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The Effect of Prolonged Exercise and Drug-Induced Variation of Non-Esterified Fatty Acids on Glycogen Repletion in Selected Rat Skeletal Muscles.

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter I

THE PROBLEM

1.1 INTRODUCTION

Research concerning metabolism during muscular activity was initiated late in the 19th century (Chauveau, 1896). Throughout this early period of research many features of carbohydrate and fat metabolism were elucidated. However it was not until the 1960's and 1970's that researchers, led by Scandinavian physiologists, clarified the roles of fats and carbohydrates during prolonged physical activity. This contemporary research thrust was aided by the development of the needle biopsy technique (Bergstrom, 1962) and the use of radioisotopes in the determination of substrate utilization (Bragdon & Gordon, 1958). The results of this research emphasized the importance of muscle glycogen stores in prolonged activity.

It was found that the initial stores of glycogen in the working muscles of an individual determined, to a large extent, the capability of the individual to sustain work for long periods of time (> 1 hour) at work intensities requiring a substantial fractional utilization of maximal oxygen uptake (66 to 85% MVO2). High initial levels of glycogen in

the working muscles of an individual led to a greater ability to sustain physical activity whereas low initial levels of glycogen in the working muscles of the same individual led to a decreased ability to sustain physical activity (Hermansen et al., 1967,1979, Ahlborg et al. 1967, Bergstrom et al. 1967, Hultman, 1967, Karlsson & Saltin, 1971, Saltin & Hermansen, 1967).

In light of this research, exercise and dietary regimens were devised to maximize the amount of glycogen stored in the muscles to be used in the activity (Bergstrom et al., 1967, Bergstrom & Hultman, 1972, Sherman et al., 1981, Costill et al., 1981). This type of regimen became known as carbohydrate loading (Forgac, 1979).

Although the application of carbohydrate loading to endurance activities does seem useful and has therefore been used since its conception, the mechanism by which the glycogen stores replete has not been elucidated (Poland, 1980). If this mechanism were known it may prove possible to increase the rate and/or level of glycogen repletion. Subsequently a more complete understanding of post-exercise metabolism, as well as metabolism in general, will emerge. There would then be immediate practical benefits for endurance athletes.

One possible factor which may contribute to the regulation of post-exercise glycogen repletion is the post-exercise rise in non-esterified fatty acids (NEFA) (Poland et al., 1980). Poland et al. (1980) observed that NEFA were the only major substrate whose concentration changes while glycogenesis is occurring. Based on this observation Poland et al. (1980) postulated a role for NEFA in post-exercise glycogen repletion. These researchers did not provide a theoretical mechanism for this hypothesis.

It is clear that the uptake of NEFA in skeletal muscle is not regulated by a cellular transport mechanism although the transport into the muscle cell may be limited by capillary endothelial cells (Rose & Goresky, 1977). Despite this potential endothelial barrier there is an increased removal of NEFA from the blood in the post-exercise state as long as the arterial concentration of NEFA is augmented (Hagenfeldt & Wahren, 1975).

Once inside the muscle cell fatty acids can contribute to the post-exercise energy demand. This fat oxidation is thought to lead to a relative inhibition of carbohydrate oxidation through the inhibition of phosphofructokinase and pyruvate dehydrogenase (Randle, 1981). Pyruvate dehydrogenase is inhibited by the increase in the intramitochondrial acetyl CoA/CoA ratio which accompanies increased fatty acid oxidation (Randle et al., 1978), while phosphofructokinase

is postulated to be inhibited by an increase in cytoplasmic citrate concentration which also accompanies increased fatty acid oxidation.

Citrate is the only known metabolic effector of PFK which is relatively independent of the energy state of the cell. It follows that if the oxidation of fatty acids were increased throughout a period where the energy requirement is constant and the oxygen supply adequate, the cell's reliance on carbohydrates might decrease. This would provide a mechanism for carbohydrate sparing. However, it is important to note that this mechanism would only cause a relative inhibition of the catabolic fate of glucose; it would not inhibit the anabolic process of glycogenesis. Thus, when glycogen stores are depleted, the increased availability and utilization of fatty acids may enhance the rate and extent of glycogen repletion. These facts and propositions support the theory of a relationship between NEFA and glycogenesis in the post-exercise state.

1.2 RATIONALE

As diet and activity level are important to the glycogen storing capability of muscle an animal model was chosen for this study. In this way a more precise control of diet and activity level was attained.

Since the metabolic capabilities and charateristics of a particular muscle may influence the effect of NEFA on post-exercise glycogen repletion, the proposed effect was studied in the three skeletal muscles of distinctly different fiber type populations of the rat. The muscles under investigation were the red vastus lateralis, the white vastus lateralis, and the soleus. These muscles had previously been shown to be good representatives of the fast oxidative glycolytic (FOG) muscle fiber, the fast glycolytic (FG muscle fiber, and the slow oxidative (SO) muscle fiber (Ariano et al., 1973, Barnard et al., 1971).

The levels of NEFA in the post-exercise state were altered through the use of nicotinic acid and heparin. These substances had previously been shown to be potent antilipolytic and lipolytic agents respectively (Carlson, 1965; Rennie et al., 1976). Pilot work indicated that any possible effect of varying NEFA levels on post-exercise glycogen repletion would be noticeable in early post-exercise glycogen repletion. Furthermore, pilot work indicated that in the protocol used in this study glycogen stores are largely repleted after three hours. For these reasons the influence of varying levels of NEFA on post-exercise glycogen repletion was studied for the first three post-exercise hours.

1.3 PURPOSE

The purpose of the present study was to determine the effect of varying levels of NEFA via heparin/corn oil and nicotinic acid administration on the rate and extent of post-prolonged exercise glycogen repletion at one hour and three hours following prolonged exercise in three skeletal muscles of the rat. These muscles were the red vastus lateralis, the white vastus lateralis, and the soleus. The effect of exercise per se on glycogen and NEFA levels was also determined.

1.4 LIMITATIONS

The rats used in this study were not all equally adept at running. For this reason the poor runners were assigned to non-running control groups, while the good runners were randomly assigned to running groups. Thus, the results of this study can only be applied to groups of rats with similar running characteristics.

1.5 ABBREVIATIONS AND DEFINITIONS

AMP	:	Adenosine Monophosphate
ADP	:	Adenosine Diphosphate
ATP	:	Adenosine Triphosphate
cAMP	:	cyclic Adenosine Monophosphate
CoA	:	Coenzyme A
E.C.	:	Enzyme Commission

FG	:	Fast Glycolytic
FOG	:	Fast Oxidative Glycolytic
g	:	gram(s)
G-6-P	:	Glucose-6-Phosphate
IMP	:	Inosine Monophosphate
IP	:	intraperitoneal
Km	:	Michaelis-Menten constant
l	:	liter
PDH	:	Pyruvate Dehydrogenase
PFK	:	Phosphofructokinase
Pi	:	Inorganic Phosphate
NAD/NADH	:	Nicotinamide Diphosphate oxidized/reduced
NEFA	:	Plasma nonesterified fatty acids
SO	:	Slow Oxidative
subcut.	:	subcutaneous
ueq	:	microequivalents
umol	:	micromoles

Chapter II

REVIEW OF RELATED LITERATURE

2.1 INTRODUCTION

The purpose of this chapter was to review 1) glycogen metabolism, 2) current concepts in carbohydrate and fat metabolism, 3) the metabolic characteristics and capabilities of rat muscle fiber types, 4) the actions of nicotinic acid and heparin as they relate to exercise and lipolysis and 5) relate this knowledge to the post-exhaustive exercise glycogen repletion process.

2.2 GLYCOGEN METABOLISM

The regulation of glycogen metabolism is governed by the relative activity of two regulatory enzymes, glycogen phosphorylase (alpha 1,4 glucan:orthophosphate glycosyl transferase E.C. 2.4.1.1.) and glycogen synthase (UDP glucose alpha 1,4 glucan-alpha-4-glucosyl transferase E.C. 2.4.1.11.) (Larner et al. 1967).

The relative abundance of allosteric effectors as well as the extent of covalent modification is known to govern the activity of the enzymes (Larner et al., 1976). The covalent

modification of the enzymes is largely due to the hormonal milieu impinging on the cell. The concentration of cellular effectors is determined by both hormonal and non-hormonal means (Larner et al., 1967). The sensitivity of the muscle cell to the phosphorylation state of the regulatory enzymes is modulated by the allosteric effectors of each enzyme (Roach & Larner, 1976, Fischer et al., 1971). The control of glycogenolysis and glycogenesis is illustrated in Figure 1.

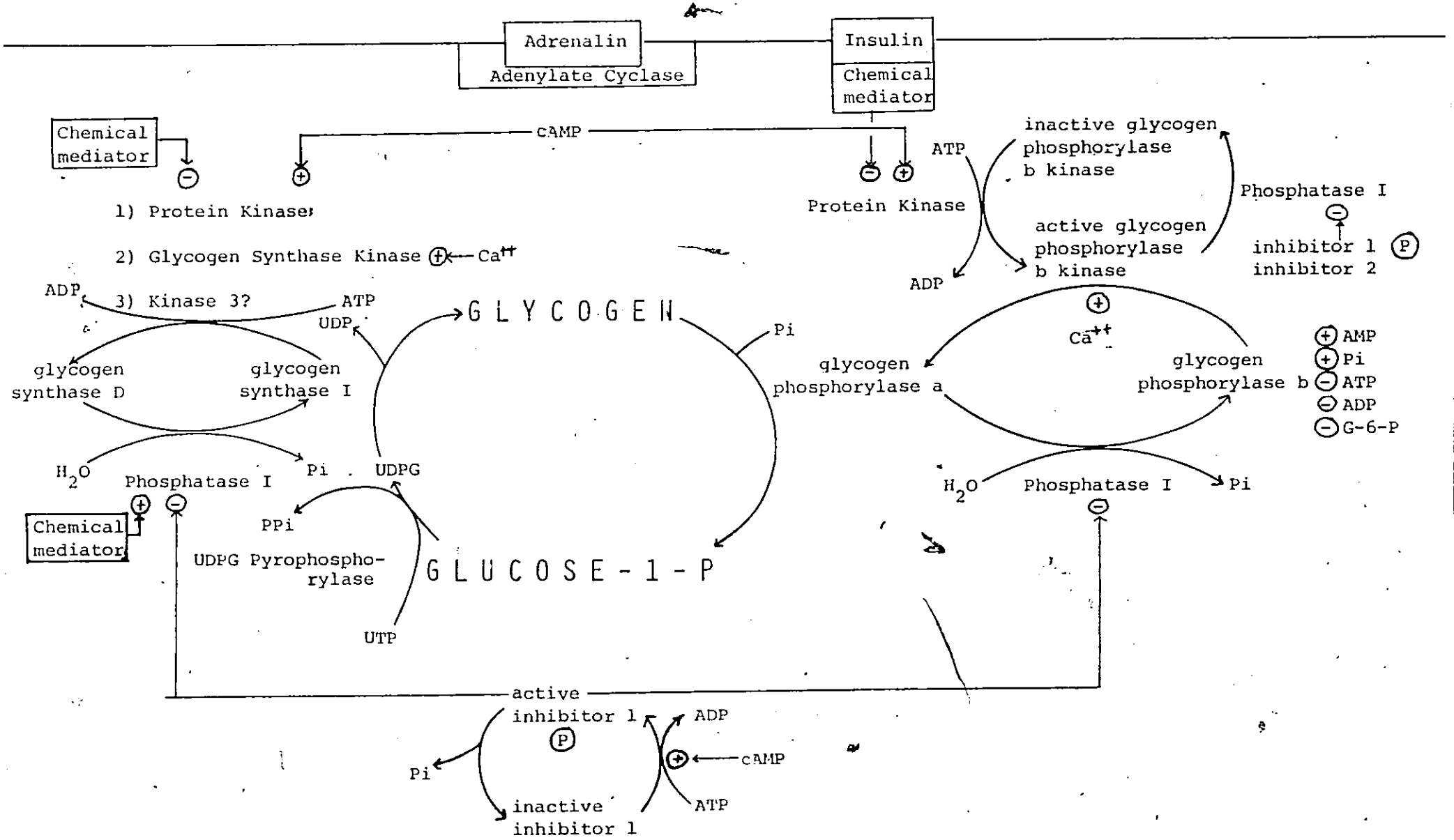


Figure 1: Glycogen Metabolism

Specifically, the glycogenolytic catecholamines (epinephrine and norepinephrine) once released into the blood or released at nerve endings (norepinephrine) bind to receptor sites on the muscle cell causing an increase in intracellular cAMP (Cohen, 1978a, Dietz et al., 1980). The increased cAMP level mediates the activation of glycogen phosphorylase and the inactivation of glycogen synthase (Cohen, 1978b). The activation of glycogen phosphorylase has been found to involve the addition of one phosphate (Pi) per monomer to the relatively inactive dephosphorylated glycogen phosphorylase b to form the active phosphorylated glycogen phosphorylase a (Fischer et al., 1971, Jenkins et al., 1981). Calcium ions are an obligatory cofactor for the activity of phosphorylase b kinase and are thus thought to provide a link between muscular contraction and glycogenolysis (Cohen et al., 1979, Brostrom et al., 1971). In contrast glycogen synthase is active in the dephosphorylated state (glycogen synthase a) and largely inactive in the phosphorylated state (glycogen synthase b) (Soderling, 1979). Glycogen synthase has been shown to contain multiple phosphorylation sites and is thought to contain 2 molecules of phosphate per subunit in the fed resting state and 3-3.5 molecules of phosphate under the influence of epinephrine (Roach & Larner, 1976, Cohen, 1979, Chiasson et al., 1981). cAMP mediates the phosphorylation of glycogen synthase a by increasing the activity of cAMP

dependent protein kinase (Dietz et al., 1980). When this study was undertaken there were two additional protein kinases which were known; these kinases were independent of cAMP (Cohen, 1979). The second kinase to be discovered, cAMP dependent protein kinase being the first, was named glycogen synthase kinase-2 (Cohen, 1979). This second kinase has been shown to contain calmodulin, a calcium binding protein, as does phosphorylase kinase (Cohen, 1979). This led to the suggestion that glycogen synthase kinase-2 and phosphorylase kinase were the same enzyme (Cohen, 1979, Srivastava et al., 1980). The third kinase which is known to exist is cAMP and calcium ion independent (Cohen, 1979). This kinase has been named glycogen synthase kinase-3 or protein kinase C (Cohen, 1983; Berridge, 1984). Each kinase is known to phosphorylate different sites on glycogen synthase so it has been proposed that each kinase has a unique physiological role (Cohen, 1979). Glycogen synthase kinase-3 is thought to be inhibited during the action of insulin, making it the most important regulator of glycogen synthase (Cohen, 1983). However, glycogen synthase kinase-3 has also been shown to be an activator of Mg-ATP dependent protein phosphatase; so that at present the physiological role of glycogen synthase kinase-3 is unclear (Cohen, 1983).

Through the actions of cAMP and calcium ions the processes of glycogenolysis and glycogenesis are precisely and reciprocally controlled. The inhibition of the action

and secretion of insulin by epinephrine (Richter et al., 1981) and norepinephrine (Gerich, 1976) ensures rapid glycogenolysis in situations requiring immediate energy production.

Glycogenesis is stimulated in the presence of insulin provided there is not sufficient catecholamine present to inhibit this effect (Bannister & Griffiths, 1972). Insulin is postulated to stimulate the dephosphorylation of glycogen synthase and the dephosphorylation of glycogen phosphorylase (Larner, 1972) thereby promoting glycogenesis. Insulin achieves this effect without altering the cAMP level of the cell (Larner, 1972). The action of insulin is thought to involve an intracellular chemical mediator which inhibits cAMP dependent protein kinase and/or the stimulation of glycogen synthase (Larner et al., 1979).

Protein phosphatase 1 is known to catalyze the dephosphorylation of glycogen synthase b, glycogen phosphorylase a and phosphorylase kinase (Cohen,1979). The action of protein phosphatase 1 is known to be inhibited by two factors known as inhibitors 1 and 2 (Huang & Glinsman, 1976). Inhibitor 1 is active in the phosphorylated state and is phosphorylated by cAMP dependent protein kinase (Cohen, 1979). Thus glycogenolysis under the influence of catecholamines is thought to "turn on" the phosphorylation of the glycogen metabolism enzymes and "turn off" their

dephosphorylation (Cohen,1978b). This latter function is thought to involve the inhibition of protein phosphatase 1 by phosphorylated inhibitor (Cohen, 1978b).

Thus based on this review of literature it appears that both insulin and the catecholamines create a hormonal cascade action thereby stimulating glycogenolysis or glycogenesis respectively.

Glycogen phosphorylase b is known to be strongly activated by AMP and Pi and weakly activated by IMP (Cohen, 1978a). Glycogen phosphorylase b is known to be inactive without AMP (Fischer et al 1971, Gross & Mayer, 1974). Glycogen phosphorylase b was further demonstrated to to be inhibited by ATP, ADP and G-6-P (Cohen, 1978a). Unlike glycogen phosphorylase b, glycogen phosphorylase a is known to achieve maximal activity without molecular effectors (Cohen, 1978a).

Glycogen synthase is allosterically activated by G-6-P (Villar-Palasi & Larner 1961 a & b). The extent to which glycogen synthase is activated is dependent on its phosphorylation state (Villar-Palasi & Larner, 1961 a & b). The phosphorylated form, glycogen synthase b, is very active in the presence of G-6-P. It was termed glycogen synthase D or glycogen synthase Dependent as it is dependent on G-6-P for activity (Cohen, 1978a). The phosphorylated form, glycogen synthase a is largely independent of G-6-P for its

activity and was named glycogen synthase I or glycogen synthase Independent (Cohen, 1978a).

The activity of glycogen synthase is decreased by a wide range of cellular effectors, such as ATP, ADP, AMP, UDP, and Pi (Roach & Larner, 1976). Cohen (1978) felt that the degree of allosteric stimulation/inhibition could be adequately represented by the ratio G-6-P/ATP,ADP,Pi. Thus although the intracellular G-6-P concentration is often above the Km for glycogen synthase D, glycogen synthesis is inhibited due to the presence of other allosteric effectors (Dietz et al., 1980). The overall control of glycogen synthase activity is proposed to involve the integration of the action of cellular effectors with the action of hormonal control (Roach & Larner, 1976).

Glycogen synthase D is observed to be the predominant form in the resting muscle (Dietz et al., 1980). The literature suggested that glycogen synthase I is the form of synthase which is largely responsible for glycogen synthesis in vivo (Conlee et al., 1978, Piras et al., 1968).

In summary, glycogen metabolism is governed by the relative activity of glycogen synthase and glycogen phosphorylase. Glycogen synthase promotes the anabolic process of glycogenesis while glycogen phosphorylase promotes the catabolic process of glycogenolysis. The relative activity of these two regulatory enzymes is

achieved by both hormonal and non-hormonal effectors. In general, effectors that cause phosphorylation will "turn-on" glycogenolysis and "turn-off" glycogenesis while those effectors which cause dephosphorylation will "turn-off" glycogenolysis and "turn-on" glycogenesis.

2.3 CURRENT CONCEPTS IN FAT AND CARBOHYDRATE METABOLISM

Currently the nature of fat and carbohydrate metabolism is regarded as a reciprocal, not a dependent relationship (Randle, 1981). That is, there is no preferred substrate; the use of metabolic fuels is dependent on substrate availability at the site of utilization rather than substrate preference. Of course substrate utilization is also dependent on the intracellular state. These principles have evolved from the study of the glucose-fatty acid cycle (Randle et al. 1963). In its present form it is more accurately described as the glucose- fatty acid-ketone body cycle (Stanley, 1981).

The cycle is based on the observation that high levels of fat oxidation in a tissue inhibits the rate of carbohydrate utilization in that tissue (Randle, 1963). Randle (1981) explained the concept of the glucose-fatty acid cycle as (p109) :

"1)the release for oxidation of fatty acids (and of ketone bodies formed from them) imposes restrictions on glucose metabolism in muscle. 2) uptake of glucose by cells imposes restrictions on fatty acid release and oxidation, 3) there is a tissue phase based on the esterification-lipolysis cy-

cle and a blood phase based on consequential modulations in FFA and ketone body concentrations, and 4) the primitive cycle is subject to modulation by hormones through effects on rates of lipolysis, glucose transport, and esterification of fatty acids."

This cycle provided an explanation for the increased myocardial glycogen concentration which accompanies starvation and alloxan diabetes (Garland & Randle, 1964a & b). Later studies showed that it was possible to restore normal rates of glucose uptake, glycolysis and pyruvate oxidation in the hearts of diabetic rats by inhibiting fatty acid oxidation with 2-bromostearate (Burges et al., 1968).

These findings laid the foundation of the glucose-fatty acid cycle. However since the original research utilized the rat heart and diaphragm as experimental tissues there was some question regarding the applicability of the results to skeletal muscle. Indeed there are many studies which indicated the glucose-fatty acid cycle did not exist in skeletal muscle (Jefferson et al., 1972, Goodman et al., 1974, Reimer et al., 1975, Beatty & Bocek, 1971). In retrospect many of these studies could be seen to have methodological flaws (Rennie & Holloszy, 1977). Later studies supported the existence of the glucose-fatty acid cycle in skeletal muscle (Rennie & Holloszy, 1977, Cuendet et al., 1975, Maizel et al., 1977, Newsholme et al., 1977). The majority of these studies (Rennie & Holloszy, 1977, Cuendet et al., 1975, Maizels et al., 1977) supported the existence of the

glucose-fatty acid cycle only in red oxidative muscle. Only Newsholme et al. (1977) proposed that the cycle was operational in all vertebrate skeletal muscle. Based on these later studies it seemed safe to conclude that the cycle was operational in skeletal muscle per se; the question of the degree of operation in different types of skeletal muscle is discussed in Section IV of this chapter.

The available evidence suggests that fatty acid oxidation produces its 'carbohydrate sparing' effect through the direct inhibition of at least two regulatory enzymes, pyruvate dehydrogenase and phosphofructokinase (Randle, 1981).

Pyruvate dehydrogenase is a mitochondrial multienzyme complex (Randle et al., 1978). This multienzyme complex has three catalytic subunits which are known to catalyze the formation of carbon dioxide, acetyl CoA, and NADH respectively (Randle et al., 1978). The overall reaction is irreversible, having a Keq of 8.4 to the sixth power of base 10 (Randle et al., 1981).

Pyruvate dehydrogenase is inhibited by an increase in the intramitochondrial acetyl CoA/CoA which accompanies increased fatty acid oxidation (Randle et al., 1978). The inhibition of pyruvate dehydrogenase by increased acetyl CoA/coA ratio occurs by covalent modification. This modification involves the phosphorylation of the enzyme component of the pyruvate dehydrogenase complex which catalyzes

the formation of carbon dioxide (Barrera et al., 1972). This phosphorylation decreases the activity of the complex (Barrera et al, 1972, Pettit et al., 1975).

Furthermore the pyruvate dehydrogenase complex has been shown to be inhibited, again through phosphorylation, by increased mitochondrial ratios of ATP/ADP and NADH/NAD+. Both of these nucleotides are intimately concerned with the energy charge of the cell (Atkinson, 1968). Thus in a situation where the energy requirements of a cell are constant but the availalabilty of acyl CoA (and hence acetyl CoA) are increased, the amount of carbohydrate oxidized may be expected to decrease, providing the cell has sufficient metabolic machinery to oxidize fat. It may be argued that competition for Coenzyme A between the pyruvate dehydrogenase catalyzed reaction and the thiolase catalyzed reaction slows the rate of glucose oxidation in and by itself. However if this were true one would expect to detect a rise in lactate release from a given muscle with an increased fatty acid oxidation rate. This is not the case in either fast glycolytic or slow oxidative rat skeletal muscle (Maizels et al., 1977). In fact, lactate release is actually decreased when the slow oxidative soleus is perfused with acetoacetate and insulin compared to the amount of lactate release when soleus muscle is perfused with only insulin in the perfusion medium (Maizels et al., 1977). This indicates that an ealier reaction of glycolysis is involved in the carbohydrate sparing pro-

cess. The available research indicates that the phosphofructokinase catalyzed reaction is the most likely point of regulation.

Phosphofructokinase (PFK) is the main regulatory enzyme in the glycolytic pathway (Hofman, 1976, Racker, 1974). PFK catalyzes the irreversible (Keq = 8.0 to the second power of base 10) reaction of fructose-6-phosphate + ATP to fructose-1,6-bisphosphate + ADP (Hofmann, 1976). It is regulated by a number of allosteric modifiers including ATP and citrate (inhibitors) and ADP, AMP, inorganic phosphate (Pi), and cyclic adenosine monophosphate (cAMP)(activators) (Hofmann, 1976). Muscle PFK is also strongly activated in vitro by fructose-2,6-bisphosphate but the in vivo role of this effector is unclear at the present time (Smith et al., 1983).

The intramuscular concentration of citrate has been shown to be increased with augmented fatty acid oxidation (Garland et al., 1963, Rennie et al., 1976). The increased citrate concentration is suspected to cause a decrease in glycolysis by inhibition of PFK (Garland et al., 1963, Rennie et al., 1976).

Citrate is the only known metabolic effector of PFK which is relatively independent of the energy state of the cell. It follows that if the oxidation of fatty acids, or other metabolic products of fatty acid degradation, were increased

throughout a period where the energy requirement is constant and the oxygen supply adequate, the cell's reliance on carbohydrates might decrease. Maizels et al. (1977) noted that the ability of ketone bodies and fatty acids to diminish glycolysis in a tissue seems to depend on the tissue having a high relative rate of glycolysis. For example Maizels et al. (1977) noted that ketone bodies decrease the rate of glycolysis in soleus muscle from 12.4 umol/g/hour to 8.6 umol/g/hour. However when the rate of glycolysis was lower, ketone bodies did not cause a statistically significant drop in glycolysis (2.1 umol/g/hour to 2.0 umol/g/hour). Maizels et al. (1977) suggested that two conditions where increased levels of fat oxidation might cause a significant decrease in the rate of glycolysis were mild or moderate physical activity and post-exercise recovery.

In order to inhibit the cytoplasmic PFK, citrate, which is formed in the mitochondrion, must gain access to the cytosol. The exact mechanism of citrate transport from the mitochondrion to the cytoplasm is not known. Maizels et al. (1977) proposed three mechanisms to account for the increased cytosolic citrate during periods of increased glycolysis (see Figure 2). All three mechanisms have some experimental support (Cheema-Dhaldi, 1975, Safer & Williamson, 1973, Randle et al., 1970).

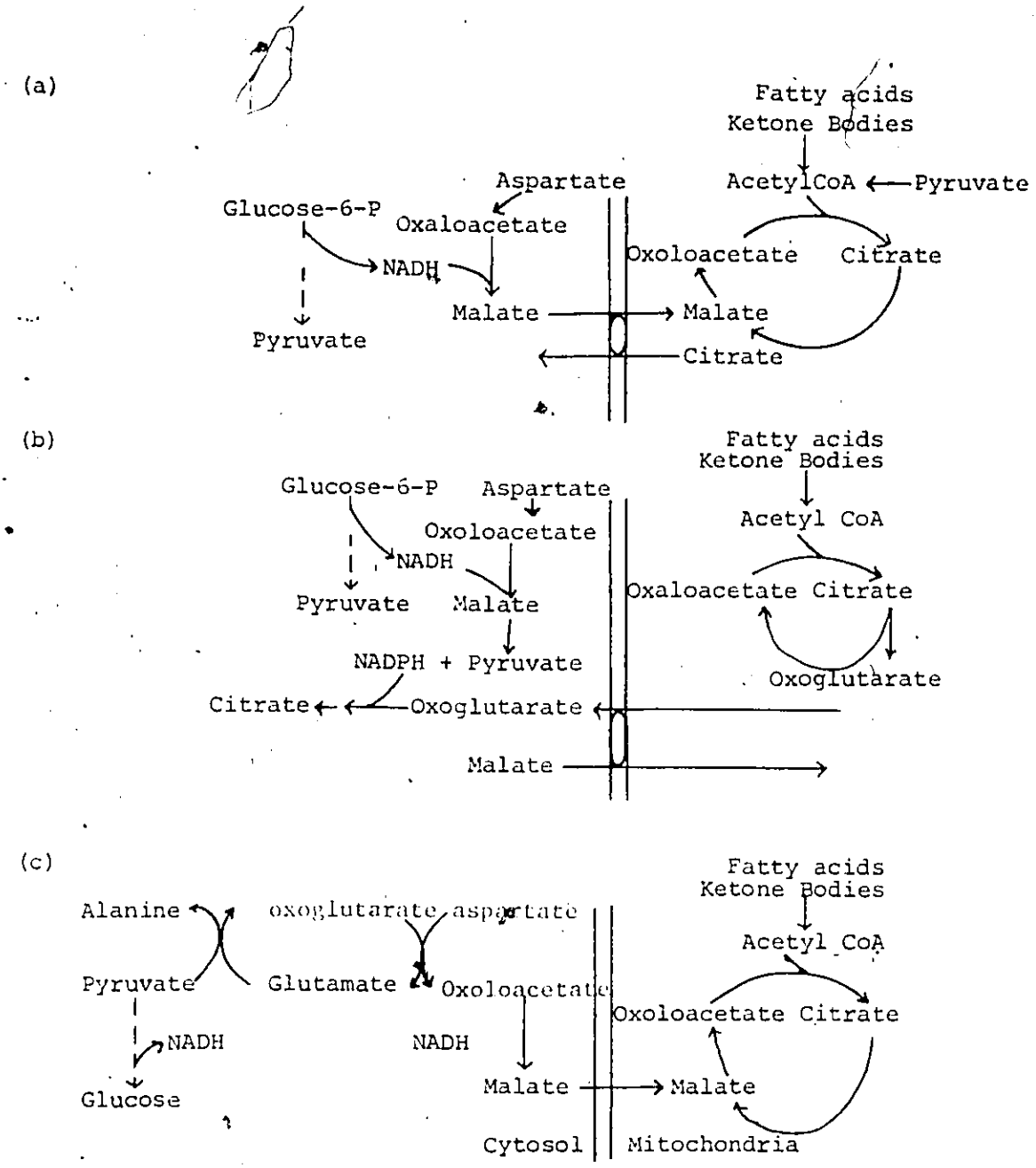


Figure 2: Hypothetical bases for relationship between glycolysis and citrate accumulation in muscle cytosol (a) NADH generated during glycolysis is used for the formulation of malate, in which form the carbon skeleton of aspartate and oxaloacetate can enter the mitochondria. Malate may also be the antiport for citrate efflux from muscle mitochondria (b) Conversion of malate into pyruvate provides NADPH for the conversion of oxoglutarate into citrate in the cytosol (c) Aspartate in the cytosol must be converted into malate in order to enter the mitochondria. In addition to generating NADH for this process, glycolysis provides pyruvate, which accepts the amino group of aspartate.

FROM: MAIZELS E.Z. et al EFFECT OF ACETOACETATE ON GLUCOSE METABOLISM IN THE SOLEUS AND EXTENSOR DIGITORUM LONGUS MUSCLES OF THE RAT. BIOCHEM. J. 162: 557-568, 1977.

Figure 2: Possible Mechanisms of Citrate Formation and Efflux

In addition to its effect on PDH and PFK, an increase in fat availabilty and oxidation could be expected to regulate carbohydrate utlilization by controlling the availability of glucose to the glycolytic pathway. This regulation could occur at three points 1) at the glucose specific transport mechanism 2) at the hexokinase catalyzed reaction and/or 3)at the phosphorylase catalyzed reaction. However if all the effects caused by the regulation of PDH and PFK are accounted for, direct control of these reactions and of the transport can, theoretically, be discounted. Thus, the remainder of this section is devoted to the development of a theoretical model which explains the carbohydrate sparing effect of fatty acid oxidation through the inhibition of PDH and PFK.

As noted previously the rise in citrate which accompanies fatty acid oxidation inhibits PFK. This would cause the intracellular concentration of fructose-6-phosphate to rise. As the Keq of phosphoglucoisomerase is near 1.0 the level of G-6-P will also be increased. G-6-P is known to be an inhibitor of hexokinase (Ozand et al., 1962). This inhibition of hexokinase caused by increased fat oxidation is thus thought to be a consequence of the direct inhibition of PFK (England & Randle, 1967). If such an indirect inhibition occurs it follows that the intracellular free glucose concentration would increase provided the transport mechanism for glucose was not directly inhibited by citrate

or another product of fatty acid metabolism. Experimentally intracellular glucose increases with the addition of free fatty acid to a perfusion medium surrounding a contracting skeletal muscle (Rennie & Holloszy, 1977). Furthermore Maizels et al. (1977) noted that the inhibition of glucose uptake by acetoacetate in soleus muscle causes an increase in intracellular glucose concentration which is ten times greater than the Km for muscle hexokinase. This suggested that hexokinase was inhibited but did not preclude the concurrent inhibition of glucose transport. However Cuendet et al. (1975) found that the uptake of non-metabolizable 3-methyl glucose was not decreased in soleus muscle when the muscle is perfused with fatty acids or ketone bodies.

The effect of this increased intracellualr free glucose concentration may be two-fold. Firstly, the glucose transport mechanism could be indirectly inhibited due to high intracellular free glucose concentration (Maizels et al., 1977). Secondly the rate of glycogenolysis could decrease as an increased intracellular free glucose concentration increases the susceptibilty of glycogen phosphorylase a to hydrolysis by phosphatase (Holmes & Mansour, 1968, Stalman et al., 1972). The decrease in the ratio glycogen phosphorylase a: glycogen phosphorylase b would result in decreased glycogenolysis, as phosphorylase a is the most active form of glycogen phosphorylase (Fischer et al., 1971). In this theoretical model of the

relationship between fatty acid oxidation and carbohydrate utilization the carbohydrate sparing effect of increased fatty acid oxidation can be explained adequately by the regulation of two regulatory enzymes, PDH and PFK. Other potentially important sites of regulation (glucose transport, hexokinase catalyzed phosphorylation and glycogen phosphorylase catalyzed phosphorlysis) were hypothesized to be controlled indirectly as a consequence of the PDH and PFK regulation.

Finally the most important aspect of this model is that the transport of glucose into a muscle cell oxidizing increased amounts of fat is only restricted if the disposal of glucose in the cell is impaired. If the muscle is able to utilize glucose (G-6-P) the inhibition of glucose transport would be eliminated. Thus when glycogen stores are depleted glucose transport per se should not be affected by high NEFA levels whereas anabolic glucose disposal will be enhanced due to increased glycogen synthesis. Cuendet et al. (1975) provided evidence that net glucose utilization in perfused soleus muscle (sum of glycolysis and glycogenesis) is not affected during increased fat availability and oxidation but the relative amounts going to each process are affected. During periods of increased fat oxidation more glucose is used in glycogenesis than is used in glycolysis. Thus, glycogen repletion may be affected in both rate and extent by high rates of increased fatty acid oxidation. The

possible mechanisms for this effect are illustrated in Figure 3.

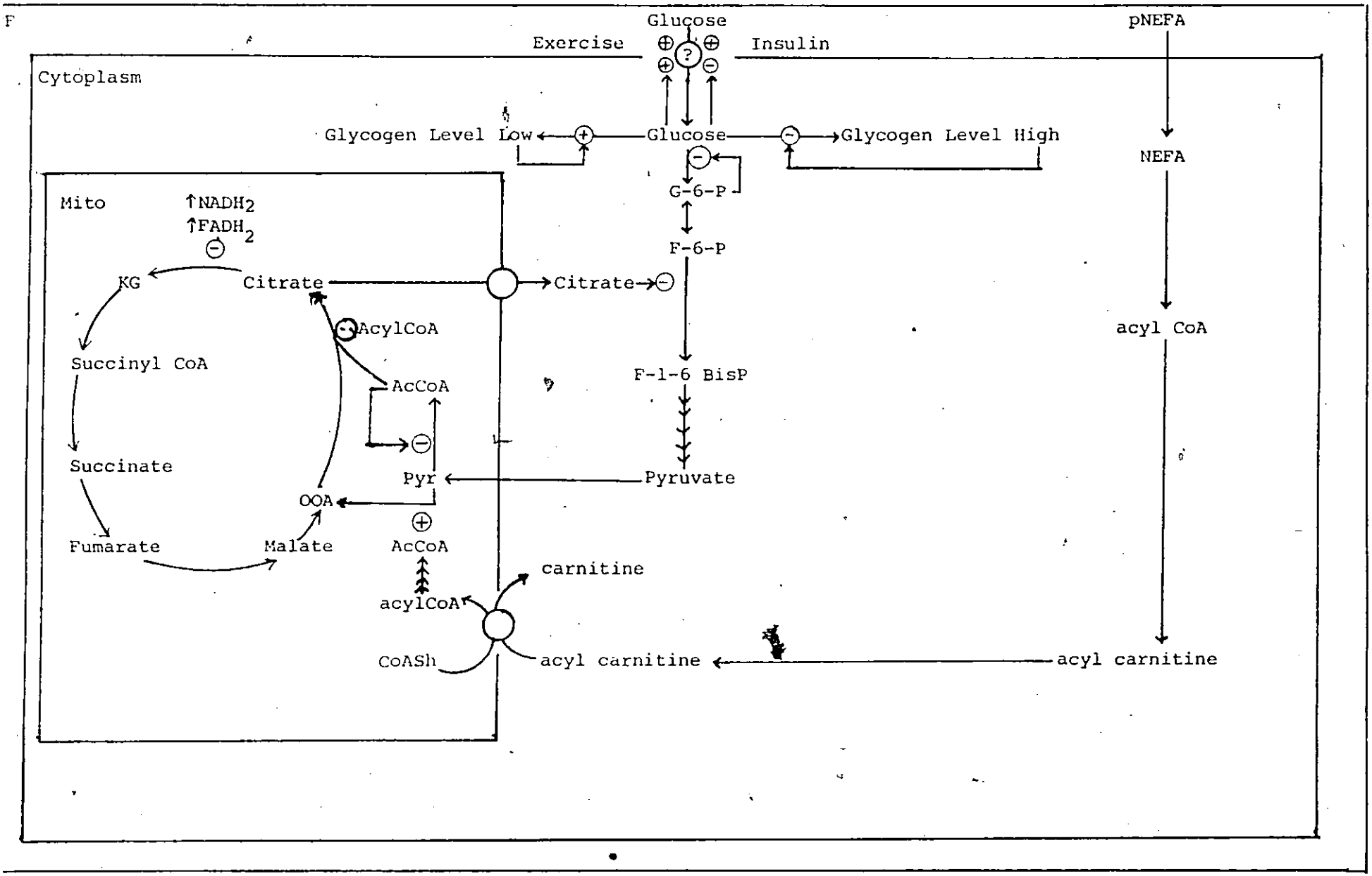


Figure 3: Possible Factors Affecting Glucose Transport and Utilization

2.4 LIPID SUBSTRATES OTHER THAN NEFA IN CARBOHYDRATE METABOLISM

Since NEFA may exert an effect on post-exhaustive exercise glycogen repletion, a logical corollary is that other lipid substrates can exert an effect on post-exercise glycogen repletion. For example, substrates such as plasma triglycerides, phospholipids, and lipoproteins may exert such an effect. However, many studies have shown that these substrates are minor contributors to the energy demand of muscle cells (Froberg, 1971; Paul, 1973). However, there is a relatively large body of research which indicates that intramuscular triglycerides are used extensively in moderate exercise of long duration (Paul, 1973). If this is so one would think that intramuscular triglycerides could create the same carbohydrate sparing effect that has been ascribed to NEFA. A brief review of the intramuscular triglyceride literature will clarify this point.

Experiments on electrically stimulated muscle preparations have demonstrated that the rate of electrical stimulation has to be great enough to increase the metabolic rate of the stimulated muscle 15-20 times that of rest to find a significant decrease in intramuscular triglycerides (Barclay, 1972). At lower stimulation rates (10 times resting rate) intramuscular triglycerides are not mobilized because there is enough plasma substrate to supply the energy to sustain the work (Barclay, 1972). Thus, in experiments us-

ing 1 twitch/sec at tetanic frequencies, which increases the metabolic rate of the muscle approximately ten times, there was no significant depletion in intramuscular triglycerides (Masoro et al. 1966; Gemmell, 1940). In essence, it is not necessary for the cell to mobilize intramuscular triglycerides in the post-exercise state if there are sufficient amounts of other substrates to meet the energy demand. This conclusion is supported by the study of Conlee et al. (1978). Conlee et al. (1978) observed that post-exercise myocardial glycogen repletion was inhibited when NEFA levels were decreased with nicotinic acid. In this study it is plausible that the lack of NEFA diverted glucose away from glycogenesis to glycolysis. Intramuscular triglycerides were not used to spare the glucose as the glucose was a more readily available substrate.

In summary, the glucose-fatty acid cycle is thought to be operational in mammalian skeletal muscle. It is suggested that the phenomenon of carbohydrate sparing could be attributed to the regulation of two key enzymes, PDH and PFK. These enzymes are affected by changes in metabolite concentrations which accompany increased fatty acid oxidation. Glucose transport per se may not be affected by an increased rate of fatty acid oxidation. Thus the repletion of glycogen stores may be somewhat dependent on the availability of alternative substrates for oxidation in the post-exercise state. The most likely alternative substrates are NEFA.

As the glycogen or glucose sparing effect of fatty acids relies on their oxidation in the tissue where they exert their action, the ability to oxidize fat is a crucial factor in the existence of a glucose sparing effect in skeletal muscle.

2.5 RELATIONSHIP OF FIBER TYPES TO THE GLUCOSE-FATTY ACID CYCLE

In the rat three distinct muscle fiber types have been recognized (Baldwin & Winder, 1977). These are 1) fast-twitch, high oxidative, moderate glycogenolytic (FOG); 2) fast-twitch, low oxidative, high glycogenolytic (FG) and 3) slow-twitch, moderate oxidative, low glycogenolytic (SO) (Baldwin & Winder, 1977). The FOG fiber has a high level of myofibrillar ATPase activity (Burke & Edgerton, 1975) as well as high oxidative potential (Peter et al., 1972). In rat skeletal muscle, the FOG fiber has a higher oxidative enzyme capacity than even the SO fiber (Nolte and Pette, 1972). Thus, in rats the respiratory capacity of skeletal muscle is greatest in the FOG fiber, intermediate in the SO fiber, and least in the FG fiber. These fiber types also have different glycogen synthase activities. In rat skeletal muscle, the total glycogen synthase activity of FOG muscle fibers has been shown to be 110% greater than in FG muscle fibers and 35% greater than in SO muscle fibers (Conlee et al., 1978). The actual glycogen synthase activities in

the three muscle fiber types were 4.46, 2.10, and 3.31 umol of glucose residues/min/g muscle for FOG, FG, and SO muscle fibers respectively (Conlee et al., 1978). In the pre-exercise state approximately 20% of the total glycogen synthase activity within a given muscle fiber type was in the glycogen synthase I form. In the post-exercise state the activity of glycogen synthase I rose to about 40% of the total glycogen synthase activity within a given muscle fiber type (Conlee et al., 1978). Since the initial total glycogen synthase activity was greatest in the FOG muscle fibers, lowest in FG muscle fibers, and intermediate in SO muscle fibers it was to be expected that the rate of glycogen repletion would be greatest in FOG muscle fibers, lowest in FG muscle fibers and intermediate in SO muscle fibers. This trend has been substantiated experimentally (Terjung et al., 1974, Poland et al., 1980, Conlee et al., 1978). The respiratory capacity of the different muscle fibers also follows this trend. The possible interrelationship of glycogen synthase activity and availabilty of fatty acids within a muscle fiber is discussed in section VI.

In summary, rat skeletal muscle is composed of three distinct muscle fiber types. These muscle fibers differ in their enzymatic profiles and hence also differ in their metabolic capacities. These differences may affect the ability of NEFA to exert a glucose or glycogen sparing effect. It is probable that any glucose or glycogen sparing

effect that is dependent on NEFA would be most clearly seen in the highly oxidative FOG fiber and least clearly seen in the slightly oxidative FG fiber.

2.6 USE OF PHARMACOLOGICAL AGENTS IN THE INTACT SYSTEM

2.6.1 Effects of Nicotinic Acid

Carlson (1962) was the first investigator to demonstrate that nicotinic acid is a potent antilipolytic agent. Nicotinic acid was observed to cause a decrease in NEFA concentration in fasting men and an inhibition of the noradrenaline induced increase of NEFA concentration in dogs (Carlson, 1965). Nicotinic acid decreases plasma NEFA levels by inhibiting the adipocyte adenylate cyclase system (D'Costa et al., 1979). This research corroborated earlier research showing a rapid accumulation of labelled nicotinic acid in the adipocytes of mice but no such accumulation in their skeletal muscle (Carlson, 1945).

Nicotinic acid is a potent antilipolytic agent whether it is administered orally or by injection (Carlson, 1965). In resting rats, a dose of 250 mg/kg of sodium nicotinate administered subcutaneously produced a statistically significant decrease in NEFA at two and four hours post-injection (Carlson, 1966). The values for the control rats were 0.88 +/- 0.04 mM and 0.91 +/- 0.05 mM at two and four hours respectively. The values for the nicotinic acid injected rats

were 0.50 +/- 0.03 mM and 0.48 +/- 0.02 mM at two and four hours respectively (Carlson, 1966).

The action of nicotinic acid in the post-exercise state is very rapid and very potent. Carlson (1962) observed that nicotinic acid pretreatment (100 mg/kg body weight) almost completely inhibited the effect of norepinephrine infusion in anaesthetized dogs. There is also substantial evidence that an injection and/or oral administration of nicotinic acid will cause a rapid decrease (< 60 min) in post-exercise NEFA levels (Bergstrom et al., 1969; Göllnick et al., 1981). The effect of nicotinic acid in the post-exercise state despite high levels of NEFA is probably due to its potent inhibition of lipolysis coupled with the short half-life of NEFA in the plasma.

The infusion of nicotinic acid also affected insulin levels although plasma glucose concentration was unaltered (Balasse & Ooms, 1973). Nicotinic acid does not exert a direct inhibitory effect on the pancreatic secretion of insulin (Malaisse et al., 1967) so the observed fall in insulin is thought to occur through a reduction in NEFA levels (Balasse & Ooms, 1973). If a decrease in NEFA were to cause a similar decrease in insulin levels in the post-exercise state one could argue that glycogenesis would be detrimentally affected. However Gerber et al. (1978) have shown that insulin concentration does not bear a strong relationship to the

rate of glycogen repletion in the post-exercise state. Berger et al. (1975) have suggested that insulin is needed in small amounts to promote normal glycogen repletion in the post-exercise state. This postulate is supported by research which has shown that the circulating insulin concentration is depressed below pre-exercise values for at least the first four hours of recovery (Terblanche et al., 1981; Conlee et al., 1978). Conlee et al. (1981) suggested that post-exercise insulin secretion in the rat is suppressed due to high levels of catecholamines and glucagon. Post-exercise insulin secretion was suppressed even when the rats were given a .5 g oral glucose load immediately after exercise (Terblanche et al., 1981). Thus, it is not likely that changes in post-exercise NEFA concentration produced by nicotinic acid administration would significantly affect post-exercise insulin concentration.

In summary, nicotinic acid is a potent antilipolytic agent. Its action is specific to the adipocyte adenylate cyclase system. Nicotinic acid was chosen to decrease the post-exercise NEFA concentration as it was shown to be a specific and potent agent in lowering NEFA levels.

2.6.2 Effects of Heparin

Heparin is classified as a linear anionic polyelectrolyte (Jacques, 1980). Commercially available heparin is a mixture of these linear anionic polyelectrolytes (Engelberg,

1978). Heparin is composed primarily of sulfated glucosamine residues and sulfated uronic acid residues (Jacques, 1980).

An injection of heparin reduces the concentration of circulating triglycerides (Engelberg; 1978). This decrease is reflected by an increase in NEFA (Rennie et al., 1976, Costill et al., 1977, Hickson et al., 1977). Heparin exerts its effect by increasing the plasma lipoprotein lipase activity (Engelberg, 1978). Lipoprotein lipase hydrolyses triglycerides in chylomicrons and very low density lipoproteins (Nilsson-Ehle et al., 1980). Lipoprotein lipase readily binds heparin (Olivecrona et al., 1971) thereby stabilizing the enzyme (Iverius et al.,1979) and making it more soluble (Bengtsson & Olivecrona, 1977). Apolipoprotein C II, the activator protein for lipoprotein lipase can stimulate lipoprotein lipase activity without affecting the binding of the lipoprotein lipase to heparin (Bengtsson & Olivecrona, 1977). Thus lipoprotein lipase, freed from the vascular endothelium by exogenous heparin, is catalytically active and can be stimulated by apolipoprotein C II. It is by this mechanism that heparin increases NEFA.

Care is needed when assessing NEFA after heparin administration as significant changes in NEFA concentration can occur if samples are left at room temperature for periods as short as 5 minutes (Giacomini et al., 1980). Freezing the

samples terminates the lipase activity but upon thawing lipase activity is restored (Giacomini et al., 1980). Lipase activity is reduced if the samples are incubated at 0 C during analysis (Giacomini et al., 1980). These results indicate that after heparin administration NEFA assays should be carried out immediately at low incubation temperatures. Alternatively, the samples could be frozen and assayed later using low incubation temperatures.

The decrease in plasma triglycerides with proportional increase in NEFA is dependent on the initial level of TG in the blood (Engelberg, 1978). Thus in exercise studies where an increased NEFA level was desired an exogenous fat source was given followed by an injection of heparin (Rennie et al., 1976, Costill et al., 1976, Hickson et al., 1977). In rat studies this exogenous fat source is usually corn oil, delivered by stomach tube. A subcutaneous injection of sodium heparin (200 U) is administered after allowing time for digestion (3 hours) (Hickson et al., 1977, Rennie et al., 1976). Significant elevation of NEFA was attained 10 minutes after the injection of heparin (Rennie et al., 1976). NEFA level was observed to be significantly elevated in exercising rats which received corn oil and heparin compared to exercising rats which did not receive corn oil and heparin (Hickson et al., 1977). Poland et al. (1983) achieved the same effect by giving an intraperitoneal injection of corn oil followed by a subcutaneous injection of sodium heparin.

Heparin is also known to be an anticoagulant and is thought to be physiologically important in this regard (Engleberg, 1978). A search of the literature failed to reveal any injurious association between acute heparin injections and bleeding in normal rats.

The increased NEFA levels caused by heparin and triglyceride administration were observed to increase the insulin concentration in resting dogs (Madison et al., 1968). However since circulating insulin does not bear a strong relationship to post-exercise glycogenesis (Gerber et al., 1978) the results of Madison et al. (1968) cannot be extrapolated to the post-exercise state. This is verified by Conlee et al. (1978) who observed that the post-exercise rise in NEFA did not cause a similar increase in circulating insulin.

In summary, heparin causes an increase in NEFA at the expense of plasma triglycerides. Exercise studies which utilized heparin without detrimental effects were noted. Thus, heparin administration in conjuction with corn oil administration is is a safe and effective method of increasing plasma NEFA levels in the post-exercise state.

2.7 POST-EXHAUSTIVE EXERCISE GLYCOGEN REPLETION

In the recovery state after long duration exhaustive exercise anabolic processes are expected to predominate in all fiber types. In this state the muscles which were used in the activity are severely depleted of glycogen (Fell et al., 1980, Gaesser & Brooks, 1980 Terjung et al., 1974, Terblanche et al., 1981). The extent of glycogen depletion is dependent on the exercise type. In rats, skeletal muscle glycogen depletion is greater when a running protocol as opposed to a swimming protocol is used as the means of exercise to exhaustion (Terjung et al., 1974).

Regardless of the type of activity, skeletal muscle glycogen stores are never completely depleted (Blawacka et al., 1977, Lambert et al., 1969). It is thought that this is a protective mechanism which exists to provide a glycogen primer for the future resynthesis of glycogen stores (Blawacka et al., 1981). Thus, the repletion of glycogen following exhaustive exercise is not dependent upon reaching complete glycogen depletion.

Rat skeletal muscle has been observed to supercompensate its glycogen stores quite quickly; often within 4 hours of the cessation of exercise (Terjung et al.,.1974, Terblanche et al., 1981). The available literature suggests that glycogen repletion in the first 30 minutes following exhaustive exercise is very slow, while the fastest rate occurs in the

second 30 minutes (Conlee et al., 1978, Terjung et al., 1974). Terjung et al. (1974) suggested that decreased substrate availability causes a decreased glycogen synthesis in the first half hour. This postulation is supported by the observation that most studies utilizing exhaustive exercise protocols report marked hypoglycemia immediately after exercise (Terjung et al., 1974, Conlee et al., 1978). These studies, which provided exogenous substrate, also observed a significant rise in blood glucose in the first 30 minutes following exercise. Therefore although NEFA levels were at their highest in this initial post-exercise period and glycogen synthase was predominantly in the independent form, significant glycogen repletion did not take place, due to lack of substrate (Conlee et al., 1978, Terjung et al. 1974). As substrate became available in the second half hour glycogen repletion was stimulated.

In the absence of exogenous carbohydrate, skeletal muscle repletes to near resting levels but does not exhibit glycogen supercompensation (Gaesser & Brooks, 1980, Fell et al., 1980). When carbohydrate is supplied, the rate of glycogen synthesis is increased; which results in glycogen supercompensation (Terjung et al, 1974, Terblanche et al., 1981).

However the degree of supercompensation may be biased if the rats are fasted before the exercise treatment. The studies of Fell et al. (1980), Conlee et al. (1978), Terjung

et al., (1974) provided a resting fed control group to compare with fasted exercised experimental groups. This method could cause misinterpretation of results as a fasting-refeeding cycle alone has been shown to induce glycogen supercompensation in skeletal muscle of resting control rats (Poland et al., 1980, Gaesser & Brooks, 1980). Thus fasting rats before exercise overlooks the bias of interpreting a fasting-exercise effect as a pure exercise effect. Although the inclusion of a fasting segment in an exercise study simplifies the experimental logistics by reducing the time to exhaustion the practise is unacceptable if a goal of the experiment is to isolate the effects of exercise.

It was of interest to note that recent evidence has suggested that glycogen repletion in rats takes place in a hormonal enviroment which would normally be conducive to glycogenolysis (Terblanche et al., 1981 Conlee et al., 1978). In these studies insulin levels remained below control values during four hours of recovery despite a glucose load which was imposed on the animal immediately after exercise. Conlee et al. (1978) suggested that the prolonged elevation of plasma norepinephrine, caused by the severe exercise stress, inhibited insulin secretion. Epinephrine and glucagon were also significantly elevated after exhaustive exercise (Fell et al., 1980) which could further suppress insulin action and secretion. Post exercise glucose entry in the presence of decreased insulin is explained by recent studies which

have demonstrated that a potent stimulus for increased glucose transport into the muscle cell is a low muscle glycogen content (Ivy & Holloszy, 1981, Fell et al., 1982). This increased transport occurrs concurrently with an increased ability to dispose of the glucose. The increased disposal of glucose occurs through the repletion of glycogen stores in the skeletal muscle (Fell et al., 1982).

After exhaustive exercise the potential for glycogenesis is increased due to an increased concentration of glycogen synthase I (Conlee et al., 1978). Many studies have demonstrated that the concentration of glycogen synthase I is inversely correlated with glycogen concentration (Bergstrom et al., 1972, Danforth, 1965, Conlee et al., 1978). This increase in glycogen synthase I is thought to occur by dephosphorylation of glycogen synthase D as acute exercise did not alter the total glycogen synthase activity (i.e. glycogen synthase I + D) (Conlee et al., 1978).

However glycogen repletion does not solely depend on glycogen synthase I activity. As noted previously the regulatory enzymes of glycogen metabolism are controlled by both phosphorylation-dephosphorylation reactions and the relative abundance of allosteric effectors. The allosteric effectors appear to be very relevant to the post-exercise state as Conlee et al. (1978) noted "the rate of glycogen repletion at a given level of glycogen synthase I activity was faster

during the period 30 and 60 minutes after exercise than it was 60 and 120 minutes post-exercise." That is the phosphorylation state of glycogen synthase did not change, yet the synthesis rate was altered. This suggested that the concentrations of one or more of the allosteric modifiers had changed causing a decreased rate of glycogen synthesis. As the energy charge of the cell is probably constant at this point (minimum 30 minutes after exercise) it seemed possible that the decrease in the amount or availability of G-6-P might have been the cause of the decreased synthesis rate.

The action of NEFA during post-exercise recovery may involve the diversion of available glucose from degradative pathways to synthetic pathways; thereby increasing substrate availability. It is well established that increased NEFA levels during strenuous exercise can exert a glycogen sparing effect in rodents (Rennie et al., 1976 Hickson et al., 1977) and humans (Costill et al., 1977). Furthermore there is evidence which suggests that NEFA levels exert a direct effect on glycogen recovery in the post-exercise state. Conlee et al. (1981) studied the effects of NEFA on glycogen recovery after exercise. The exercise stress was a 1 hour swim; untrained rats were used. A fed control group was included in the study and the rats were fed ad libitum until the experiment. Conlee et al. (1981) suppressed lipolysis in one group of exercising rats by injecting them with nicotinic acid (25 mg/kg), a potent antilipolyitic drug. The

rats which received the nicotinic acid were injected 15 minutes before the one hour swim, again immediately after the swim and finally two hours post-exercise. A second group of exercising rats received saline injections at the appropriate times. Control animals received the appropriate injection of nicotinic acid or saline. In the post-exercise state a similar concentration in myocardial glycogen concentration (35-38%) was noted in both groups of exercised animals. There was a significant depression of NEFA concentration in the nicotinic acid treated animals. In the first two hours, myocardial glycogen repleted faster in non-nicotinic acid injected animals than in the nicotinic acid injected animals. After 2 hours the NEFA levels of the two groups were quite similar. After this point glycogen concentrations also became quite similar in the two groups (nicotinic and saline injected). At 4 hours both groups had a glycogen concentration of approximately 5.58 mg/g wet weight. Thus, it would appear that decreased NEFA levels can cause at least a transient decrease in myocardial glycogenesis during recovery from a moderate exercise stress.

Studies detailing post-exercise skeletal muscle recovery have observed that early post-exercise glycogen recovery takes place when NEFA levels are significantly elevated (see Table 1). The relationship between plasma NEFA levels and early post-exercise glycogen repletion is not strict as evidenced by the study of Conlee et al. (1978). Conlee et al.

(1978) observed that NEFA levels decreased from 760 umol/l plasma at exhaustion to 380 umol/l plasma in the first 30 minutes of recovery. From this point NEFA levels remained significantly elevated throughout the remainder of the 4 hour measurement period. On the other hand glycogen concentrations in the different tissues gradually increased throughout the 4 hour observation period (see Table 1). However the proposed relationship between NEFA and glycogen repletion may still have exerted an effect. For example if the NEFA level was over an "inhibitory threshold" the proposed relationship would be a factor during glycogen repletion. Even if this were not found to be true the result would not be incongruent with the thesis at hand as NEFA can only exert an inhibitory effect when they are being utilized by the tissue. As suggested by Maizels et al. (1977) the importance of NEFA as a glucose sparing substrate may only be important when the tissue in question has a high glycolytic flux. As the flux decreases in the post-exercise state the importance of fatty acids in this role will decrease regardless of their intracellular or extracellular concentration.

Study	Muscle	pre-ex glycogen conc'n mg/ Net weight	post-ex glycogen conc'n 0 hr	½ hr	1 hr	2 hr	3 hr	4 hr	pre-ex NEFA conc'n unol/l	post-ex NEFA conc'n 0 hr	½hr	1hr	2 hr	3hr	4 hr
Terblanche	Plantaris	9.37	2.55		5.45	8.30		7.73							
1981	Soleus	4.90	1.68		4.28	6.14		4.68	480	1080	880	660	520		490
Terjung	RVL	8.33	2.01	3.94	7.68	9.79		11.28							
	WVL	8.38	2.47	2.54	3.86	4.34		6.24							
	Soleus	5.84	1.55	2.14	4.10	5.44		6.68							
1974	Heart	5.25	2.69	3.33	8.03	7.94		9.74							
Armstrong	Soleus	4.29	1.23			3.05		3.97							
&	Plantoris	6.16	3.13			4.45		5.48							
Ianuzzo	Red Gastroc	5.68	2.39			4.54		4.96							
1977	W. Gastroc	6.57	4.34			4.73		5.42							
Bagby	RVL	5.36	1.88												
et al	Soleus	5.31	1.59						210	684					
1978	W. Gastroc	6.19	1.42												
Blawacka 1981	VL (R&W)	9.50	1.10	1.60	1.90		4.90	6.80							
Clark &	RVL	9.00	1.80												
Conlee	WVL	6.84	3.42												
1979	Soleus	6.30	1.98												
Conlee et	Heart	4.86	3.24		5.58	5.94		5.58	100	510		370	120		130
al 1981	Heart (NA)	4.86	3.24		4.50	5.04		5.58	70	210		95	120		110

Table 1: Exercise Studies Involving Glycogen Repletion, Depletion and/or pNEFA Levels

Study	Muscle	pre-ex glycogen conc'n mg/ Net weight	post-ex glycogen conc'n 0 hr	½ hr	1 hr	2 hr	3 hr	4 hr	pre-ex NEFA conc'n unol/l	post-ex NEFA conc'n 0 hr	½hr	1hr	2 hr	3hr	4 hr
Conlee et al 1978	RVL	7.52	1.33	2.95	4.18	5.10		7.27	60	760	380	300	390		280
	WVL	6.87	1.73	1.95	2.35	2.88		4.16							
	Soleus	6.09	1.74	2.15	3.14	4.24		5.55							
Fell et al 1980	Plantaris	9.32	.98				4.44		230	500				380	
	Soleus	4.87	1.04				3.88								
	RVL	7.78	1.31				3.61								
	WVL	7.06	1.14				3.07								
Gaesser & Brooks 1980	Quadriceps	12.84	.40	1.22	1.44	1.67		2.86							
Poland et al 1980	Heart	4.26	2.75		5.15	5.66		4.87		140 % of control			222 % of control		180 % of control
	Soleus	5.54	1.92		2.96	3.02		4.05							
	RVL	4.40	1.59		3.40	3.73		3.75							
	WVL	3.98	1.80		2.30	3.02		3.23							

Table 1(Con't): Exercise Studies Involving Glycogen Repletion, Depletion and/or pNEFA Levels

One way to determine if the plasma concentration of NEFA has any effect on post-exercise glycogen repletion in skeletal muscle is to modify the NEFA levels. There has been only one study (Poland, 1983) which has attempted to use this means to resolve the question. Poland et al. (1983) ran trained and untrained rats on a rodent treadmill set at 1600 m/h. The untrained rats ran for approximately 15 minutes and the trained rats ran for 30 minutes. Some rats were made lipemic 30 minutes prior to exercise by administering corn oil intraperitoneally and heparin subcutaneously. This procedure produced the desired effect as demonstrated by NEFA levels of 2850 ueq/l and 1891 ueq/l at 2 and 4 hours post-injection respectively compared to control values of approximately 239 ueq/l. The exercise also produced the desired effect of significantly reducing muscle glycogen in both untrained and trained rats. Despite these sustained high NEFA levels both muscle citrate and muscle glycogen levels were relatively stable. The only significant difference in muscle occurred at 4 hours when muscle citrate was significantly elevated in vastus lateralis (33.5 vs 29.6 ug/g). However Poland et al. (1983) homogenized vastus lateralis to determine citrate and glycogen levels. This method does not allow one to observe significant changes which may have occurred in a particular fiber type. It has been shown in section IV that the metabolic characteristics of the different fiber types are vastly different and that

this may affect the ability of NEFA to produce an effect in a particular fiber. Thus unfortunately the study of Poland et al. (1983) does not resolve the question of whether or not NEFA levels influence the rate and extent of early post-exercise glycogen repletion. The present investigation has studied the effect of NEFA on early post-exercise glycogen repletion in the FOG, FG, and SO fiber types in order to determine if varying the level of NEFA has any effect on early post-exercise glycogen repletion.

Chapter III

METHODOLOGY

3.1 INTRODUCTION

This chapter presents the methodology that was used in this study. The methodology is described in chronological order commencing with the description of subjects, followed by acclimitization procedure, experimental procedure, and analysis of blood and tissue. The chapter concludes with the statistical procedures used in analysing the results.

3.2 DESCRIPTION OF SUBJECTS

The subject population was composed of 80 male Wistar rats. The animals were approximately 10 weeks old when they arrived at the University of Ottawa's Main Campus Animal Care Facility. Their body weight upon arrival was between 180-200 grams. The animals were purchased from Charles River animal breeding laboratories.

3.3 ANIMAL ACCLIMITIZATION PROCEDURE

Upon arrival the animals were housed in individual cages. They were maintained on standard laboratory rat chow and water ad libitum. A light /dark cycle was maintained so that the time 6:00 am - 6:00 pm was dark and the time 6:01 pm - 5:59 am was light. During the initial acclimitization period (5 days) the rats were allowed to adapt to their new environment and food. On the 6th day all of the rats began a program to condition them to run on a calibrated rodent treadmill (Quinton model 42-15). The rats were conditioned until all the rats could run at 22 m/min for 40 min. The rats ran five consecutive days/week. The treadmill was always set at an incline of 15%. Once all rats could run at 22 m/min for 40 min the experimental protocol was begun.

3.4 SELECTION AND DESCRIPTION OF EXPERIMENTAL GROUPS

Prior to the experimental day, the rats were assigned to one of ten groups: pre-exercise (PRE), post-exercise (POST), saline injected-exercise 1 h (SE1), saline injected-exercise 3 h (SE3), saline injected-non-exercise 1 h (SNE1), saline injected-non-exercise 3 h (SNE3) nicotinic acid injected-exercise 1 h (NAE1), nicotinic acid injected-exercise 3 h (NAE3), heparin injected-exercise 1 h (HE1), heparin injected-exercise 3 h (HE3). The groups SE, NAE, HE and POST were exercised to exhaustion, while SNE and PRE provided non-ex-

ercised trained control values. In addition to the drug treatments described below all groups received two ml of a solution containing .5 g dextrose/ml water at the point of exhaustion. The dextrose solution was administered via a gavage needle. The non-exercised trained control group received their dextrose solution approximately ten minutes after the second experimental rat became exhausted. This procedure ensured that the control groups would receive their dextrose at the approximate mean time of the experimental groups. Of course this was an arbitrary procedure but it was thought to be necessary in order to standardize the dextrose administration as much as possible. All of the saline groups received a one ml intraperitoneal injection of .9% saline. All heparin injected animals received a one ml intraperitoneal injection of corn oil and a .5 ml subcutaneous injection of .9% saline solution containing 400 U of heparin/ ml saline solution. All nicotinic acid injected animals received a one ml intraperitoneal injection of a .9% saline solution containing 16.6 mg nicotinic acid/ ml saline solution. This solution was buffered with NaOH to a pH of 7.4. The nicotinic acid injected rats also received 33.2 mg of nicotinic acid in their 50% dextrose solution. This solution was buffered with NaOH to a pH of 7.0. The rats in groups HE, SE, and NAE received their appropriate injections as soon as exhaustion had been determined. Exhaustion was defined as the point when the rat was unable to maintain the

pace and avoid the shock grid at the rear of the treadmill. The resting trained control rats received an intraperitoneal injection of one ml .9% saline approximately ten minutes after the second experimental rat became exhausted. See Table 2 for an illustration of the injection regimens of the different groups.

			0h. individual exhaustion	1h.	3h.
PRE	sacrifice at onset of exercise				
POST		exercise	sacrifice at exhaustion		
HE		exercise	corn oil by IP. heparin by subcut. dextrose by gavage	sacrifice HE1	sacrifice HE3
SE		exercise	saline by IP. dextrose by gavage	sacrifice SE1	sacrifice SE3
NAE		exercise	nicotinic acid by IP and gavage dextrose by gavage	sacrifice NAE1	sacrifice NAE3
SNE		rest	saline by IP. dextrose by gavage	sacrifice SNE1	sacrifice SNE3

Table 2: Injection Regimens

3.5 EXPERIMENTAL DAY

The experimental day always began at 9:30 am by placing four animals in four lanes of the rodent treadmill. The running protocol of Hickson et al. (1972) was followed which called for 40 min of exercise with the treadmill speed set at 22 m/min. After this time the treadmill speed was increased to 27 m/min for 5-min periods which were separated by 5-min intervals of running at 22 m/min. As the animals became exhausted they were removed from the treadmill and given the appropriate injection according to their treatment group. All animals also received 2 mls of a solution containing .5 g dextrose/ml via the gavage needle.

3.6 SACRIFICE METHODOLOGY

All rats received a .4 ml injection of sodium pentobarbital (65 mg/ml) at the times corresponding to their experimental group classification. After the anesthetic had taken effect blood and muscle tissues were taken

3.7 BLOOD COLLECTION

As soon as the anesthetic had taken effect, as determined by the absence of eye reflexes, the abdominal cavity was opened using stainless steel scissors. The scissors were then used to cut an incision from mid-abdomen to mid-sternum. The incision was reflected to expose the heart and a vacutainer was used to collect the blood directly from the heart. The

vacutainer contained .07 ml of 15% ethylene diamine tetraacetate (EDTA) as an anticoagulant. Once the blood had been collected it was stored in ice until it could be put in a refridgerated centrifuge (4 degrees Celsius). The maximum length of time between blood collection and centrifugation was two minutes. The blood was centrifuged until the plasma was separated from the red blood cells. Three fifty microliter samples of the plasma thus obtained were analyzed by the NEFA assay of Laurell and Tibbling (1966). The NEFA assay of Laurell and Tibbling is presented in Appendix A.

3.8 MUSCLE DISSECTION AND PREPARATION

Immediately after the blood sample had been taken the leg muscles were rapidly exposed. The RVL, WVL and soleus muscles were dissected out. All the visible fat, connective tissue, and blood were removed with a probe and forceps. Muscle samples weighing 35-50 g. were taken from the dissected muscle and frozen in isopentane pre-cooled in liquid nitrogen. The frozen muscle tissue was then transferred into a pre-cooled test tube. The test tubes were stored in liquid nitrogen until they could be transferred for storage to a laboratory freezer kept at -50 degrees Celsius. After removal from frozen storage, the muscle samples were kept on solid CO_2 until they were weighed. Weighing was done on a Mettler H balance. The acid hydrolysis procedure of Lo et al. (1970) was used to determine the amount of glycogen in

the samples of rat skeletal muscle. The methodology of the glycogen assay is presented in Appendix B.

3.9 STATISTICAL ANALYSES

The statistical analyses were performed in six phases.

The first phase used the ANOVA technique to determine if the experimental groups were similar with respect to mean body weight on the experimental day and mean run times to exhaustion.

The second phase consisted of determining the means and standard deviations for all the groups with regards to glycogen concentration and NEFA concentration.

The third phase consisted of two one way analyses of variance. The independent variable was the exercise treatment. The respective dependent variables were glycogen concentration and NEFA concentration. This third phase determined the effect of the exercise treatment on glycogen concentration and NEFA concentration.

The fourth phase consisted of a two way ANOVA. The independent variables were drug treatment and time. The dependent variable was NEFA level. The drug treatment factor had four levels. These were heparin-injected exercised, saline-injected exercised, nicotinic acid- injected exercised and saline-injected non-exercised. The time factor had two lev-

els. These were one hour post-exercise and three hour post-exercise. This fourth phase determined if the drug treatments were effective in altering the NEFA levels.

The fifth phase consisted of a three way ANOVA. The first two factors were the same as above. The third factor was muscle type. There were three levels in this factor. These were red vastus lateralis, white vastus lateralis, and soleus muscles. The dependent variable was glycogen concentration.

The sixth phase consisted of simple main effects analyses and Tukey post-hoc analyses. This phase isolated areas of significance within significant interactions and main effects respectively. The probability level of 0.05 was used for all statistical analyses.

Chapter IV

RESULTS AND DISCUSSION

4.1 RESULTS

The purpose of this study was to determine the effect of varying levels of NEFA via heparin and nicotinic acid administration on the rate and extent of post-exhaustive exercise glycogen repletion in the first three hours following exhaustive exercise in three skeletal muscles of the rat. The results will be analyzed as follows: a) training program results including a subjective evaluation of the training program, the mean body weights of the various groups on the experimental day and the mean times to exhaustion of the various groups. b) means and standard deviations of all groups with regards to glycogen concentration and NEFA concentration. c) one-way ANOVA to determine the effect of exhaustive exercise on NEFA concentration and two-way ANOVA to determine the effect of exhaustive exercise and muscle type on glycogen concentration. d) two-way ANOVA to determine the effect of time and treatment on NEFA concentration. e) three-way ANOVA to determine the effect of muscle, drug treatment, and time on glycogen repletion.

a) subjective evaluation of acclimitization program

The acclimitization program was successful in bringing the rats to a level where they all could run on the rodent treadmill at 15% incline and 22 m/min for 40 minutes. The progress of the rats in reaching this objective was quite erratic. A group of rats which were good runners one week might turn into bad runners the next week. Some rats exhibited respiratory difficulty during parts of their acclimitization. The symptoms included a wheezing sound upon exertion, wet snout and sneezing after exertion and difficulty in maintaining the pace during an acclimitization run. When the rats exhibited these symptoms they were not run. Generally, after a few days rest they were able to resume their acclimitization program. Rats which exhibited symptoms two days before the experimental day were assigned to non-running experimental groups.

TABLE 3

Mean body weights of groups on the experimental day

Experimental group	n=	MEAN +/- S. D. (in grams)
PRE-EXERCISE	8	336 +/- 18
POST-EXERCISE	8	318 +/- 28
HEPARIN-EXERCISE (1 h)	8	343 +/- 26
SALINE-EXERCISE (1 h)	8	345 +/- 23
NICOTINIC-EXERCISE (1 h)	8	330 +/- 17
SALINE-NON-EXERCISE (1 h)	8	326 +/- 19
HEPARIN-EXERCISE (3 h)	8	342 +/- 26
SALINE-EXERCISE (3 h)	8	348 +/- 30
NICOTINIC-EXERCISE (3 h)	8	341 +/- 26
SALINE-NON-EXERCISE (3 h)	8	334 +/- 31

There was some variation in the mean weights of the experimental groups on the experimental day (Table 3). A one way ANOVA was run to examine the effects of the experimental groupings with regards to body weight. There were no significant differences between the body weights of the experimental groups on the experimental day ($p > 0.05$).

TABLE 4

Mean run times to exhaustion for experimental groups

Experimental group	n=	MEAN +/- S. D. (mins.)
POST	8	98 +/- 17
HE (1 h)	8	93 +/- 19
SE (1 h)	8	90 +/- 47
NAE(1 h)	8	83 +/- 31
HE (3 h)	8	79 +/- 22
SE (3 h)	8	72 +/- 19
NAE(3 h)	8	83 +/- 33

Table 4 illustrates the range in run times to exhaustion that were obtained in this study. A one way ANOVA was used to examine the effect of the experimental groupings on the run times to exhaustion. The results of this one way ANOVA indicate that the experimental groups were similar with regards to their run times to exhaustion. However, it is interesting to note that the groups with the lowest and highest weights (POST and SE3 respectively) were the groups with the longest and shortest run times to exhaustion respectively.

TABLE 5

Mean glycogen and NEFA concentrations

Group	N=	RVL	WVL	SOL	NEFA
PRE	8	5.26 +/- 1.90	5.73 +/- 1.24	6.83 +/- 1.67	234 +/- 38
POST	8	0.62 +/- 0.32	1.58 +/- 1.09	1.24 +/- 0.31	620 +/- 254
HE 1 h	8	5.59 +/- 1.80	2.97 +/- 0.69	4.78 +/- 1.15	508 +/- 13
SE 1 h	8	4.00 +/- 1.51	2.99 +/- 0.80	3.79 +/- 1.44	404 +/- 131
NAE1 h	8	3.75 +/- 1.32	3.47 +/- 0.82	4.30 +/- 2.46	239 +/- 62
SNE1 h	8	5.98 +/- 1.71	5.96 +/- 1.11	7.53 +/- 2.42	266 +/- 50
HE 3 h	8	5.30 +/- 1.25	3.66 +/- 0.45	7.14 +/- 0.91	288 +/- 100
SE 3 h	8	5.25 +/- 1.49	3.60 +/- 1.28	6.55 +/- 1.69	282 +/- 66
NAE3 h	8	4.95 +/- 1.20	3.29 +/- 1.27	7.32 +/- 2.42	303 +/- 94
SNE3 h	8	6.69 +/- 2.75	5.79 +/- 2.32	8.88 +/- 1.82	219 +/- 56

Glycogen concentrations and the associated standard deviations are expressed in milligrams of glycogen/gram of muscle.
NEFA concentrations and the associated standard deviations are expressed in microequivalents/ liter of plasma.

Table 5 illustrates the mean glycogen and NEFA concentrations of all the experimental groups. By observing the values of the PRE and POST groups the reader will note that the exercise treatment caused the NEFA concentration to rise to a level approximately three times greater than the resting NEFA value of the PRE group.

By studying the NEFA values the reader will observe that at one hour post-exercise all groups had lower NEFA values than the NEFA value of the POST group. Of the one hour groups, the HE1 group had the highest NEFA concentration while the NAE1 group had the lowest NEFA concentration and the SE1 group had a NEFA concentration between the HE1 and NAE1 groups. At three hours post-exercise all treatment groups were quite similar. The NEFA concentrations of all groups decreased from one hour to three hours post-exercise with the exception of the groups NAE1 and NAE3. These groups exhibited an increase in NEFA concentration from one hour to three hours post-exercise. The reader will also note that the exercise treatment caused a five to ten fold decrease in glycogen concentration. The largest decrease occurred in RVL muscle, the smallest decrease in WVL muscle, while the decrease in SOL muscle was intermediate.

TABLE 6

NEFA concentration as a function of exercise

Source	Sum of squares	df	Mean square	F ratio
Exercise	596756.25	1	596756.25	18.098*
Residual	461641.75	14	32974.41	

* significant at the $p < 0.05$ level

Table 6 indicates that the three fold increase in NEFA concentrationn caused by the exercise treatment was statistically significant. This result is consistent with previous reports in the literature as can be seen in Table 1. As all of the experimental groups underwent the same exhaustive exercise treatment it is highly probable that all exercised groups had elevated NEFA concentrations at exhaustion. Thus, all of the drug treatments, which were given at exhaustion, began to exert their effects at a time when the NEFA concentrations of all the exercised groups were significantly elevated.

TABLE 7

Glycogen concentration as a function of muscle type and exercise

Source	Sum of squares	df	Mean square	F ratio
muscle	9.775	2	4.888	3.178
exercise	276.049	1	276.049	179.496*
2-way interaction				
A X B	4.244	2	2.122	0.263
Residual	64.592	42	1.538	

* significant at $p < 0.05$ level

The exercise treatment caused a significant depletion in the glycogen content of the muscles. All three muscle types were depleted to statistically similar levels at the end of exhaustive exercise. However, the effect of the exercise on glycogen concentration was not completely uniform across the three muscle types. As noted previously RVL was depleted the most, WVL the least, and SOL intermediate. This differential effect of muscle type on glycogen concentration was close to being sta-

tistically significant (p = 0.052). In a physiological sense this result may be significant; this possibility is discussed in the next section.

TABLE 8

NEFA as a function of drug treatment and time

Source	Sum of squares	df	Mean square	F ratio
Drug (A)	238651.75	3	79550.563	9.292*
A at 1 h.	377196.	3	125732.	14.687*
A at 3 h.	31064.	3	10355.	1,21
Time (B)	104895.00	1	104895.00	12.253*
B at HE	213448.	1	213448.	24.93*
B at SE	59536.	1	59536.	6.95*
B at NAE	16384.	1	16384.	1.91
B at SNE	9216.	1	9216.	1.08
2-way interaction				
A x B	172863.375	3	57621.125	6.731*
Residual	479422.313	56	8561.109	

* significant at the p < 0.05 level

As noted previously the drug treatments used in this study caused variations in the NEFA levels. However these variations were only significant at the one hour measurement period. The simple main effects analysis revealed that at this time the NEFA of the HE1 group was significantly greater than the NEFA of the other one hour groups. Furthermore, the SE1 group had significantly greater NEFA levels than the NAE1 and SNE1 groups. The NAE and SNE NEFA levels were not significantly different from each other at the one hour measurement period. There were no significant differences between drug treatment groups at the three hour measurement period.

The NEFA of both the HE and SE groups decreased significantly from one hour to three hours post-exercise. The NEFA levels of NAE and SNE did not change significantly from one hour to three hours post-exercise.

There was a significant interaction between drug treatment and time. This interaction occurred as a result of the differential effect of time on the NEFA levels of the drug treatment groups. An analysis of simple main effects showed that the interaction occurred as a result of large significant differences between the means of the one hour groups which decreased to non-significant differences at the three measurement period. This interaction is seen in Figure 4.

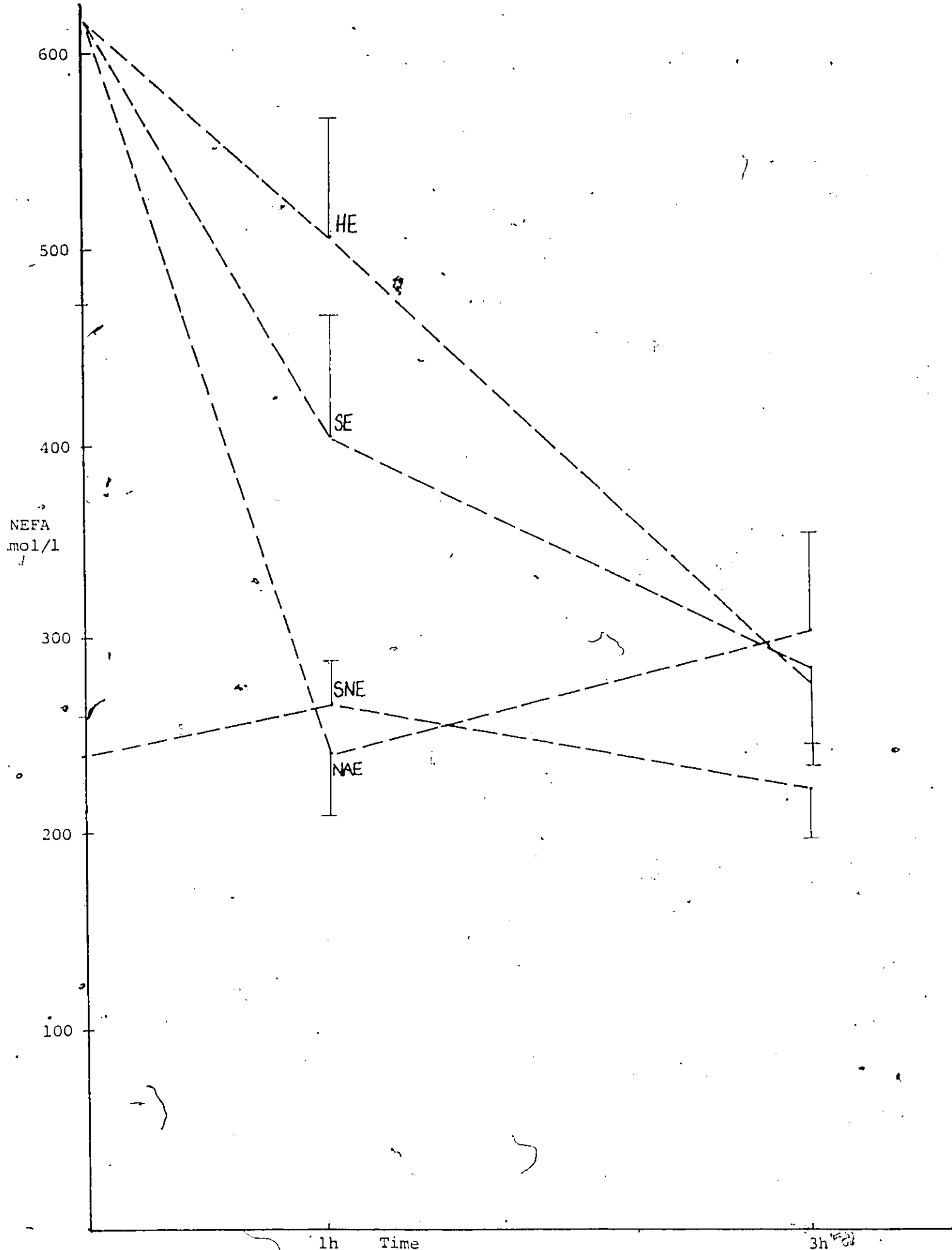


Figure 4: NEFA Concentration vs Time

TABLE 9

Glycogen as a function of exercise, drug treatment, and time

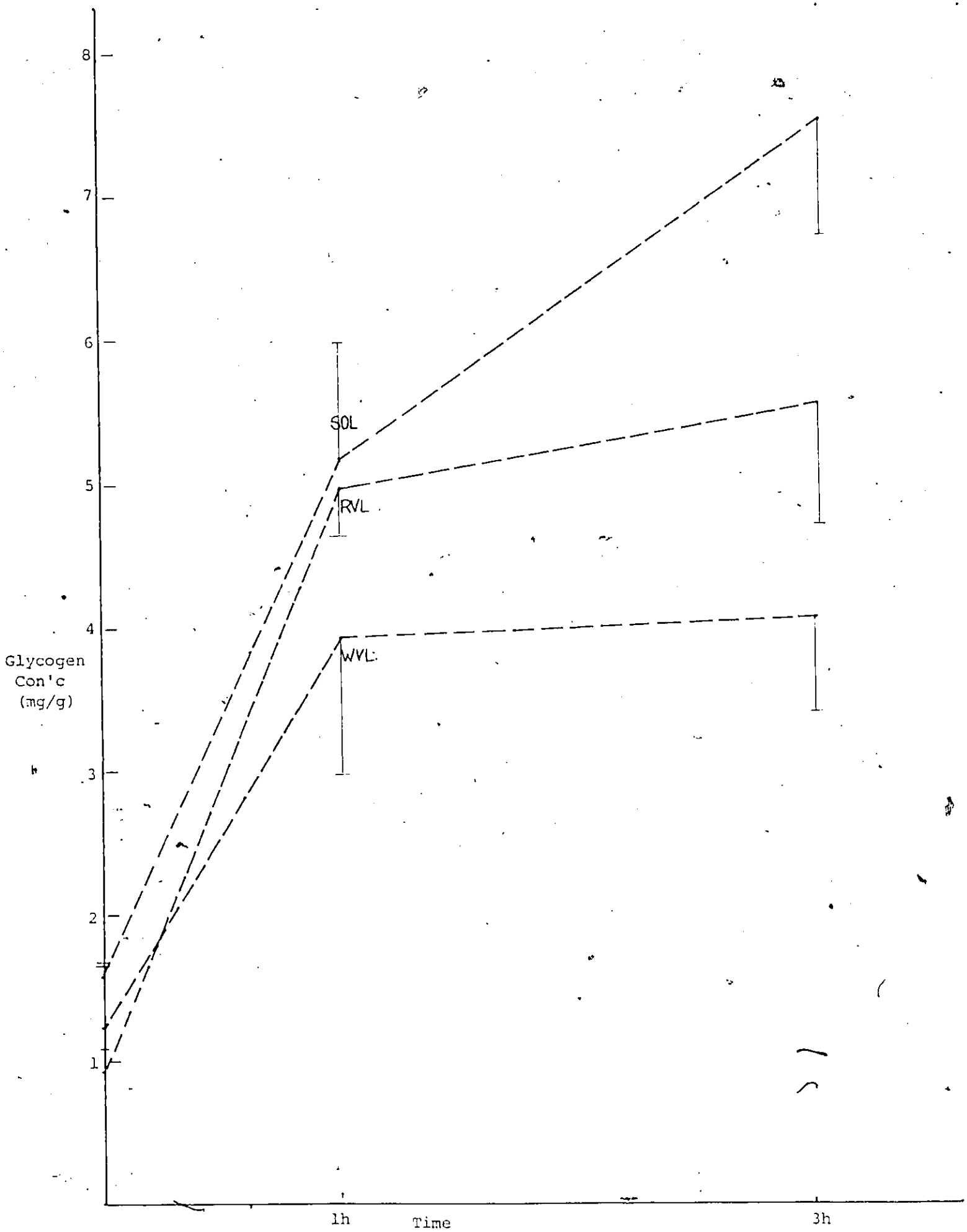
Source	Sum of squares	df	Mean square	F ratio
Muscle (A)	172.468	2	86.234	32.859*
A at 1 h.	27.69	2	13.84	5.28*
A at 3 h.	141.34	2	70.67	26.93*
Drug (B)	183.253	3	61.084	23.276*
Time (C)	58.918	1	58.918	22.450*
C at RVL	8.07	1	8.07	3.07
C at WVL	.85	1	.85	< 1
C at SOL	89.87	1	89.87	34.25*
2-way interactions				
A x B	16.096	6	5.736	2.186
A x C	40.244	2	20.122	7.667*
B x C	6.069	3	2.023	0.771
3-way interactions				
A x B x C	9.264	6	1.544	0.588
Residual	440.895	23	2.624	

* significant at the $p < 0.05$ level

Table 9 shows that all three main effects were significant. The Tukey post-hoc analyses (Appendix D) for drug treatments revealed that at one hour the glycogen concentration of all drug treatment/exercise groups was significantly lower than the corresponding SNE1 value with the exception of HE1 versus SNE1 in RVL. For the case of HE1 versus SNE1 there was no significant difference in glycogen concentration.

The glycogen concentration of RVL in HE1 group was significantly higher than the glycogen concentration of RVL in both the SE1 and NAE1 groups. There were no other significant differences between the means of any other drug treatment/exercise groups within one muscle at one hour post-exercise.

There was a significant interaction between muscle and time in regards to glycogen repletion. This interaction was caused by a significantly greater repletion rate in SOL from one hour to three hour as opposed to the repletion rate in both RVL and WVL from one hour to three hours and a significantly greater repletion rate in RVL from 0 hour to one hour as opposed to the repletion rate in WVL at the same time. This effect can also be seen in Figure 5.


8
7
6
5
4
3
2
1
SOL
RVL
WVL
Glycogen
Con'c
(mg/g)
1h
Time
3h

In summary, the exercise treatment caused a significant increase in NEFA concentration and a significant decrease in glycogen concentration. The drug treatments caused a significant variation in NEFA levels at the one hour measurement period. This variation was transient and had disappeared by three hours post-exercise. The drug/exercise treatments caused a significant variation in the glycogen concentration at both the one and three hour measurement periods. The Tukey post-hoc procedure revealed that the significant main effect of drug treatment was largely due to the comparison of the SNE groups with the exercised groups. However, the Tukey post-hoc procedure also revealed that the glycogen concentration of RVL in the HE1 group was significantly greater than the glycogen concentration of RVL in the SE1 and NAE1 groups.

4.2 DISCUSSION

Although all rats could run the required 40 minute acclimitization period by the time of the experimental day, it was quite easy to distinguish between rats which were excellent runners and rats which were poor runners. The rats which were excellent runners ran almost continuously during the acclimitization program whereas the rats which were poor runners ran intermittently during the acclimitization program. The rats which were deemed as poor runners were placed in experimental groups where they would not have to

run to exhaustion. The other rats were randomly assigned to the experimental groups which ran to exhaustion.

By referring to Table 5 the reader will note that the non-exercised rats (groups PRE, SNE1 and SNE) exhibit an increase in glycogen content of their muscles throughout the study. The existence of this trend can be explained in two ways. Firstly, it may indicate the presence of a diurnal rhythm. Secondly, it may indicate that the oral glucose load given to groups SNE1 and SNE3 created a stimulus to synthesize more glycogen. Resting muscle does not have a strong impetus to synthesize glycogen but the possibility that the glucose load accounted for part of the increase cannot be discounted. Of course, these two possibilities are not mutually exclusive.

As noted previously, the protocol used in this study may have favoured the utilization of glycogen from RVL muscle. This is an important point to consider as the activity of glycogen synthase varies inversely with the concentration of glycogen within the muscle cell (Bergstrom et al.,1972, Danforth, 1965, Conlee et al., 1978). Even though the decrease in glycogen concentration in RVL was not significantly different from the decrease in glycogen concentration in WVL and SOL, the fact that the RVL decrease tended toward lower levels may indicate that RVL had a stronger impetus to synthesize glycogen in the early part of post-exercise glycogen

repletion. If this is so, RVL was the most likely muscle to exhibit any possible associations between early post-exercise glycogen repletion and NEFA levels. In light of this rationale there are some very interesting results in evidence for RVL muscle at one hour post-exercise. At this time the rats with high NEFA (HE1) had repleted significantly more glycogen than had the rats with moderate or low NEFA (SE1 and NAE1 respectively). In addition, a similar trend in glycogen repletion values can be seen in SOL muscle at one hour post-exercise (Table 5). This trend would be more apparent if the glycogen content of the soleus in rat #204 is considered to be an aberrant value (Appendix C). This may be considered a fair assumption as the glycogen content of the soleus in rat #204 is more than twice the average glycogen content of the rest of the NAE1 SOL group. If the glycogen value of rat #204 is excluded from the group, the group mean decreases from 4.30 ug glycogen/g muscle to 3.55 ug glycogen/g muscle. The group means for high, moderate, and low NEFA (HE1, SE1, and NAE1 respectively) would then be 4.78, 3.79, 3.55 ug glycogen/g muscle. In contrast, the group means for WVL glycogen content for the drug treatment/exercise groups are all very similar at one hour post-exercise (Table 5). Thus, based on the results of this study it seems that there is an association between NEFA levels and glycogen repletion in RVL from immediately post-exercise to one hour post-exercise. There appears to be the possibility

of the same association for SOL. The results of this study indicate that there is no such association between NEFA concentration and glycogen repletion for WVL in the first three hours post-exercise.

At three hours post-exercise there were no statistically significant differences in NEFA between the different drug treatment/exercise groups. The NEFA of the NAE group appears to rise from one hour post-exercise to three hours post-exercise. This increase in NEFA was not statistically significant. However, such a result would be consistent with the literature as (Svedmyr et al. 1969) have indicated that a 'rebound effect' can occur if nicotinic acid is used to suppress NEFA. In this 'rebound effect' quantities of NEFA are released from the adipocytes as the circulating concentration of nicotinic acid begins to fall. The NEFA of both the HE and the SE groups decreased significantly from one hour to three hours post-exercise. The decrease in the NEFA of SE is consistent with previous reports in the literature as outlined in Table 1. The decrease in the NEFA of the HE group was expected as pilot work indicated that this decrease would occur. However, the literature suggested that the NEFA would stay elevated for well over three hours (Hickson et al. 1977, Poland et al., 1983). The difference between the results of this study and the results of the literature with regards to the effects of heparin and corn oil administration on NEFA concentration may be due to methodological variations.

The first difference occurs as a result of the blood collection procedure. In this study, the tube containing the blood was placed in ice immediately after collection for a period of time not longer than two minutes. After this time the blood was centrifuged at 4 degrees Celsius. Immediately after separation aliquots of the plasma were placed in an organic solvent which completely inhibits the activity of lipoprotein lipase (Giacomini, 1980). If blood is allowed to stand at room temperature for periods as short as five minutes significant increases in NEFA can occur (Giacomini, 1980). This occurs because the test tube is a closed system; NEFA are produce by the action of lipoprotein lipase but they are not removed. Thus, the NEFA results in this study should resemble the in vivo NEFA concentration more closely than the results of a study where the collected blood was allowed to stand at room temperature for several minutes. It is difficult to say if this is a problem in the literature as very few researchers elaborate on their collection procedures.

The second difference is in the administration of the heparin and corn oil. The heparin was administered with the same technique and in the same quantity (subcutaneously and 200 Units) as in the study of Hickson et al. (1977) but the corn oil administration was different with respect to both technique and quantity. In this study one ml of corn oil was administered intraperitoneally whereas other studies ad-

ministered three mls of corn oil by stomach tube (Hickson et al.,1977, Rennie et al.,1976). The intraperitoneal method was used with success in one study (Poland et al.,1983) but the amount of corn oil used was not mentioned. Thus, by the process of elimihation the amount of corn oil administered may have been responsible for the decrease in NEFA in the HE group from one hour to three hours post-exercise. The method of blood collection may have been responsible for the lower NEFA values in the HE group as compared to the values seen in the studies of Hickson et al. (1977), Rennie et al. (1976) and Poland et al. (1983).

Although the drug treatments were effective in creating statistically significant differences between drug treatment/exercise groups at one hour these differences had disappeared between one hour and three hour post-exercise. Coincidentally, there were no statistically significant differences in glycogen content between drug treatment/exercise groups at three hours post-exercise.

Independent of drug treatments, the RVL repleted at a faster rate than the SOL which in turn repleted at a faster rate than the WVL from immediately post-exercise to one hour post-exercise. This result is consistent with reports in the literature (Table 1). However, from one hour to three hour post-exercise SOL repleted the fastest, followed by RVL and WVL. It is not possible to postulate any effect of NEFA

on the glycogen repletion rates from one hour to three hours post-exercise since NEFA levels at three hours post-exercise were similar in all drug treatment/exercise groups at the three hour measurement period.

At one hour post-exercise the results of this study indicate that NEFA levels are related to glycogen repletion in RVL muscle. This relationship is clearly not in evidence for WVL. This suggests that the availability of NEFA exerts a moderate influence o the glycogen repletion rate in the highly oxidative RVL whereas the availability of NEFA does not exert a comparable influence on the glycogen repletion rate in WVL which has a very low oxidative capacity. Since the drug treatments caused a differential effect on the repletion rate in the same muscle (RVL) the bias of comparing treatment effects across muscles with different activities of glycogen synthase is eliminated. Thus, these results support the theory that NEFA promote glycogen synthesis by sparing glucose from oxidation. It may be argued that the high oxidative capacity of RVL allows this muscle to take full advantage of alternate fuel sources, in this case NEFA, during the early part of post-exercise glycogen repletion. By using alternate fuel sources, muscles may be able to store more glycogen in a shorter space of time.

These results also suggest a possible explanation for the results of Poland (1983). As described in Chapter 3 Poland

(1983) followed the glycogen repletion pattern in rats with high and low NEFA levels. The results of Poland (1983) indicated that high levels of NEFA do not affect the glycogen repletion rate in Vastus Lateralis muscle. However, Poland (1983) analyzed the entire VL instead of separately analyzing its high oxidative and low oxidative components (RVL and WVL respectively). Thus any effect of NEFA on the glycogen repletion pattern of VL would be an average of its effect in RVL and WVL. Both the literature and the results of this study suggest that the glycogen repletion pattern in WVL is not related to NEFA levels in the same way as it is in RVL. Thus the non-significant findings of Poland (1983) may be attributable to the differences in the metabolic characteristics of the different muscle fibers which compose the VL muscle.

In summary, the results of this study indicate that there is a relationship between varying levels of NEFA in the rate of glycogen repletion in RVL from immediately post-exercise to one hour post-exercise. The means of drug treatment/exercise groups for glycogen in WVL were not significantly different from each other. Thus, the results indicate that there is not a strong relationship between varying NEFA levels and glycogen repletion in WVL. The results indicate that a relationship similar to the one observed for RVL may exist in SOL. However, the results of this study indicate that if such a relationship between NEFA and glycogen reple-

tion does exist in SOL it is not as strong a relationship as the one observed between NEFA and glycogen repletion in RVL.

Chapter V

CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSION

The purpose of this study was to investigate the effect of varying levels of NEFA, via heparin and nicotinic acid administration, on the rate and extent of glycogen repletion in the first three hours following exhaustive exercise in three skeletal muscles of adult male Wistar rats. The results indicate that moderately high levels of NEFA are associated with an increased rate of post-exercise glycogen repletion in red vastus lateralis muscle in the first hour following exhaustive exercise relative to the repletion rate observed in RVL from animals which had had medium or low levels of NEFA in the first hour following exhaustive exercise. Conversely, NEFA concentration is not associated with glycogen repletion in white vastus lateralis muscle in the first hour following exxhaustive exercise.

The drug treatments used in this study created significant differences in NEFA levels at the one hour measurement period. These significant differences had disappeared by the three hour measurement period. Thus, it is not possible to draw conclusions concerning the possible association be-

tween NEFA and three hour post-exhaustive exercise glycogen repletion values in any of the muscles. In conclusion, the results of this study indicate that NEFA are associated with post-exhaustive exercise glycogen repletion in red vastus lateralis. These results support the contention that NEFA promote glycogen repletion by sparing glucose from oxidation within highly oxidative muscles possibly by freeing the glucose for glycogenesis.

5.2 RECOMMENDATIONS

If this protocol is duplicated two recommendations should be considered. Firstly, the number of rats in each cell should be increased. Secondly, a larger volume of corn oil should be used to ensure significant elevation of NEFA at the three hour measurement period. Three milliliters of corn oil should be sufficient to achieve this effect.

An improvement in the design of this experiment would be to use specific inhibitors and promoters of fatty acid oxidation to investigate the role of NEFA in post-exercise glycogen repletion.

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Appendix A

A.1 APPENDIX A: PLASMA NEFA ASSAY

Reagents
CHM solution: chloroform-heptane 4:3 (v/v) containing 2% methanol, reagent grades.

Copper-TEA solution: 10 ml of 1 M triethanolamine (TEA), 10 ml of 0.5 M $Cu(NO3)2$ and 3.5 ml of 1 N NaOH are diluted with distilled water to 100 ml. 33 g of NaCl is dissolved in this solution and the pH is adjusted to 8.1. To be prepared daily.

DPC solution: 0.4% diphenylcarbazide, reagent grade in ethanol. To be prepared daily. Immediately before use 0.1 ml of 1 M TEA is added to 10 ml of 0.4% DPC.

Stock standard solution:Dissolve 512 mg palmitic acid in 1000 ml chloroform (2000 umoles/l). Working standard solutions: Dilute 5 ml stock standard to 100 ml. with CHM and dilute further 5, 10, 15, and 20 20 mls of this solution to 100 mls to obtain 5, 10, 15, 20 umoles/l standards.

Procedure
1) To stoppered test tubes 250 +/- 50 mg of silicic acid is added with a suitable spoon followed by 6 ml of CHM solution.

2) 50 ul of plasma is transferred to each tube, which is immediately stoppered and shaken 30 times. 6 ml of CHM solution (blank), 10 umoles/l and 20 umoles/l palmitic acid standards are treated in the same way.
3) The tubes are centrifuged at 4000X g for 20 minutes.

4)5 ml of the supernatant is transferred to another stoppered test tube containing 2 ml of Cu-TEA solution.

5) These tubes are shaken horizontally in a mechanical shaker for 5 min.

6)These tubes are then centrifuged at 4000X g for 20 minutes.

7)3 ml of the upper phase is transferred with a volumetric pipette to another test tube containing 0.5 ml of DPC solution.
The pipette must not touch the inner wall of either test tube.

8)After careful mixing the colour is developed for 15 min and read in a spectrophotometer set at 550 nanometers.

All glassware was purified with a dichromic-sulphuric acid mixture.

A standard curve was calculated each time the assay was conducted, using the mean absorbance of three samples at each concentration.

The equation used to calculate the fatty acid content of the plasma is presented below:

$$\text{umol fatty acids/l plasma} = \frac{(A\ 550 - A)}{B} \times 120$$

where: A 550= Absorbance of sample at 550 nanometers.
A = Y intercept
B = slope of standard curve.

A.2 APPENDIX B: MUSCLE GLYCOGEN ASSAY

Reagents:

30% KOH solution saturated with sodium sulfate.
300 g potassium hydroxide pellets were dissolved in distilled water to one liter and saturated with sodium sulfate.

95% ethanol

5% phenol
50 g phenol crystals were dissolved in distilled water

to one liter.

96-98% sulfuric acid

50 mg glycogen powder was dissolved in distilled water to 10 ml.

working standard solutions: .5, 1.0, 2.0, 3.0, and 5.0 mls of stock solution was diluted to 10 ml in a volumetric flask to obtain working standard solutions of 10, 20, 40, 80, 100 ug glycogen/ml.

sodium sulfate saturated aqueous solution.

Procedure:
1)After removal from frozen storage, the muscle samples were kept on solid CO_2 until they were weighed on a Mettler H balance.

2)Samples were transferred individually to stoppered tubes containing 0.5 ml of 30% potassium hydroxide saturated with sodium sulfate. Special care was taken to ensure that the samples were completely immersed in the solution.

The tubes were capped and placed in a boiling water bath for 30 minutes until a homogenous solution was obtained.

3)The tubes were removed from the water bath and cooled in ice for ten minutes.

4)1:1 to 1.2 volumes of 95% alcohol was added to precipitate the glycogen from the alkaline digestate.

6)The samples were vortexed and then cooled in ice for 60 minutes.

7)The samples were then centrifuged at four degrees Celsius and 3000X g for 60 minutes.

8)The supernatants were carefully aspirated using disposable Pasteur pipettes.

9)The tubes were then inverted and drained by gravity for fifteen minutes.

10)The precipitated glycogen was then redissoved in 3 mls of distilled water.

11)A one ml aliquot of the above glycogen solution was pipetted into a clean test tube.

12)One ml of five percent phenol solution was added to this aliquot.

Five ml of 96-98% sulfuric acid were rapidly added the stream of acid being directed against the liquid surface to ensure thorough mixing.

The tubes were allowed to cool for ten minutes after which they were mixed in a vortex.

15)The tubes were then placed in a water bath at 25-30 degrees Celsius before readings were taken.

16)Blanks were prepared by using one ml of distilled water instead of glycogen solution.

17)The absorbance was read on a Bausch and Lomb Spectropic 20 Spectrophotometer at 490 millimicrons.

18)All tests were carried out in duplicate.

Whenever samples were analyzed, two samples of standard glycogen solution were subjected to the same procedure; to evaluate recovery and accuracy.

The equation used to calculate the glycogen content of the tissue is presented below:

$$\text{mg glycogen/g wet tissue weight} = \frac{(A_{490} - A)}{B} \times \frac{V}{v} \times \frac{1}{W} \times \frac{1}{R}$$

where: A_{490} = Absorbance of sample at 490 nanometers.
A = Y intercept.
B = slope of standard curve.
V = total volume of glycogen solution.
v = volume of aliquot used in the colour reaction.
W = weight of muscle sample in mg.
R = recovery

A.3 APPENDIX C: RAW DATA

NEFA GROUP	ANIMAL #	MUSCLE RVL	WVL		SOL
PRE-EX.	1	6.65	4.83	4.38	193
	12	3.66	7.88	8.29	280
	17	5.02	5.35	8.36	257
	19	2.39	3.92	5.78	257
	210	8.53	7.02	9.03	194
	216	4.50	5.94	7.10	266
	223	5.95	5.71	6.59	244
	227	5.44	5.22	5.10	181
POST-EX	3	0.43	2.91	0.91	542
	8	1.04	0.70	1.64	423
	16	0.33	0.33	1.06	543
	20	0.46	3.04	1.21	597
	206	0.39	2.36	1.72	1152
	215	1.21	0.91	1.14	836
	219	0.54	0.61	1.34	408
	224	0.59	1.76	0.90	461
HE 1	31	3.87	2.33	4.49	750
	45	4.57	3.40	3.21	650
	203	6.16	3.96	7.04	336
	207	8.83	2.82	4.95	538
	213	7.57	1.76	4.02	398
	234	3.83	3.49	5.03	418
	238	4.83	2.95	4.07	481
	246	5.05	3.08	5.39	496
SE 1	5	5.54	3.31	6.54	444
	10	2.68	1.38	1.91	563
	23	3.47	3.42	2.68	631
	47	2.73	4.13	3.04	298
	48	6.69	2.55	4.68	346
	220	4.86	2.85	4.56	340
	225	3.27	3.23	3.56	267
	233	2.77	3.07	3.42	342
NAE 1	4	3.49	3.13	4.63	140
	9	3.64	3.71	5.98	288
	32	2.53	5.14	3.84	333
	46	2.44	2.71	2.54	237
	204	4.69	3.07	9.53	210
	231	3.43	2.86	3.01	229
	237	6.51	3.02	2.52	287
	243	3.29	4.14	2.33	188
SNE 1	6	6.34	7.04	12.73	225
	11	3.93	7.17	6.53	253
	18	8.66	6.95	9.22	280
	24	7.95	6.23	5.80	304
	36	5.94	4.14	5.80	365
	44	4.98	6.03	6.19	244
	241	6.11	4.98	7.93	247
	247	3.96	5.11	6.05	209

HE 3	30	5.91	3.65	8.28	449
	42	5.07	3.95	8.13	356
	201	4.44	3.71	8.24	188
	208	6.53	4.30	6.33	276
	212	7.44	4.01	6.79	126
	221	3.81	2.90	6.62	280
	228	4.14	3.18	6.55	341
	230	5.02	3.54	6.21	292
SE 3	29	5.99	6.46	9.88	341
	39	5.72	2.03	5.91	399
	236	5.83	3.40	6.17	301
	239	6.54	3.09	6.66	297
	240	6.59	2.98	8.09	258
	242	5.48	3.44	5.78	192
	244	2.63	3.55	4.66	238
	245	3.22	3.85	5.23	232
NAE 3	28	3.69	3.15	7.81	377
	38	5.72	2.03	5.91	399
	202	5.78	5.64	13.03	131
	209	4.12	2.89	6.95	370
	214	6.53	2.60	5.63	280
	218	5.85	3.59	6.86	225
	226	4.79	4.52	6.59	267
	232	3.15	1.92	5.81	377
SNE 3	37	2.03	2.30	6.03	267
	41	5.81	3.38	8.71	302
	205	5.75	6.27	9.06	117
	211	10.12	5.99	10.41	233
	217	6.29	4.96	7.46	234
	229	10.70	8.90	12.14	173
	235	5.58	8.83	8.64	206
	222	7.24	5.69	8.58	222

A.4 APPENDIX D: RESULTS OF POST-HOC ANALYSES

Tukey Post-hoc Analyses

RVL		WVL		SOL	
SNE1 vs NAE1	2.23*	SNE1 vs HE1	2.99*	SNE1 vs SE1	3.74*
SNE1 vs SE1	1.98*	SNE1 vs SE1	2.97*	SNE1 vs NAE1	3.23*
HE1 vs NAE1	1.84*	SNE3 vs NAE3	2.50*	SNE1 vs HE1	2.75*
SNE3 vs NAE3	1.74*	SNE1 vs NAE1	2.49*	SNE3 vs SE3	2.33*
HE1 vs SE1	1.59*	SNE3 vs SE3	2.19*	SNE3 vs HE3	1.74*
SNE3 vs SE3	1.44	SNE3 vs HE3	2.13*	SNE3 vs NAE3	1.56
SNE3 vs HE3	1.39	NAE1 vs SE1	<1	HE1 vs SE1	<1
HE3 vs NAE3	<1	SE1 vs NAE1	<1	NAE1 vs SE1	<1
SE3 vs NAE3	<1	HE3 vs NAE3	<1	NAE3 vs SE3	<1
SE1 vs NAE1	<1	SE3 vs NAE3	<1	HE3 vs SE3	<1
SNE1 vs HE1	<1	HE3 vs SE3	<1	NAE3 vs HE3	<1
HE3 vs SE3	<1	SE1 vs HE1	<1	HE1 vs NAE1	<1

Significance based on .05 level.