 ± 0.225 mg/g muscle, which was higher than that in resting soleus muscle. The direct synthesis of glycogen was 0.14 ± 0.063 mg/g muscle. In the fasting rested state, the glycogen synthesis in white gastrocnemius muscle was extremely low as compared with that in soleus muscle.

Figure 3.12: White gastrocnemius glycogen contents after rest or postexercise recovery in rats infused with ^{3}H-glucose and ^{14}C-U-threonine.

White gastrocnemius glycogen contents in ^{3}H-glucose and ^{14}C-threonine infused rested or exercised rats were determined after three hours of postexercise recovery. Upper panel: open bars are total ^{14}C-glycogen d.p.m. counts in white gastrocnemius muscle, the filled bars are the ^{14}C-glycogen d.p.m. counts arising from the direct uptake of ^{14}C-glucose from plasma in white gastrocnemius muscle. Lower panel: open bars are total glycogen concentrations in white gastrocnemius muscle, the filled bars are the glycogen synthesized via the direct uptake of glucose from plasma in white gastrocnemius muscle. All data are expressed as means ± SEM. *significantly different from the corresponding total ^{14}C-glycogen counts or total glycogen content, respectively ($P < 0.05$). +significantly different from the resting controls ($P < 0.05$). $n = 5$ for the exercise group, and $n = 6$ for the resting group.

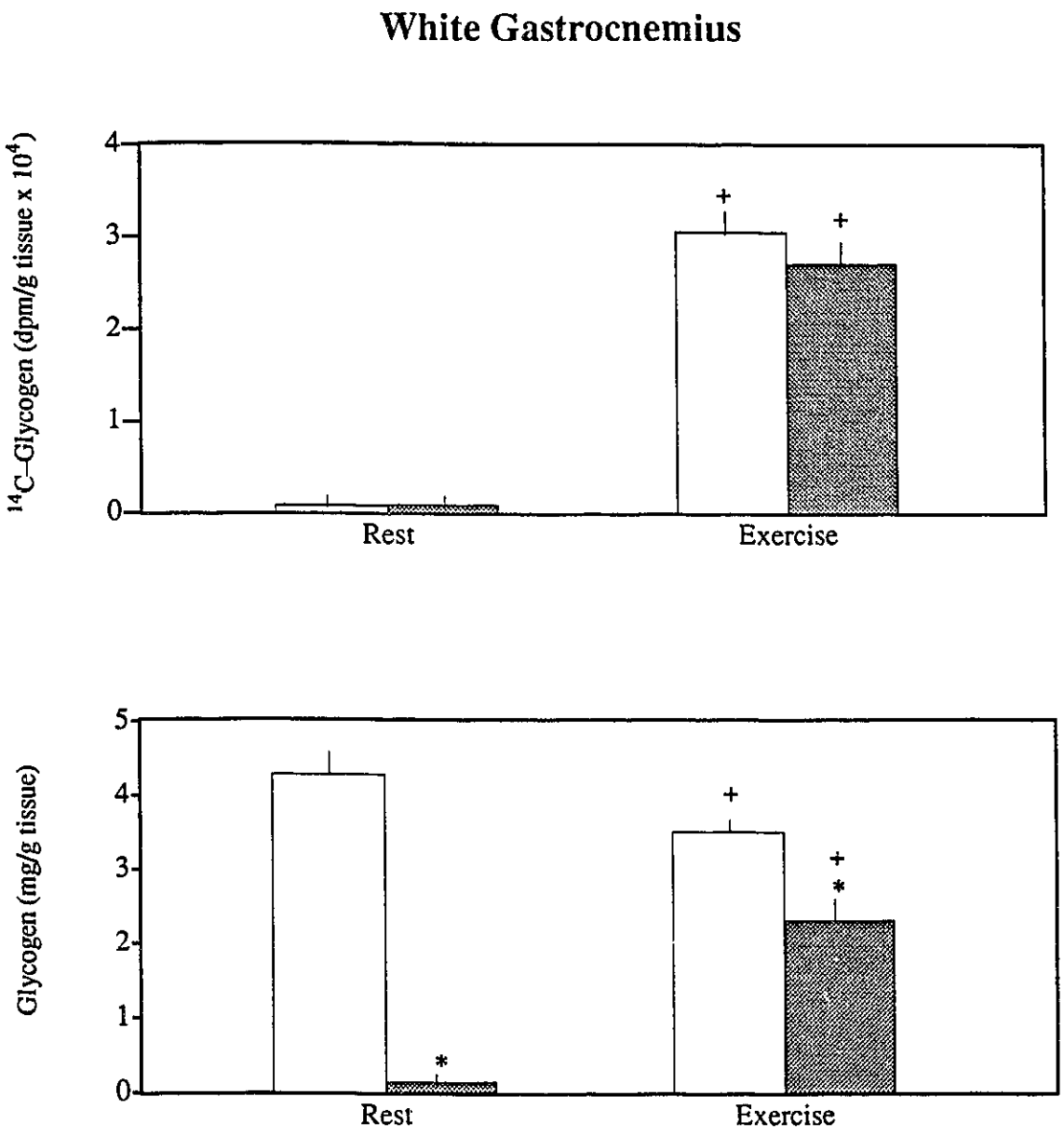


Figure 3.12 : White gastrocnemius glycogen contents after rest and postexercise recovery in rats infused with[^{3}H–6]–glucose and [^{14}C–U]–threonine

EXERCISE GROUP. Exercise and recovery induced a large increase in the incorporation of ^{14}C into glycogen in white gastrocnemius muscle. Total ^{14}C-glycogen rose to $3.058x10^4 \pm 0.410x10^4$ dpm/g muscle (upper panel, $P < 0.05$), which was 37-times that in resting white gastrocnemius muscle. The direct ^{14}C-glycogen rose to $2.717x10^4 \pm 0.281x10^4$ dpm/g muscle, which was 30-times that in rested white gastrocnemius muscle. This indicates a significant increase of glycogen synthesis in white gastrocnemius muscle during recovery from exercise. The direct ^{14}C-glycogen was lower than total ^{14}C-glycogen in exercised white gastrocnemius muscle, but this difference was not statistically significant. The direct ^{14}C-glycogen could account for 90% ± 2.4% of total ^{14}C-glycogen in this muscle. The total glycogen concentration in exercised white gastrocnemius muscle was 3.51 ± 0.097 mg/g muscle after three hours of postexercise recovery, and remained significantly lower (18% lower) than that in the control group (lower panel). The direct synthesis of glycogen in exercised white muscle, however, was 16-fold higher than that in resting controls. The direct glycogen concentration was 34% lower ($P < 0.05$) than the total glycogen concentration in exercised white gastrocnemius muscle (lower panel).

3.4.3. Red Gastrocnemius Glycogen in ^{14}C-Threonine Infused Rats (Figure 3.13).

RESTING CONTROL. Similarly to the white gastrocnemius muscle, the glycogen turnover in red gastrocnemius muscle in ^{14}C-threonine infused rats was low in the fasting rested state. This glycogen turnover was higher than that in the white gastrocnemius muscle and was lower than that in the soleus muscle as represented by the intermediate amounts of ^{14}C incorporation into glycogen (upper panel) and the intermediate amounts

of the direct glycogen synthesis (lower panel) in red gastrocnemius muscle as compared with that in white gastrocnemius and soleus muscles. There was no significant difference between the direct and total ^{14}C-glycogen counts in rested red gastrocnemius muscle ($0.349 \times 10^4 \pm 0.056 \times 10^4$ dpm/g muscle *versus* $0.381 \times 10^4 \pm 0.077 \times 10^4$ dpm/g muscle, respectively, upper panel). This suggested that there was no significant glyconeogenesis from threonine in rested red gastrocnemius muscle in ^{14}C-threonine infused rats. Total glycogen concentration in red gastrocnemius muscle in resting controls was 3.83 ± 0.298 mg/g muscle, which was between soleus and white gastrocnemius glycogen concentrations (lower panel). The synthesis of glycogen by the direct uptake of plasma glucose was 0.56 ± 0.115 mg/g muscle, a value between that obtained in the soleus and white gastrocnemius muscle.

EXERCISE GROUP. The incorporation of ^{14}C into glycogen in red gastrocnemius muscle was significantly stimulated by the prolonged swimming and recovery. Total ^{14}C-glycogen in exercised red gastrocnemius muscle ($2.783 \times 10^4 \pm 0.091 \times 10^4$ dpm/g muscle) was eight-times that in the resting controls (upper panel). The direct ^{14}C-glycogen in red gastrocnemius muscle ($2.483 \times 10^4 \pm 0.067 \times 10^4$ dpm/g muscle) was 6.5-times that in the resting controls. The ^{14}C-glycogen synthesized by the direct route was significantly lower than the total ^{14}C-glycogen in red gastrocnemius muscle (upper panel, $P < 0.05$). This direct ^{14}C-glycogen could account for $89.4\% \pm 2.4\%$ of total ^{14}C-glycogen. These data suggested that glyconeogenesis from threonine did occur during recovery in exercised red gastrocnemius muscle, in contrast to the situation at rest. The total glycogen concentration

Figure 3.13: Red gastrocnemius glycogen contents after rest or postexercise recovery in rats infused with ^{3}H-glucose and ^{14}C-U-threonine.

Red gastrocnemius glycogen contents in ^{3}H-glucose and ^{14}C-threonine infused rested or exercised rats were determined after three hours of postexercise recovery. Upper panel: open bars are total ^{14}C-glycogen d.p.m. counts in red gastrocnemius muscle, the filled bars are the ^{14}C-glycogen d.p.m. counts arising from the direct uptake of ^{14}C-glucose from plasma in red gastrocnemius muscle. Lower panel: open bars are total glycogen concentrations in red gastrocnemius muscle, the filled bars are the glycogen synthesized via the direct uptake of glucose from plasma in red gastrocnemius muscle. All data are expressed as means ± SEM. *significantly different from the corresponding total ^{14}C-glycogen counts or total glycogen content, respectively ($P < 0.05$). +significantly different from the resting controls ($P < 0.05$). $n = 5$ for the exercise group, and $n = 6$ for the resting group.

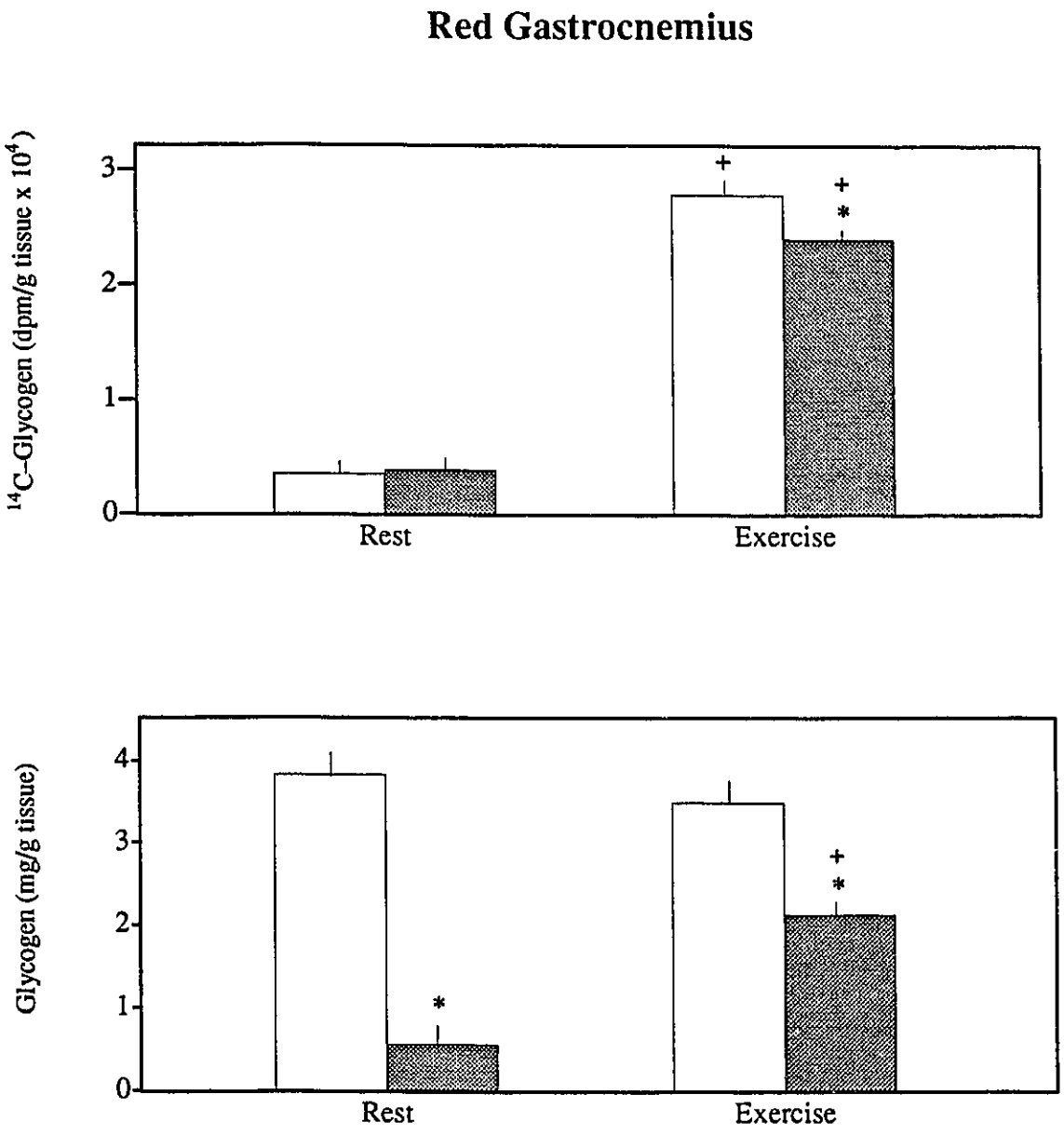


Figure 3.13 : Red gastrocnemius glycogen contents after rest and postexercise recovery in rats infused with[^{3}H–6]–glucose and [^{14}C–U]–threonine

in red gastrocnemius muscle after three-hour postexercise recovery was 3.49 ± 0.171 mg/g muscle (lower panel), a value which was slightly lower than that in the resting controls, but not statistically significant. The direct synthesis of glycogen in exercised red muscle was 2.26 ± 0.089 mg/g muscle, which was four-times higher than that in the resting muscle, and was significantly lower (35% lower) than total glycogen in exercised red gastrocnemius muscle after the three-hour recovery (lower panel, $P < 0.05$).

3.5. ROLE OF FAT: GLYCEROL AS A PUTATIVE SUBSTRATE FOR MUSCLE GLYCONEOGENESIS

3.5.1. Soleus Glycogen in ^{14}C-Glycerol Infused Rats (Figure 3.14).

The upper panel of Figure 3.14. indicates the total d.p.m. of ^{14}C-glycogen in soleus and that formed from the direct uptake of ^{14}C-glucose from plasma. The lower panel shows the total soleus glycogen concentrations and the glycogen synthesized by the direct uptake of plasma glucose in either the resting control group after a nine-hour rest, or the exercise group after the three-hour postexercise recovery in rats infused with ^{3}H-glucose and ^{14}C-glycerol as the tracers.

RESTING CONTROL. Similar to ^{14}C-threonine infused rats, there was a high glycogen turnover in the rested soleus muscle as evidenced by the high ^{14}C incorporation into glycogen (upper panel) and the relatively large amount of the direct glycogen synthesis (lower panel) in the rested soleus, as compared with that in white and red gastrocnemius muscles in ^{14}C-glycerol infused rats (see Fig. 3.15 and Fig 3.16). There was no difference between the total and the direct ^{14}C-glycogen in the resting soleus muscle (upper panel).

Figure 3.14: Soleus glycogen contents after rest or postexercise recovery in rats infused with ^{3}H-glucose and ^{14}C-U-glycerol.

Soleus glycogen contents in ^{3}H-glucose and ^{14}C-glycerol infused rested or exercised rats were determined after three hours of postexercise recovery. Upper panel: open bars are total ^{14}C-glycogen d.p.m. counts in soleus muscle, the filled bars are the ^{14}C-glycogen d.p.m. counts arising from the direct uptake of ^{14}C-glucose from plasma in soleus muscle. Lower panel: open bars are total glycogen concentrations in soleus muscle, the filled bars are the glycogen synthesized via the direct uptake of glucose from plasma in soleus muscle. All data are expressed as means ± SEM. *significantly different from the corresponding total ^{14}C-glycogen counts or total glycogen content, respectively ($P < 0.05$). +significantly different from the resting controls ($P < 0.05$). $n = 5$ for both exercise and the resting groups.

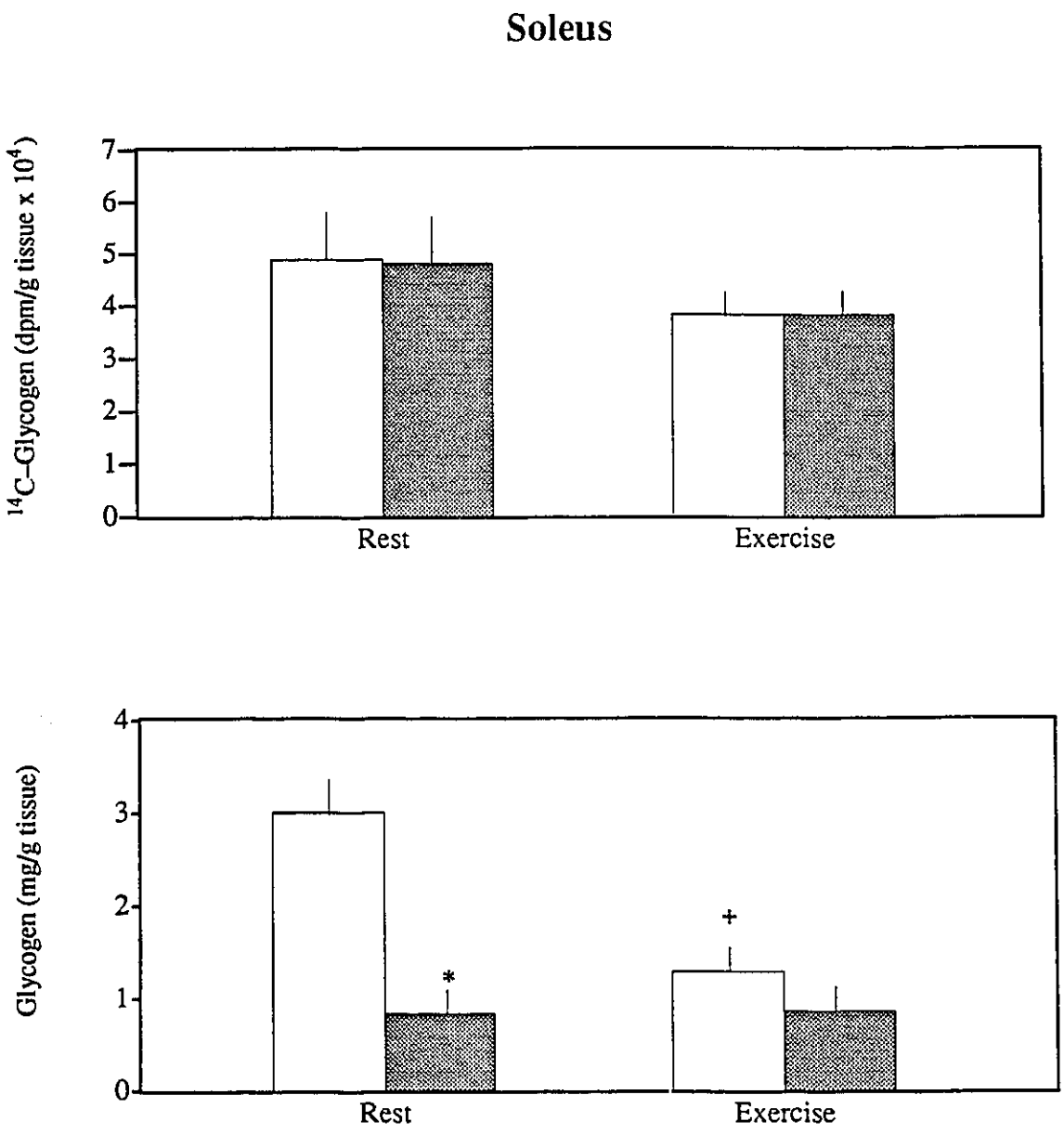


Figure 3.14 : Soleus glycogen contents after rest and postexercise recovery in rats infused with[^{3}H-6]-glucose and [^{14}C-U]-Glycerol

Total soleus glycogen concentration at rest was 3.01 ± 0.206 mg/g muscle and the direct glycogen synthesis in soleus muscle was 0.84 ± 0.069 mg/g muscle (lower panel).

EXERCISE GROUP. There was a small (not significant) decrease in both total and the direct ^{14}C-glycogen counts in exercised soleus muscle in rats infused with ^{14}C-glycerol (upper panel). There was no significant difference between total and the direct ^{14}C-glycogen in exercised soleus muscle. These data indicate there was no significant glyconeogenesis from glycerol occurring in exercised soleus muscle. The total glycogen concentration in exercised soleus was 1.30 ± 0.192 mg/g muscle, which was significantly lower (57% lower) than that in the resting muscle (lower panel, $P < 0.05$). The direct glycogen synthesis in exercised soleus was not different from that at rest (0.84 ± 0.169 mg/g muscle vs. 0.87 ± 0.111 mg/g muscle) and was 32% lower than the total glycogen in the exercised soleus, but not statistically significant.

3.5.2. White Gastrocnemius Glycogen in ^{14}C-Glycerol Infused Rats (Figure 3.15).

RESTING CONTROL. As in ^{14}C-threonine infused animals, the glycogen turnover in white gastrocnemius muscle was very low in the resting state. The low turnover was depicted by a very low ^{14}C incorporation and the small amount of the direct glycogen synthesis in rested white gastrocnemius muscle as compared with that in soleus and red gastrocnemius muscles in the resting state. There was no significant difference between total ^{14}C-glycogen and the direct ^{14}C-glycogen in rested white gastrocnemius muscle (upper panel). These data suggest that it is unlikely that there was significant

Figure 3.15: White gastrocnemius glycogen contents after rest or postexercise recovery in rats infused with ^{3}H-glucose and ^{14}C-U-glycerol.

White gastrocnemius glycogen contents in ^{3}H-glucose and ^{14}C-glycerol infused rested or exercised rats were determined after three hours of postexercise recovery. Upper panel: open bars are total ^{14}C-glycogen d.p.m. counts in white gastrocnemius muscle, the filled bars are the ^{14}C-glycogen d.p.m. counts arising from the direct uptake of ^{14}C-glucose from plasma in white gastrocnemius muscle. Lower panel: open bars are total glycogen concentrations in white gastrocnemius muscle, the filled bars are the glycogen synthesized via the direct uptake of glucose from plasma in white gastrocnemius muscle. All data are expressed as means ± SEM. *significantly different from the corresponding total ^{14}C-glycogen counts or total glycogen content, respectively ($P < 0.05$). +significantly different from the resting controls ($P < 0.05$). $n = 5$ for both exercise and the resting groups.

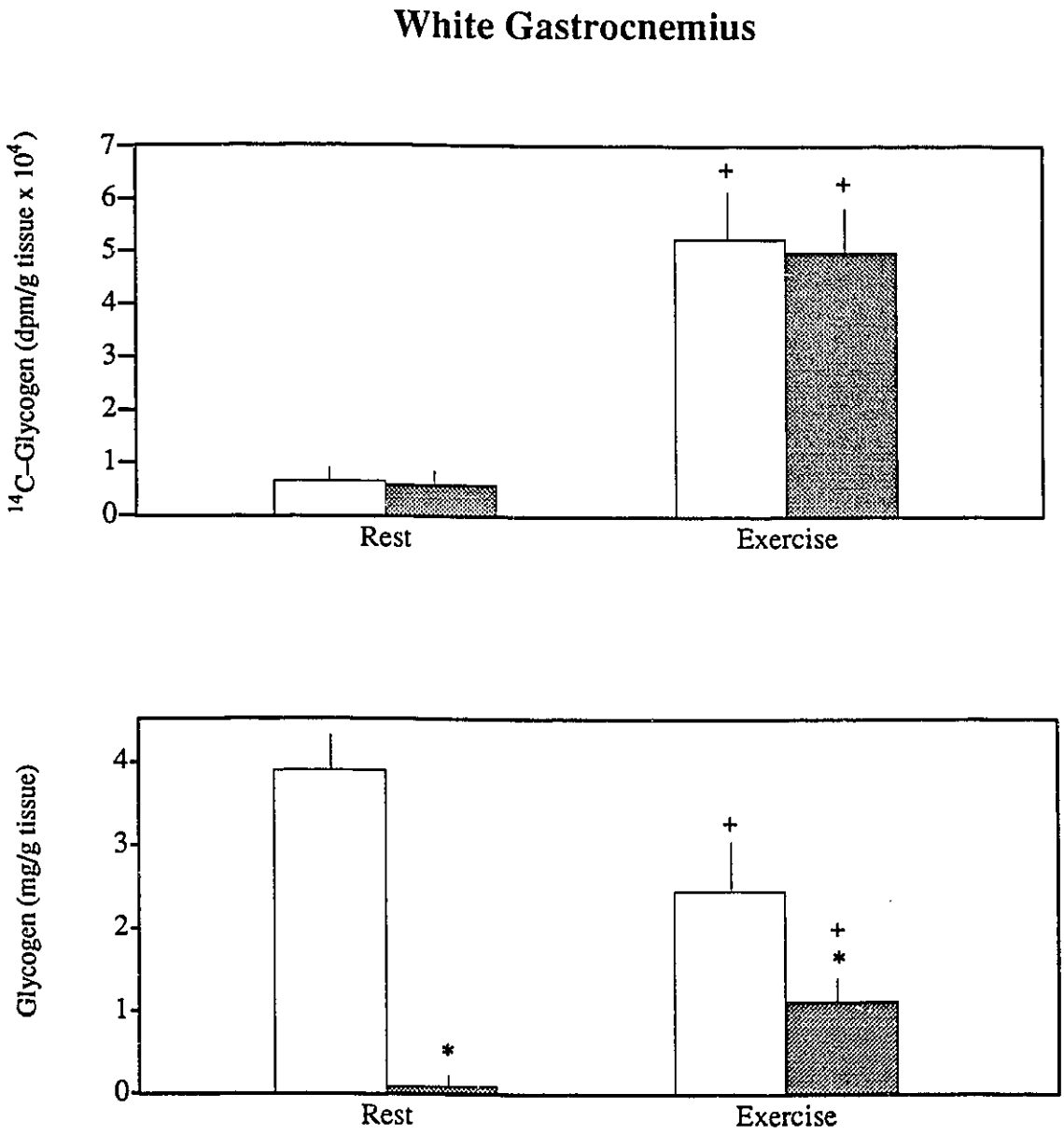


Figure 3.15: White gastrocnemius glycogen contents after rest and postexercise recovery in rats infused with[^{3}H–6]–glucose and [^{14}C–U]–Glycerol

gluconeogenic formation of glycogen from glycerol at rest in white gastrocnemius muscle. Total glycogen concentration in rested white gastrocnemius muscle was 3.90 ± 0.372 mg/g muscle which was higher than that in soleus and red gastrocnemius muscles (lower panel). The direct glycogen synthesis in rested white muscle was very small (0.10 ± 0.012 mg/g muscle) as compared with that in rested soleus and red gastrocnemius muscles in ^{14}C-glycerol infused animals.

EXERCISE GROUP. As in ^{14}C-threonine infused rats, exercise followed by recovery resulted in a significant increase in the incorporation of ^{14}C into glycogen in white gastrocnemius muscle in ^{14}C-glycerol infused rats. Both of total ^{14}C-glycogen and the direct ^{14}C-glycogen in exercised white gastrocnemius muscle were eight times higher than those in the resting controls (upper panel, $P < 0.05$). There was no significant gluconeogenic formation of glycogen from glycerol in exercised white gastrocnemius muscle as evidenced by the lack of significant difference between total and the direct ^{14}C-glycogen counts. The total glycogen content in exercised white gastrocnemius muscle was 2.46 ± 0.443 mg/g muscle, remaining 37% lower than that in rested white muscle (lower panel, $P < 0.05$). The direct glycogen synthesis in exercised white muscle was eleven-times higher than that in the resting white muscle, and was 54% lower than the total glycogen content in exercised white muscle (lower panel, $P < 0.05$).

3.5.3. Red Gastrocnemius Glycogen in ^{14}C-Glycerol Infused Rats (Figure 3.16).

RESTING CONTROL. There was a relatively low glycogen turnover in red

gastrocnemius muscle in the resting state as represented by the low ^{14}C incorporation into glycogen (upper panel) and the small amount of glycogen synthesized from the direct uptake of plasma glucose (lower panel). This glycogen turnover was higher than that in white gastrocnemius muscle and lower than that in soleus muscle at rest. There was no significant difference between total ^{14}C-glycogen and direct ^{14}C-glycogen in rested red gastrocnemius muscle (upper panel). These data suggest an insignificant glyconeogenic formation of glycogen from glycerol in rested red gastrocnemius muscle. The total glycogen concentration in the resting red gastrocnemius muscle was 3.75 ± 0.371 mg/g muscle (lower panel), a value between those in white gastrocnemius and soleus muscles. The direct glycogen synthesis was relatively small (0.54 ± 0.099 mg/g muscle), and was higher than that in white muscle, lower than that in soleus muscle at rest.

EXERCISE GROUP. As in white gastrocnemius muscle, exercise and recovery induced a significant increase in glycogen turnover in red gastrocnemius muscle. The incorporation of ^{14}C into glycogen in exercised muscle was 3.2-times higher than that in the rested red muscle (upper panel, $P < 0.05$). The lack of significant difference between total ^{14}C-glycogen and the direct ^{14}C-glycogen suggest that there was no significant glyconeogenesis from glycerol occurring in exercised red gastrocnemius muscle during recovery period. The total glycogen concentration in exercised red gastrocnemius muscle was 2.97 ± 0.169 mg/g muscle, which was lower than that in rested red muscle, but not statistically significant (lower panel). The direct glycogen synthesis was increased to 1.85 ± 0.325 mg/g muscle which was 3.4-times higher than that in rested red gastrocnemius

Figure 3.16: Red gastrocnemius glycogen contents after rest or postexercise recovery in rats infused with ^{3}H-glucose and ^{14}C-U-glycerol.

Red gastrocnemius glycogen contents in ^{3}H-glucose and ^{14}C-glycerol infused rested or exercised rats were determined after three hours of postexercise recovery. Upper panel: open bars are total ^{14}C-glycogen d.p.m. counts in red gastrocnemius muscle, the filled bars are the ^{14}C-glycogen d.p.m. counts arising from the direct uptake of ^{14}C-glucose from plasma in red gastrocnemius muscle. Lower panel: open bars are total glycogen concentrations in red gastrocnemius muscle, the filled bars are the glycogen synthesized via the direct uptake of glucose from plasma in red gastrocnemius muscle. All data are expressed as means ± SEM. *significantly different from the corresponding total ^{14}C-glycogen counts or total glycogen content, respectively ($P < 0.05$). +significantly different from the resting controls ($P < 0.05$). $n = 5$ for both exercise and the resting groups.

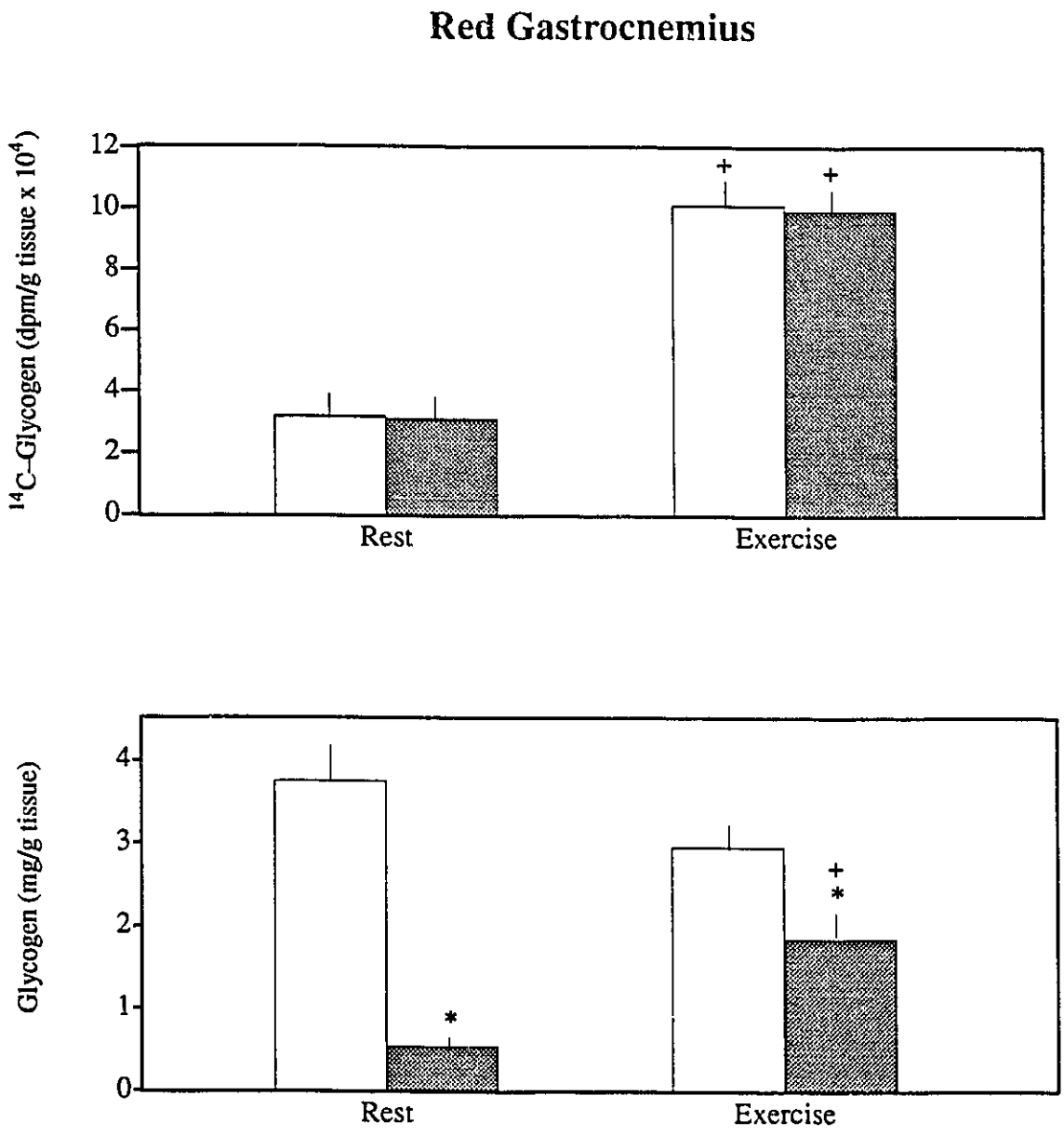


Figure 3.16: Red gastrocnemius glycogen contents after rest and postexercise recovery in rats infused with[^{3}H-6]-glucose and [^{14}C-U]-Glycerol

muscle, and it was significantly lower (37% lower) than the total glycogen content in exercised red gastrocnemius muscle.

CHAPTER FOUR

DISCUSSION

4.1. METHODOLOGY

4.1.1. Exercise Model

The exercise model we have used is identical to that used by Ryan and Radziuk, in which muscle glyconeogenesis was found to occur under physiological circumstances (Ryan and Radziuk, 1994). Glucose metabolism was measured as a "control" for our swimming model. Our results indicate that fasted untrained rats can maintain glucose homeostasis during prolonged swimming and this is accomplished by nearly matched increases (maximally two fold) in the rates of glucose production and clearance during this period. During post-exercise recovery, both Ra and MCR of glucose gradually returned to basal values after three hours of recovery. These results are consistent with the results obtained by Ryan and Radziuk (Ryan et al., 1993). In addition, similar to Ryan's study, our swimming exercise only induces a maximally 1.1-fold increase in plasma lactate concentration (1.92 mM) after the first hour of exercise. This lactate level is also similar to that of 1.90 mM found previously in a low-intensity exercise (Donovan and Sumida, 1990). Therefore, our swimming model would qualify as a low to moderate intensity exercise and it is identical with that used by Ryan and Radziuk (1993, 1994). Because lactate accumulation is minimal, this is a good model for the examination of the metabolism of substrate (other than lactate) during exercise and recovery periods without the interference of high lactate levels.

4.1.2 Tracer Approach for Urea Turnover

Isotope tracer methods have been reported to be the most accurate methods for the determination of body urea production (Wolfe, 1981). A single-compartment model for urea kinetics is appropriate when changes occur slowly. During prolonged low-intensity exercise, the metabolic changes in urea are considered to take place slowly relative to urea equilibration among the compartments in which it distributes. Thus the changes in urea can be viewed as occurring in a well mixed space. Under these circumstances, the error caused by using a single-compartment model for urea kinetics is relatively small. Moreover, the inaccurate assumption can be offset by a correction factor that has become known as a "pool fraction" (Wolfe, 1981; Wolfe et al., 1982). In this study, the number of samples is obviously limited because of the small blood volume in rats. However, the slow changes in urea metabolic flux during prolonged low-intensity swimming allow reasonable interpolation between the necessarily few data points.

Several isotope tracers, such as ^{15}N-urea, $^{15}N_2$-urea, ^{14}C (or ^{13}C)-urea have been used for urea studies. ^{15}N-urea has been found to be less useful as a tracer for urea kinetics, since urea can be catabolized by bacteria in the gut and then ^{15}N released from ^{15}N-urea can be recycled into urea pool or protein (Regoeczi et al., 1965; Long et al., 1978). $^{15}N_2$-urea is generally considered to be a non-recycling tracer, because any label that is reincorporated into urea will yield a single labelled product. This is because of $^{15}N_2$-urea is present at tracer concentrations. Therefore, of the stable isotopes only double labelled urea gives the true rate of urea synthesis. ^{14}C-urea has also been demonstrated to be a valid tracer for estimating urea kinetics (Wolfe, 1981), although it has been reported that

there may be recycling of the urea carbon back into urea (Long et al., 1978). When both $^{15}N_2$-urea and ^{14}C-urea were used simultaneously in a urea kinetic study (Wolfe, 1981), close agreement between Ra calculated with ^{15}N and ^{14}C was observed. The rate calculated from ^{14}C data were only slightly lower (less than 5%). Moreover, the distribution of the two tracers was found to be the same (Wolfe, 1981). Therefore, ^{14}C-urea was chosen in the present studies.

4.1.3. Estimation of Muscle Protein Degradation by 3-MH and Tyrosine

The urinary excretion of 3-MH is widely used as an index of muscle protein degradation (eg, Young and Munro, 1978, Dohm et al., 1982; Dohm et al., 1985) because it is a simple and valid estimation of 3-MH production. However, in our swimming model, urine samples cannot be easily collected, but changes in the kidney function during swimming and recovery can be estimated from the rate of urea clearance which appears to be unchanged in our animals. Therefore, in this study muscle myofibrillar protein degradation during swimming and recovery was estimated from plasma 3-MH concentrations. This estimation should closely reflect the changes of 3-MH production and muscle myofibrillar protein degradation. Moreover, the measurement of 3-MH contents in muscles after three hours of recovery would provide direct evidence of changes in muscle myofibrillar protein breakdown during exercise and recovery. Production of the essential amino acid, tyrosine, can be used as an indicator of net body tissue protein breakdown (Dohm, 1986). More than 50% of body protein is present in muscle (Young and Munro, 1978). Furthermore, tyrosine released from muscle protein breakdown is not

metabolized in muscle (Dohm et al., 1981). Changes in plasma tyrosine concentrations may therefore also be used as an estimate of net muscle protein breakdown (Haralambie and Berg, 1976). Thus, we have measured the time-course of changes in plasma tyrosine concentration during swimming and recovery as further indices of net body protein degradation. Tyrosine content in muscle was concurrently measured after three hours of recovery in order to obtain more information on net muscle protein degradation.

4.1.4. Tracer Approach for Muscle Glyconeogenic Substrate Study

In an attempt to test whether or not and to what extent muscle glycogen can be formed from gluconeogenic amino acids or glycerol by muscle glyconeogenesis, we infused ^{14}C-U-threonine (an essential and gluconeogenic amino acid used as a representative of amino acids) or ^{14}C-U-glycerol into rat blood circulation. Presumably, ^{14}C-glycerol or ^{14}C-U-threonine would enter the muscle, labelling the intramuscular glycerol or threonine pools, then undergo muscle glyconeogenesis via intramuscular 3-carbon substrate pool, labelling glycogen with ^{14}C. At the same time, ^{14}C-labelled threonine or glycerol would also enter the liver, undergo hepatic gluconeogensis to form ^{14}C-glucose which could then enter the plasma and be taken up directly into muscle glycogen, labelling muscle glycogen with ^{14}C as well (direct ^{14}C-glycogen). The infusion of the second tracer ^{3}H-6-glucose, a non-recycling tracer, was necessary to account for the direct ^{14}C-glycogen arising from recycled ^{14}C-glucose in plasma. The difference between the total amount of ^{14}C-glycogen in muscle and that arising from the direct uptake of plasma ^{14}C-glucose would be the ^{14}C-glycogen formed from the ^{14}C-labelled precursors

by glyconeogenesis within the muscle. The percent difference in total versus direct uptake, for example, could be used as a relative index for muscle glyconeogenesis.

The assessment of muscle glyconeogenesis from labelled precursors involves a number of assumptions. These include linearity of glycogen synthesis which allows the using of an average ratio of plasma ^{3}H-glucose / ^{14}C-glucose which is changing to some extent during the postexercise recovery. Ryan and Radziuk (1994) have tested this assumption by using $^{14}CO_2$ as a label. No muscle glyconeogenesis was seen using this label and none was expected on an enzymatic basis. This consistency in the results suggests that this assumption is valid at least in the gastrocnemius muscles. Using these methods, therefore, an average estimate of muscle glyconeogenesis during postexercise recovery is obtained. Other assumptions include the equilibration of the intravenously infused ^{14}C-labelled threonine or glycerol with the intramuscular pool. Amino acids are taken up readily by muscle since they are substrates for muscle protein synthesis and, therefore, reasonable equilibration is expected. Glycerol uptake by muscle has also been documented (Terblanche et al., 1981). The results will, however, remain qualitative in nature.

4.2. Discussion of Results

The primary goal of this study was to examine possible substrates for muscle glyconeogenesis under a particular set of physiological circumstances - during fasting recovery from prolonged swimming in rats. The intramuscular 3-carbon pool has been demonstrated to be involved in the process of muscle glyconeogenesis (Ryan and

Radziuk, 1994). Lactate, glycerol and gluconeogenic amino acids may, therefore, all play roles as substrates for muscle glyconeogenesis during post-exercise recovery. Although a number of studies have demonstrated that lactate serves as a substrate for muscle glyconeogenesis *in vitro* (McLane et al., 1979; Bonen et al., 1990) and *in vivo* (Johnson and Bagby, 1988) when lactate levels were maintained high in perfusate or blood circulation by exogenous nutritional support, direct evidence of incorporation of lactate into muscle glycogen by muscle glyconeogenic pathway under physiological circumstance has not been obtained when ^{14}C-lactate was infused as a tracer. It could be concluded, therefore, that 3-carbon substrates formed locally in muscle during exercise from metabolism of glucose do not equilibrate well with circulating lactate (Ryan and Radziuk, 1994). Substrates for muscle glyconeogenesis other than lactate have not been frequently considered. In the light of a significant utilization of body fat and a suggested increase in proteolysis during prolonged exercise (Rennie et al., 1981), we hypothesized that the glyconeogenic amino acids released from increased proteolysis or glycerol liberated from catabolism of body fat might serve as substrates for muscle glyconeogenesis after exercise.

4.2.1. Net Body Protein Breakdown During Exercise and Recovery

The results of this study have demonstrated a maximally two-fold increase in Ra of urea during prolonged swimming and postexercise recovery, starting from the first hour and thirty minutes of the swim. The Ra of urea remained close to the maximal level throughout the three hours of postexercise recovery period. The major conclusion to be

drawn from these data is that the net body protein breakdown is markedly increased not only as exercise progresses but also during the three hours of post-exercise recovery in the swimming rats.

Similar to this study, an increased urea synthesis and body protein breakdown have been reported during prolonged low-intensity running (Haralambie and Berg, 1976) in which the large rise in plasma urea concentration which was seen was much greater than could be accounted for by a reduction in urea clearance. Rennie et al. (1981) also reported that total urea production over a four-hour moderate-intensity run and a 12-hour recovery was significantly increased. Although a number of studies showed that urea excretion over a 12-hour period after prolonged exercise was increased (eg, Haralambie and Senser, 1980; Decombaz et al., 1979; Calles et al., 1984; Dohm et al., 1982, Dohm et al., 1985), the change in urea production during a relatively short recovery period was not measured in these studies. Our data indicates that urea synthesis remains elevated for at least three hours after prolonged swimming and is, therefore, in general agreement with these studies.

Different results were obtained by several groups of investigators (Wolfe et al., 1982; Stein et al., 1987; Carraro et al., 1990). In these studies, neither plasma urea concentrations nor the urea production were affected by the exercise and recovery. This is likely based on the difference in the intensity and duration (Lemon, 1987; Dohm, 1986) of these exercise protocols since changes in urea production develop relatively slowly as seen in our data. It is possible that the degree of stress involved in the exercise protocol may also influence the extent of proteolysis.

The marked increase in net protein breakdown induced by exercise such as used here, should lead to additional free amino acids available for further metabolism, gluconeogensis in the liver and glyconeogenesis in the muscle. The sites of protein breakdown therefore need to be identified.

4.2.2. Muscle Protein Degradation During Exercise and Recovery

In order to localize the tissue sites at which protein breakdown occurs during exercise and recovery, time courses of plasma 3-MH and tyrosine during the experimental period as well as their concentrations in the skeletal muscles at the end of the recovery period were determined. The results demonstrate that 1) prolonged swimming induces a significant increase in protein degradation in some skeletal muscles, and this elevated muscle proteolysis persists after exercise; 2) the enhanced muscle protein degradation takes place in exercised white and red gastrocnemii; and 3) the myofibrillar protein degradation did not change in the soleus muscle of exercising rats. These conclusions are based on the following observations: a) plasma 3-MH concentrations were significantly increased during both the exercise period (up to 39% after the first hour of the exercise) and the three hours of recovery period (up to 78% after the first hour of recovery); b) similar patterns for the increase in plasma tyrosine concentrations were also found during exercise and recovery; c) there was no significant reduction in kidney function during and after exercise if one considers the MCR of urea; and d) the total 3-MH concentration after a three-hour recovery period increased two and half-fold and two-fold in exercised white and red gastrocnemius muscles, respectively. In contrast, it did not change in

exercised soleus muscle.

Consistent with our results, several investigators reported that myofibrillar protein degradation increased after running (Dohm et al., 1982, Dohm et al., 1985; Carraro et al., 1990). Others, however, found that myofibrillar protein degradation decreased during various running protocols, and that it returned to basal after exercise without any overshoot (Rennie et al., 1981; Dohm et al., 1985). These differences are most likely due to the variation in the type, intensity and duration of the exercise involved. Most previous studies have focused on running exercise. Frisch et al. (1984), however, reported that 24-hour 3-MH excretion increased in response to a prolonged swim, and suggested that total myofibrillar protein degradation increased during this period. This is again consistent with our findings.

That the myofibrillar protein breakdown took place in muscle is strongly suggested by the presence of increased 3-MH in the gastrocnemii at the end of the recovery period. This suggestion is further strengthened by the fact that the soleus did not demonstrate increased 3-MH levels indicating that the raised 3-MH in the gastrocnemii is not simply a reflection of the increased plasma levels.

A number of studies have indicated the presence of an increased net muscle degradation during exercise. Dohm et al. (1981) demonstrated increased tyrosine concentrations in both plasma (17%) and the gastrocnemius muscle (86%) immediately after a one-hour swim in rats. MacLean et al. (1991) reported that total and total essential amino acids in muscle were significantly increased at exhaustion from cycling. A rise in tyrosine release from perfused exercised muscle or hindquarter has been observed (eg,

Kasperek and Snider, 1989; Balon et al., 1990). These findings are consistent with our observations. Muscle tyrosine contents in our swimming rats did not increase. This may be due to a gradual increase in muscle protein synthesis during recovery so that by the end of the recovery period tyrosine release and uptake by protein are nearly equal. This would not however be true for 3-MH. It is also possible that some metabolism of tyrosine takes place in the muscle. It is interesting to note that Kasperek and Snider (1989) reported that in isolated exercised soleus muscle, tyrosine release was increased (30-50%), whereas 3-MH release was unchanged. These findings suggested a non-myofibrillar protein breakdown in soleus after exercise. Our study has also shown an unchanged myofibrillar protein degradation in exercised soleus muscle as indicated by unchanged 3-MH levels. In the context of our studies, however, it should be noted that little or no glyconeogenesis has been seen in the soleus (Ryan and Radziuk, 1994), in the meantime, increased muscle protein degradation was not found in the same muscle - an observation that is suggestive of a potential linkage between proteolysis and glyconeogenesis in muscles.

4.2.3. Amino Acids as Substrate for Muscle Glyconeogenesis

During postexercise recovery, a large amount of free amino acids is available in certain muscles from enhanced muscle proteolysis. Whether amino acids can be incorporated into muscle glycogen through an intramuscular 3-carbon pool is still a question. The conversion of threonine (a representative of essential gluconeogenic amino acids) into muscle glycogen via muscle glyconeogenesis was traced using ^{14}C-threonine.

The results of the threonine study suggest that at least the red gastrocnemius muscle is capable of synthesizing a portion of its glycogen from threonine via muscle glyconeogenesis during recovery from prolonged swimming in rats. Threonine therefore does appear to serve as a substrate for muscle glyconeogenesis under this particular set of physiological circumstances. Moreover, it seems likely that muscle protein is a source of muscle glyconeogenic substrate. An estimate of the glyconeogenic contribution of threonine to muscle glycogen formation is approximately 10-11% in the red gastrocnemius muscle. It has been suggested that the assessment of muscle glyconeogenesis by ^{14}C-U-lactate (when this can be detected) is underestimated by approximately 30-50% probably due to label dilution by glycolytic intermediates (McLane et al., 1979; Bonen et al., 1990). This would also happen in the case of ^{14}C-U-threonine. Taking such an underestimate into account, the incorporation of labelled threonine may be representative of up to 15-17% of new glycogen synthesis via glyconeogenesis in the red gastrocnemius muscle. Ryan and Radziuk (1994) have suggested that maximally 40% of new muscle glycogen may be formed by glyconeogenesis in white and red gastrocnemius muscles during recovery from prolonged swimming. It should be noted that metabolized ^{14}C-glucose, ^{14}C-lactate (when applicable) and ^{14}C-threonine may all be labelling the same 3-carbon pool. It is likely, therefore, that the different substrates contribute to the label dilution.

Because the difference between total and direct uptake of ^{14}C-label in the soleus consistently stops short of significance, one may conclude that no glyconeogenesis takes place in this muscle under physiological condition.

4.2.4. The Role of Body Fat: Glycerol as A Possible Substrate for Muscle Glyconeogenesis

Our protein studies suggest that body protein, particular muscle protein may contribute to muscle glyconeogenesis via amino acids *in vivo* during recovery from prolonged swimming. In particular, threonine released from increased proteolysis during and after exercise appears to serve as a substrate for this glyconeogenesis in muscle after exercise. One further question remains: can body fat, especially intramuscular fat contribute to muscle glyconeogenesis via glycerol during postexercise recovery?

As it is well known that prolonged exercise causes a substantial catabolism of the body fat, in particular intramuscular fat (Baldwin et al., 1973; Spriet et al., 1984) and an accumulation of glycerol in both plasma and muscle (Terblanche et al., 1981; Fell et al., 1983), we have only examined whether circulating glycerol can contribute to muscle glycogen via muscle glyconeogenesis after prolonged swim. The results of this study indicate that there is no detectable incorporation of glycerol into muscle glycogen via muscle glyconeogenesis in soleus, white and red gastrocnemius muscles when ^{14}C-glycerol is infused into the blood circulation as a tracer. This suggests two possibilities: 1) glycerol released from enhanced catabolism of body fat does not serve as a substrate for muscle glyconeogenesis after exercise; or 2) glycerol formed locally in muscle during exercise does not equilibrate with that in blood circulation (a similar situation to that of lactate).

Terblanche et al. (1981) have reported that muscle itself has no capacity to synthesize glycogen from glycerol based on the insignificant incorporation of ^{14}C into muscle glycogen from ^{14}C-glycerol in perfused exercised hindquarters under either normal

or high concentration of glycerol in the perfusate. In addition, Favier et al. (1987) has claimed that glycerol does not play a role in muscle glycogen repletion during recovery from prolonged swimming. This was based on the observation that muscle glycogen resynthesis after exercise was not inhibited by the blockade of lipolysis. On the other hand, it is quite possible that the contribution of intramuscular glycerol into glycogen via muscle glyconeogenesis cannot be detected by using ^{14}C-glycerol as a tracer, equilibration between intramuscular glycerol pool and circulating glycerol may be low. Havel et al. (1967) reported a large utilization of intramuscular triglycerides during prolonged exercise but an insignificant release of glycerol after exercise. There is evidence that muscle has the capacity to actively take glycerol up from the circulation after prolonged swimming during an exogenous glycerol load. No load was administered, however, in the present study. From a methodological point of view, the negative results for glycerol provide a control for the small but positive results obtained in the threonine experiments.

CHAPTER FIVE

CONCLUSION

The principal conclusion which can be drawn from this work is that body protein, particular muscle protein, may contribute to muscle glyconeogenesis via glyconeogenic amino acids *in vivo*, under a particular set of physiological circumstances - during recovery from prolonged submaximal exercise.

The conclusion is based on the following observations 1) a significant increase in whole-body net protein breakdown as evidenced by increased urea production as well as a significant increase in muscle protein degradation, demonstrated by increased levels of 3-MH in the circulation and the gastrocnemii during exercise and recovery; 2) evidence of a significant conversion of threonine (a representative of free glyconeogenic amino acids) into muscle glycogen by a glyconeogenic pathway in at least the red gastrocnemius muscle; and 3) in contrast to this, there was no evidence of muscle glyconeogenesis when it was assessed using ^{14}C-U-glycerol as a tracer. The negative observation which was expected based on previous studies served to validate the positive results obtained with ^{14}C-threonine.

The contribution of threonine label to muscle glyconeogenesis could account for approximately 10-11% of the ^{14}C-glycogen synthesized during postexercise recovery. The observation that no muscle glyconeogenesis was detected using ^{14}C-U-glycerol as a tracer suggests that either glycerol is not a substrate for muscle glyconeogenesis or glycerol formed locally in muscle does not equilibrate with that in the circulation.

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