Accelerated Rates of Glycolysis in Cardiac Hypertrophy: Are they a Methodological Artifact?

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ABSTRACT

Glycolysis, measured by ^3H_2O production from [5-^{3}H]-glucose, is accelerated in isolated working hypertrophied rat hearts. However, non-glycolytic detritiation of [5-^{3}H]-glucose by transaldolase in the non-oxidative pentose phosphate pathway (PPP) could lead to an overestimation of true glycolytic rates. Since the PPP may be upregulated in cardiac hypertrophy, I tested the hypothesis that detritiation of [5-^{3}H]-glucose does not overestimate glycolysis in hypertrophied hearts and that accelerated rates of glycolysis reported in hypertrophied hearts are real and not artifactual. Glycolysis was measured by three independent methods in isolated working hearts from halothane-anesthetized sham-operated (Control) and aortic-constricted (Hypertrophy) rats. Two of the three methods determined glycolytic rates by quantifying the accumulation of glycolytic end products in timed collections of perfusate while the last method determined glycolytic rates by detritiation of [5-^{3}H]-glucose in the glycolytic pathway by production of ^3H_2O. The first method involved enzymatic determination of lactate and pyruvate combined with rates of glucose oxidation. The second method involved determination of radiolabeled [^{14}C]-lactate and [^{14}C]-pyruvate accumulation combined with rates of glucose oxidation. The third method, which measures glycolytic flux by metabolism of [5-^{3}H]-glucose, was in question and thus was used for comparison with the aforementioned alternative methods of determining glycolytic flux. Glycolysis was accelerated in hypertrophied hearts, regardless of the method used. There was also excellent concordance between the three methods with no significant differences in glycolysis detected between corresponding groups. Moreover, glucose-6-phosphate dehydrogenase activity and transaldolase expression, enzymes controlling key steps in the oxidative and non-oxidative PPP,

respectively, were not different between control and hypertrophied hearts. Thus, non-glycolytic loss of ^3H_2O from [5-^{3}H]-glucose is insignificant and ^3H_2O production from [5-^{3}H]-glucose is an accurate means to measure glycolysis in isolated working normal and hypertrophied rat hearts. Furthermore, the PPP does not appear to be increased in this model of cardiac hypertrophy.

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LIST OF ABBREVIATIONS

1,3-BPG:	1,3-Biphosphoglycerate
2-PG:	2-Phosphoglycerate
3-PG:	3-Phosphoglycerate
ATP:	Adenosine triphosphate
CPT1:	Carnitine palmitoyltransferase 1
CoA:	Coenzyme A
dH_2O:	Distilled water
DHAP:	Dihydroxyacetone phosphate
ETC:	Electron Transport Chain
E4P:	Erythrose 4-phosphate
$FADH_2$:	Reduced flavin adenine nucleotide
FFA:	Free Fatty Acids
F6P:	Fructose 6-phosphate
G6P:	Glucose 6-phosphate
G6PDH:	Glucose 6-phosphate dehydrogenase
GAP:	Glyeraldehyde-3-phosphate
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
HK:	Hexokinase
LPL:	Lipoprotein Lipase
LDH:	Lactate Dehydrogenase
MCT:	Monocarboxylic Acid Transporter

NAD^+:	Oxidized nicotinamide adenine nucleotide
$NADH_2$:	Reduced nicotinamide adenine nucleotide
PDC:	Pyruvate Dehydrogenase Complex
PEP:	Phosphenolpyruvate
PGI:	Phosphoglucose isomerase
PGK:	Phosphoglycerate kinase
PGM:	Phosphoglycerate mutase
PK:	Pyruvate kinase
PPP:	Pentose Phosphate Pathway
PFK-1:	Phosphofructokinase-1
R5P:	Ribose 5-phosphate
S7P:	Septulose 7-phosphate
TIM:	Triose phosphate isomerase
TCA cycle:	Tricarboxylic Cycle
VLDL:	Very-low density Lipoprotein
Xu5P:	Xylulose 5-phosphate

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I want to dedicate this work to my family, a source of inspiration and support during this degree. I greatly appreciate the devotion and faith that was placed in me during those stressful times and I pray that you guys continue to be there for me as I carry on in my studies. Finally, I want to give a big thanks to all my friends who comforted me and provided me with support during this degree. Finally, I want to thank everyone in Daniel and Phileo Fellowship and my good friends at the iCAPTUR^{4}E Centre.

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I. INTRODUCTION

A) Cardiac Hypertrophy

1. Overview

Cardiac hypertrophy is a condition where the heart adapts to prolonged increases in pressure/volume overload by an enlargement of the heart (83). This enlargement or hypertrophy of the myocardium is caused by increases in wall thickness, myocyte volume and size so that wall stress is normalized (57,83). This adaptive response also extends beyond that of a structural impact; gross mechanical function and fuel metabolism are also affected, depending on the nature of cardiac hypertrophy. There are two main types of cardiac hypertrophy: exercise-induced and pathological cardiac hypertrophy (119). This study will focus on the pathologically hypertrophied heart and the fundamental changes in its metabolism compared to the normal myocardium.

2. Clinical Significance

Pathological cardiac hypertrophy is common in Western populations, present in 15-20% of populations (76), with ~90% of hospitalized individuals in advanced stages of cardiac disease possessing a hypertrophied heart (41,75,76,74,49). Although cardiac hypertrophy resulting from arterial hypertension is an initial compensatory response, cardiac failure eventually occurs due to a number of mechanisms that are provoked by the heart's adaptation to work overload (24). Therefore, cardiac hypertrophy has emerged as a major risk factor for more severe forms of cardiovascular disease (75,76,74,49) leading to sudden death, myocardial infarction, and congestive heart failure (49). In some cases, sudden death can occur without a definite ischemic event (41). Individuals with

hypertrophied hearts also tend to demonstrate poorer recoveries from acute ischemic insults because the heart is more susceptible to injury in the event of an ischemic insult (14,50,6) compared to individuals with normal hearts. Therefore, the co-existence of cardiac hypertrophy and coronary artery disease, both of which are highly prevalent conditions, in any individual has dramatic implications.

3. *Myocardial (Mal)Adaptations in Cardiac Hypertrophy*

When the heart initially copes with the increase in workload demand, diastolic dysfunction first occurs (59,144) while dramatic changes in protein expression take place as the hemodynamic stimuli evokes growth signals, cell receptors, and transcription factors to induce structural cardiac hypertrophy (69,92). Despite the normalization of mechanical function according to workload, structural remodeling inadvertently affects other minor properties of the heart that ultimately contribute to its impending dysfunction (99). Alterations in cardiac receptors, endocrine function, contractile proteins, ion exchange and energy metabolism are observed in hypertrophied hearts (16,44,51,14) (Figure 1).

For example, altered rates of shortening velocity and delayed relaxation of cardiac muscle of the heart can be attributed to alterations and deficiencies in ion exchange and handling as the cause for these changes in contraction and relaxation (58). Another significant feature is increased Ca^{2+}accumulation in the myocytes of hypertrophied hearts; a result of a lack of Ca^{2+} handling (6) which is thought to result in a reversal of Na^{+}/Ca^{2+} exchange to regulate intracellular Ca^{2+} (105,125,93). Following this export of Ca^{2+}, greater Na^{+} accumulation is observed in the cell, which ultimately leads to ischemic

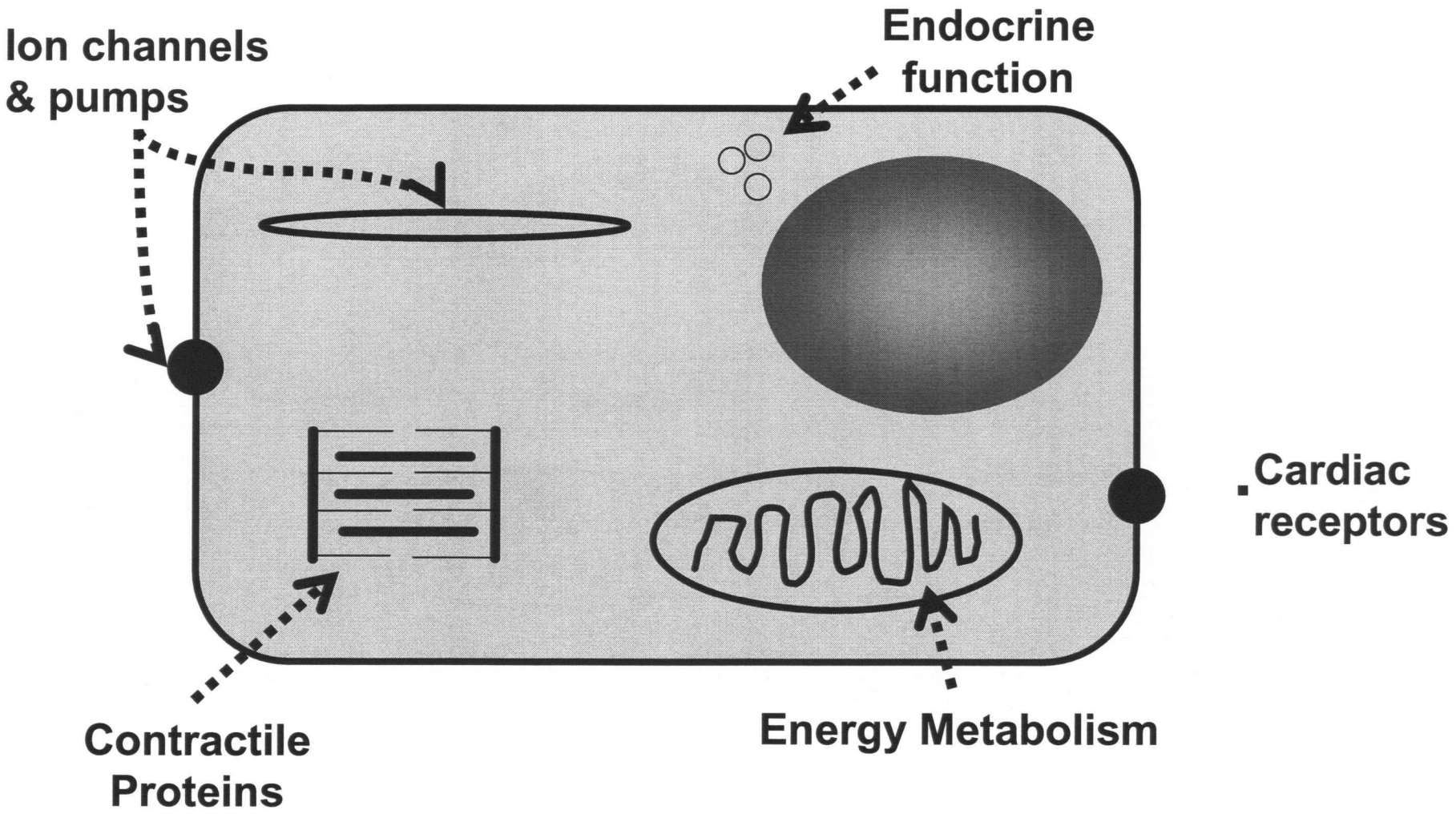


Figure 1: Myocardial adaptations in cardiac hypertrophy.

dysfunction when consequent Na^+/H^+ ion exchange results in myocardial acidosis (14,91,52). Although the effects of myocardial acidosis are not immediately detrimental in the aerobic perfusion setting, its effects become fully realized post-ischemia, and contribute to the increased susceptibility of the hypertrophied heart to injury in the event of ischemia (14,91,52).

The hypertrophied heart's response to ischemia is even more dramatic when its metabolism is considered. Increases in glycolytic enzyme levels (53), and altered properties of lactate dehydrogenase (21) have been reported in hypertrophied hearts, leading to increased glycolytic metabolism. This change becomes detrimental as the by-products of glycolytic metabolism ($NADH_2$, lactate, H^+) accumulate and contribute to the pathogenesis of myocardial ischemic damage in hypertrophied hearts (14,5).

The hypertrophied heart is distinctively susceptible to myocardial ischemia, and is observed to have decreased mechanical function upon post-ischemia reperfusion when compared to normal hearts (127,11). An alteration in energy substrate use by the hypertrophied heart has been implicated as an important cause for the pathophysiology of cardiac hypertrophy (21,28) and decreased post-ischemic function in the hypertrophied heart (11,33). Although glycolysis is beneficial in generating energy during and after ischemia, oxidative metabolism of glucose is low relative to glycolysis. As a consequence, a more acidic intracellular environment is produced that complicates Ca^{2+} handling which leads to myocardial dysfunction (94,6). Therefore, the hypertrophied heart is maladaptive in the ischemic setting because of alterations in glucose metabolism that may contribute to changes in myocardial ion exchange during and after an ischemic event.

B) Energy Metabolism of the Mammalian Heart

1. Overview

The omnivoric nature of the heart reflects its perpetual need to provide energy for the constant activity of the heart and its function in providing circulation of blood to the rest of the body. This required energy is derived from fatty acids, glucose, lactate, amino acids and ketone bodies (97,116,62). Fatty acids are the predominant source of energy for the normal heart (Figure 2), while glycolysis and the oxidation of glucose and lactate meet the remainder of the heart's energy requirements under normal circumstances. However, the extent to which all exogenous sources of energy contribute to ATP pools is entirely dependent on the availability and presence of competing energy substrates, with fatty acid oxidation and glucose catabolysis operating in an inverse relationship as outlined by the Randle cycle (111).

2. Myocardial Fatty Acid Metabolism

Oxidation of fatty acids accounts for a large majority (~70-85%) of energy production (116). In the blood, these free fatty acids are bound to either albumin or lipoprotein and enter cells by passive diffusion (1) and/or saturable protein-facilitated transport (2). Thus, high extracellular FFA levels and/or low intracellular FFA levels favour the uptake of fatty acids. The proteins FAT (fatty acid translocase/CD36) and FABP (fatty acid binding protein) (135) are the main components involved in fatty acid protein-facilitated transport.

Imported FFA's are then primed for mitochondrial β-oxidation by fatty acyl-CoA synthetase (98,123). This enzyme activates each individual fatty acyl for oxidative degradation (β-oxidation) by catalyzing the addition of a CoA group to the fatty acyl

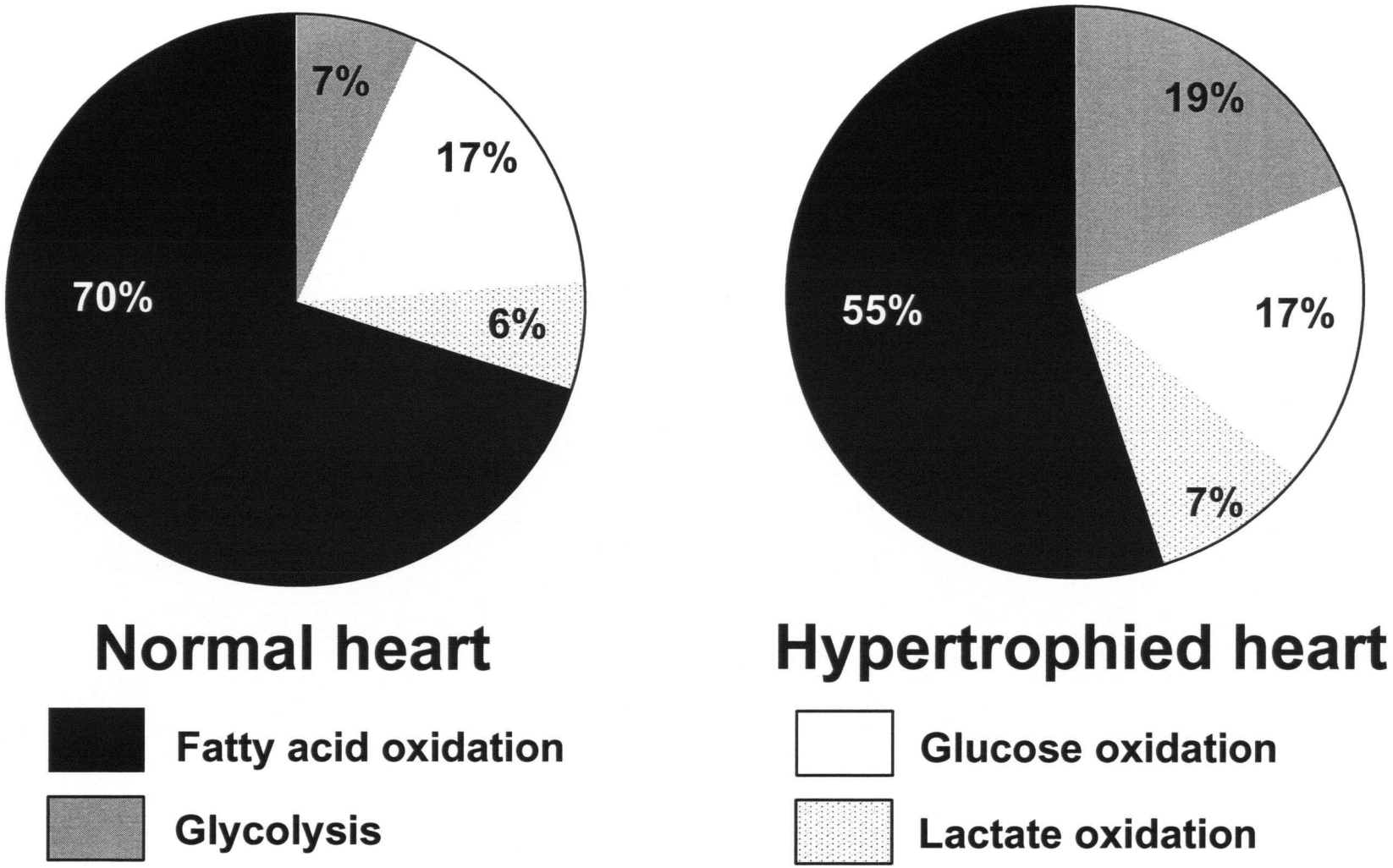


Figure 2: Myocardial energy production in normal and hypertrophied rat hearts perfused with 0.4 mM palmitate. Presented values are percentages of contribution to total ATP in each heart. Data from Allard *et al.* Am J Physiol 267: H742-750, 1994. Data reprinted by permission of American Physiological Society.

moiety. Following the formation of the fatty acyl-CoA complex, all FFA's diverge into different metabolic fates according to individual FFA structure and length, all of which is governed by an internal regulatory mechanism that favours the oxidation of shorter FFA's over longer FFA's (77,78). For small chain fatty acids, there is no specific mechanism of regulation and entry into the mitochondria is relatively unobstructed (78). Long chain fatty acids (14 carbon chain and over) do not share this fate; instead, transport into the mitochondria is mediated by the carnitine palmitoyltransferase system, which is intricately controlled by the end products of fatty acid oxidation and covalent modification of relevant enzymes (100,97,101).

This carnitine-mediated translocation process is mediated by a trimeric protein complex [*For a more detailed review of this mechanism, see McGarry*] (20,87,88,4) which is inhibited by downstream by-products of fatty acid oxidation; namely malonyl-CoA. (86). Acetyl-CoA carboxylase (ACC) is responsible for the critical conversion of acetyl-CoA to malonyl-CoA under high fatty acid/glucose oxidation levels (25). Therefore, carnitine-mediated translocation is ultimately intertwined with the β-oxidation of long-chain fatty acids (81). Once in the mitochondria, fatty acids are progressively broken down into acetyl-CoA units by β-oxidation, producing one $NADH_2$, one $FADH_2$ and one H^+ for each removal of acetyl-CoA from the parent fatty acyl-CoA (123). All acetyl-CoA derived from β-Oxidation and from pyruvate decarboxylation (by PDC) rally at the TCA Cycle, undergoing oxidation to produce reducing equivalents $NADH_2$ and $FADH_2$, which will undergo oxidative phosphorylation for ATP generation. Overall, the oxidation of fatty acid (palmitic acid) generates 131 ATP, including ATP generated by each cleavage of an Acetyl-CoA during β-oxidation (Figure 3).

β-Oxidation

7 $NADH_2$ × 3 ATP = 21 ATP
7 $FADH_2$ × 2 ATP = 14 ATP
35 ATP

Total ATP Production

8 Acetyl-CoA × 12 ATP = 96 ATP
β-Oxidation = 35 ATP
131 ATP

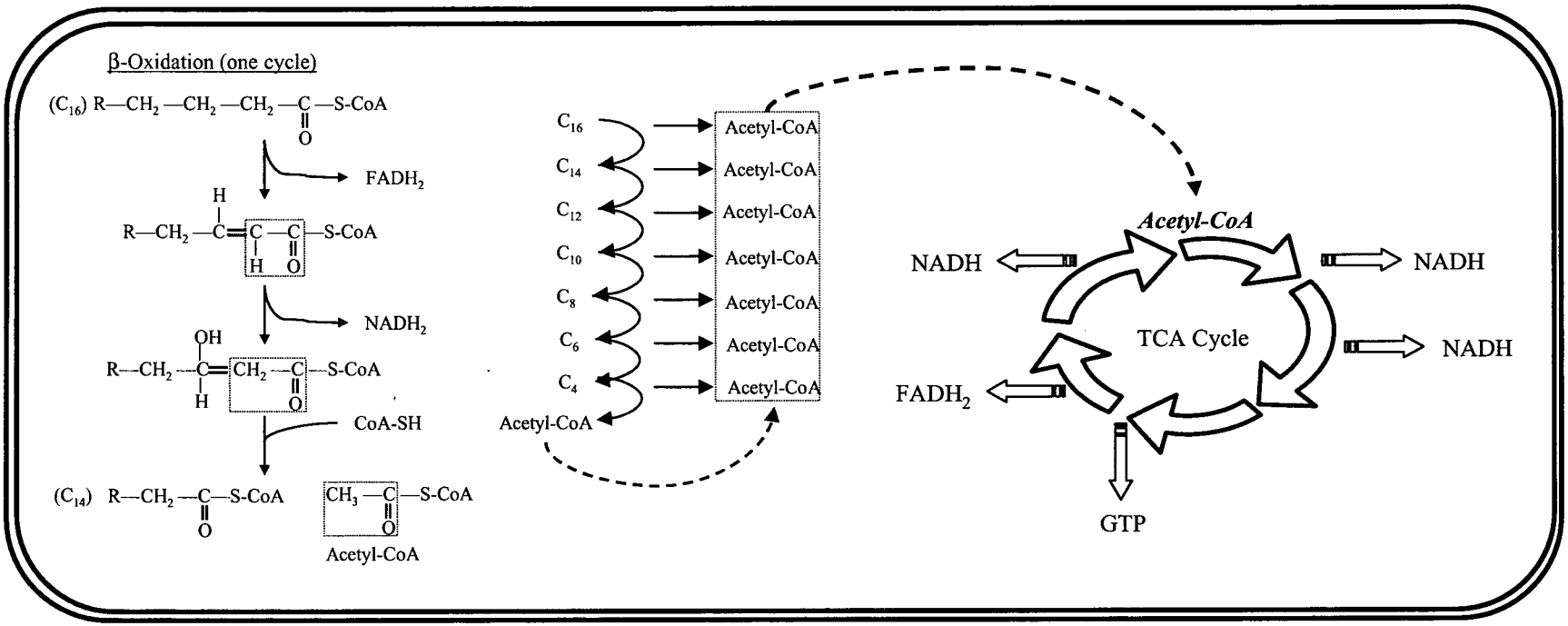


Figure 3 : Energy Production in the Myocyte Generated by Fatty Acid Oxidation. Adapted with permission from: Figure 16-8 and 15-10; Principles of Biochemistry 2nd Edition, Lehninger, Nelson and Cox, Copyright © (1993). Reprinted by permission of W. H. Freeman and Company•Worth Publishers, Inc..

Although the products of fatty acid oxidation and glucose oxidation rally at the start of the TCA cycle in the form of acetyl-CoA, competition between fatty acids and glucose as the dominant source of fuel in the heart exists in a reciprocal fashion as described by the Randle Cycle (95). As outlined, glucose oxidation decreases in response to increases in fatty acid oxidation because the buildup of acetyl-CoA and $NADH_2$ allosterically inhibit the pyruvate dehydrogenase complex (PDC) – the main regulator of glucose oxidation (129). Inversely, increases in carbohydrate metabolism result in a decrease in fatty acid oxidation. This is due to the increased production of malonyl-CoA from acetyl-CoA carboxylase (ACC) and subsequent inhibition of carnitine palmitoyltransferase I (CPT1) and fatty acid transport into the mitochondria. Balance between these metabolic pathways is also dictated by mitochondrial ratios of $NADH/NAD^+$ and acetyl-CoA/CoA, which have regulatory allosteric effects on the activity of PDC and ACC, respectively. Overall, metabolism of fatty acids and glucose is well regulated and primarily controlled by substrate availability and metabolite interplay, as well as by workload and hormonal control.

3. *Myocardial Carbohydrate Metabolism*

Carbohydrates, such as glucose and glycogen, are a significant source of energy for the heart and are first metabolized by the glycolysis pathway, which splits glucose into two molecules of pyruvate. In doing so, glycolytic degradation of one glucose molecule produces 2 ATP molecules that are involved in ion homeostasis maintenance and muscle contractility (97,129,39). The pyruvate generated from this pathway enters

the mitochondria where it is used to generate large amounts of ATP in a process known as glucose oxidation (97,129,39).

Prior to glycolysis, glucose is transported into myocytes by way of glucose transport protein carriers (97,26,129,39), and once within the myocyte cytoplasm, glucose is phosphorylated by hexokinase to glucose-6-phosphate. Glucose-6-phosphate then has two major fates: either to be stored as glycogen, or to be degraded by glycolysis (Embden-Meyerhof pathway). Under normal circumstances, the majority of glucose 6-phosphate units undergo glycolysis, where one glucose molecule is ultimately split into two pyruvate molecules (a 3 carbon glycolytic intermediate) by the glycolytic pathway. In doing so, a net 2 ATP and 4 $NADH_2$ are produced for every glucose molecule that passes through glycolysis. At the completion of the glycolytic pathway, pyruvate generated has three main fates: it can be transported out of the myocyte; be converted to lactate by lactate dehydrogenase; or undergo oxidation in the mitochondria, in a process known as glucose oxidation (Figure 4).

The oxidation of pyruvate (essentially glucose) in the mitochondria is a very industrious process, and occurs via three main components, the Pyruvate Dehydrogenase Complex (PDC) (35), the Tricarboxylic Acid Cycle (TCA Cycle) (71,70), and the Electron Transport Chain (ETC) (90,60,17). First, the PDC decarboxylates pyruvate into acetyl-CoA, thus priming it for complete oxidation by the TCA (71,70). Once within the TCA cycle, reducing power is transferred from acetyl-CoA to cofactors such as $FADH_2$ and $NADH_2$, and these reducing equivalents undergo oxidative phosphorylation at the site of the ETC to generate ATP (90,60,17). All in all, 38 ATP is generated alongside $3CO_2$ and $3H_2O$ for every glucose molecule that undergoes glycolysis and glucose

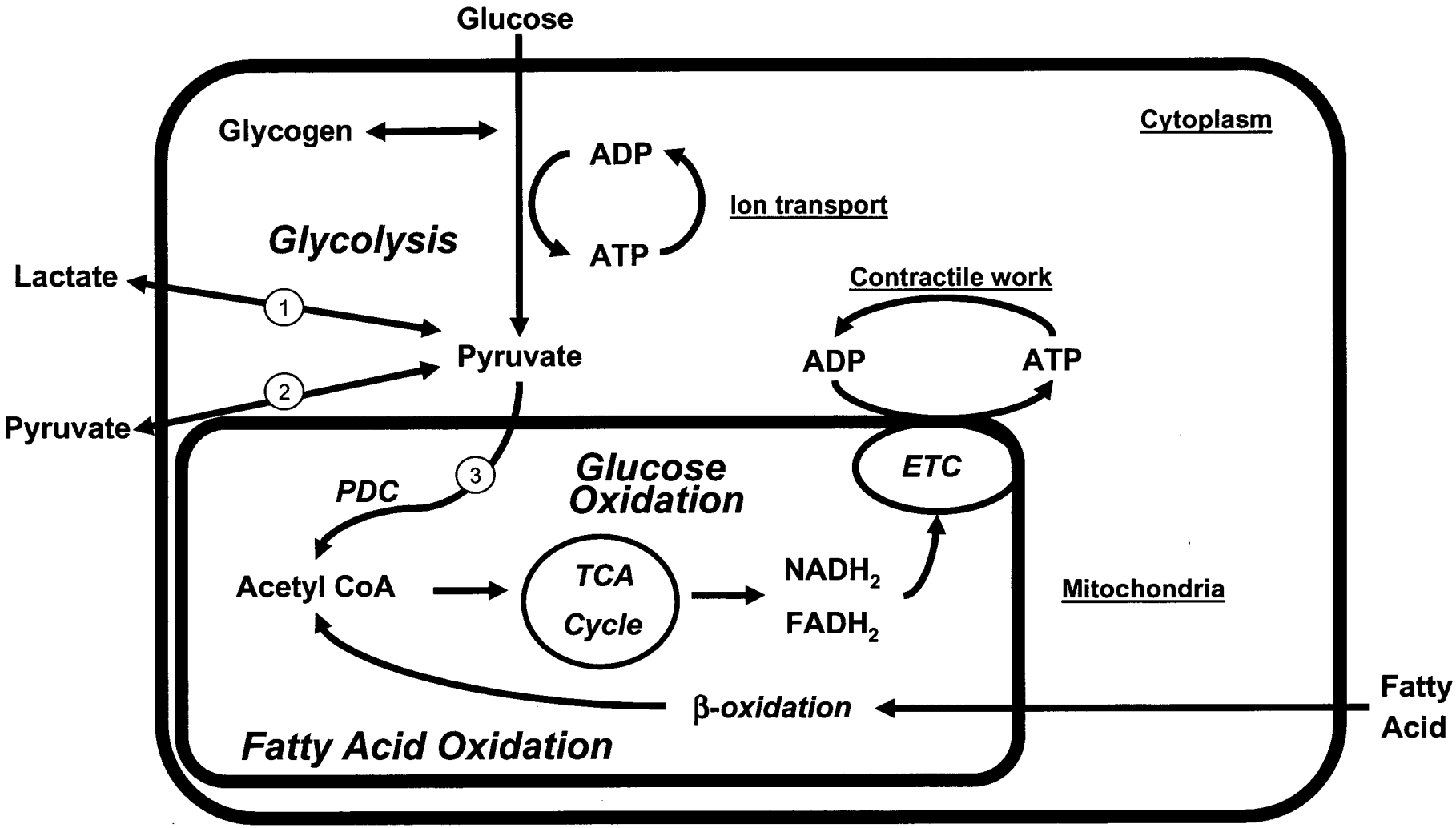
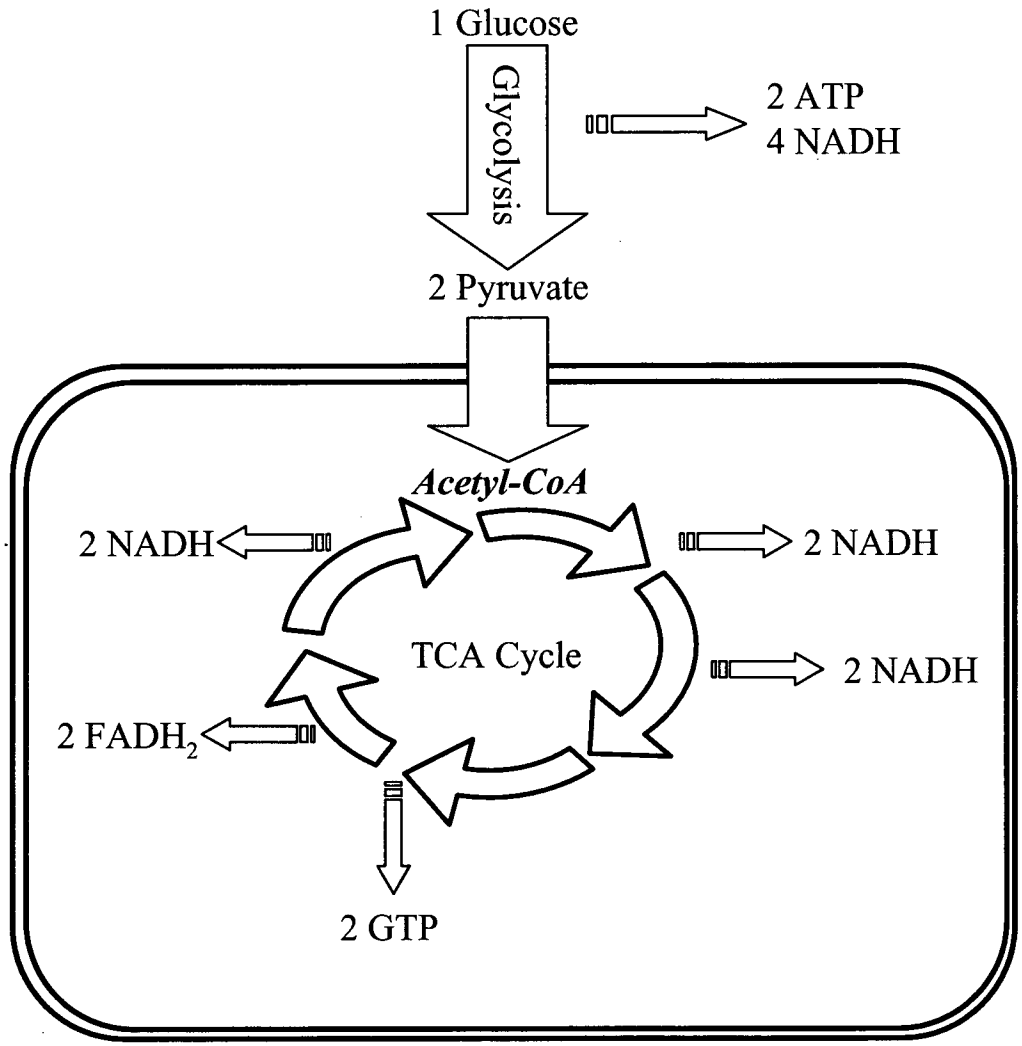


Figure 4: Summary of Metabolism- Glucose enters the cytoplasm and can either be stored as glycogen or continue into Glycolysis. Upon conversion to pyruvate, there are three fates: 1) conversion to lactate by *LDH*, 2) transport out of the myocyte by MCT carriers, 3) transport into the mitochondria for further oxidation, also known as glucose oxidation. (modified figure from review by Sambandum et al. (118))

oxidation (Figure 5). However, the fraction of pyruvate that is actually oxidized is regulated by the Pyruvate Dehydrogenase Complex (PDC). This site is the major regulatory step of glucose oxidation, a regulation that is executed by a kinase-phosphatase system (pyruvate dehydrogenase kinase/pyruvate dehydrogenase phosphatase) and intramitochondrial $NADH_2/NAD^+$ and acetyl-CoA/CoA levels (35). Phosphorylation of PDC, catalyzed by PDK (107,108,23), leads to inactivation of the complex, while dephosphorylation of PDC is catalyzed by PDP and leads to activation of the PDC. PDP is also sensitive to the intramitochondrial environment, being dependent on $Mg^{2+}$and further activated in response to increases in concentrations of Ca^{2+} in the mitochondrial matrix (34,112,103,36,19,129). For example, during exercise an increase of Ca^{2+} can cause an activation of PDH phosphatase, thereby activating the PDC.

In the isolated working rat heart, the fraction of pyruvate (derived from glycolysis) oxidized ranges from 20-40%, depending on the fatty acid condition (7,121,9,47,138,139,142). Under low fatty acid conditions (0.4 mM palmitate), the percentage of pyruvate oxidized increases upwards of ~35-40%, whereas isolated working hearts perfused with high fatty acid oxidize a much lower fraction of pyruvate (~10-20% of total pyruvate at 1.2 mM palmitate) (7,121,9,47,138,139,142). Under conditions of no FFA, pyruvate/glucose oxidation rates are near maximal because intracellular ratios of $NADH_2/NAD^+$ and acetyl-CoA/CoA are at their lowest, thus inducing an activation of PDC.

There are other secondary pathways of glucose metabolism: a small portion of pyruvate is devoted to the maintenance of the Tricarboxylic Acid Cycle. In the event of excessive carbon loss in the cycle (cataplerosis) (136), pyruvate is converted to



(A)

Glycolysis:

4 NADH × 3 ATP = 12 ATP

2 ATP = 2 ATP

14 ATP

(B)

Per turn of TCA Cycle (Glucose Oxidation):

6 NADH × 3 ATP = 18 ATP

2 $FADH_2$ × 2 ATP = 4 ATP

2 GTP → 2 ATP = 2 ATP

24 ATP

Glycolysis = 14 ATP

Glucose Oxidation = 24 ATP

38 ATP

Figure 5 : Energy Production in the myocyte Generated by (A) Glycolysis and (B) Glucose Oxidation.

oxaloacetate and shunted to the TCA cycle to replenish intermediate pools in a process known as anaplerosis (102). Pyruvate carboxylase is one major anaplerotic enzyme that catalyzes this carboxylation of pyruvate to oxaloacetate (130,131,64,32). Another anaplerotic fate of pyruvate, one of minor metabolic significance, is the conversion of pyruvate to alanine (65). Also known as the "pyruvate-alanine cycle", this process is also anaplerotic by contributing to oxaloacetate pools in a reaction catalyzed by glutamine-pyruvate tranaminase (GPT). This reaction is as follows:

$$\text{pyruvate} + \text{glutamine} \Leftrightarrow \text{alanine} + \text{oxaloacetate}$$

As well, a small portion of glycolytic intermediates are also known to participate in nucleotide metabolism through a secondary pathway called the Pentose Phosphate Pathway (PPP). In this pathway, G6P is shunted away from glycolysis and used to form the carbon backbone of nucleic acids, as well as generating reducing power (in the form of $NADPH_2$) for lipid biosynthesis processes.

C) The Pentose Phosphate Pathway (PPP)

1. Overview

Synonymously recognized as the Pentose Phosphate Shunt, or the Hexose Monophosphate Shunt, the PPP forms a link between carbohydrate metabolism (glycolysis and glucose oxidation), fatty acid metabolism and nucleotide synthesis in the cytosol (Figure 6). Normally, glucose is catabolized by glycolysis and glucose oxidation; however, the presence of the PPP yields a secondary pathway of glucose oxidation that seeks to replenish $NADPH_2$ and ribose-5-phosphate pools (145,148,147), both of which serve functions unrelated to glucose and fatty acid metabolism. In terms of overall

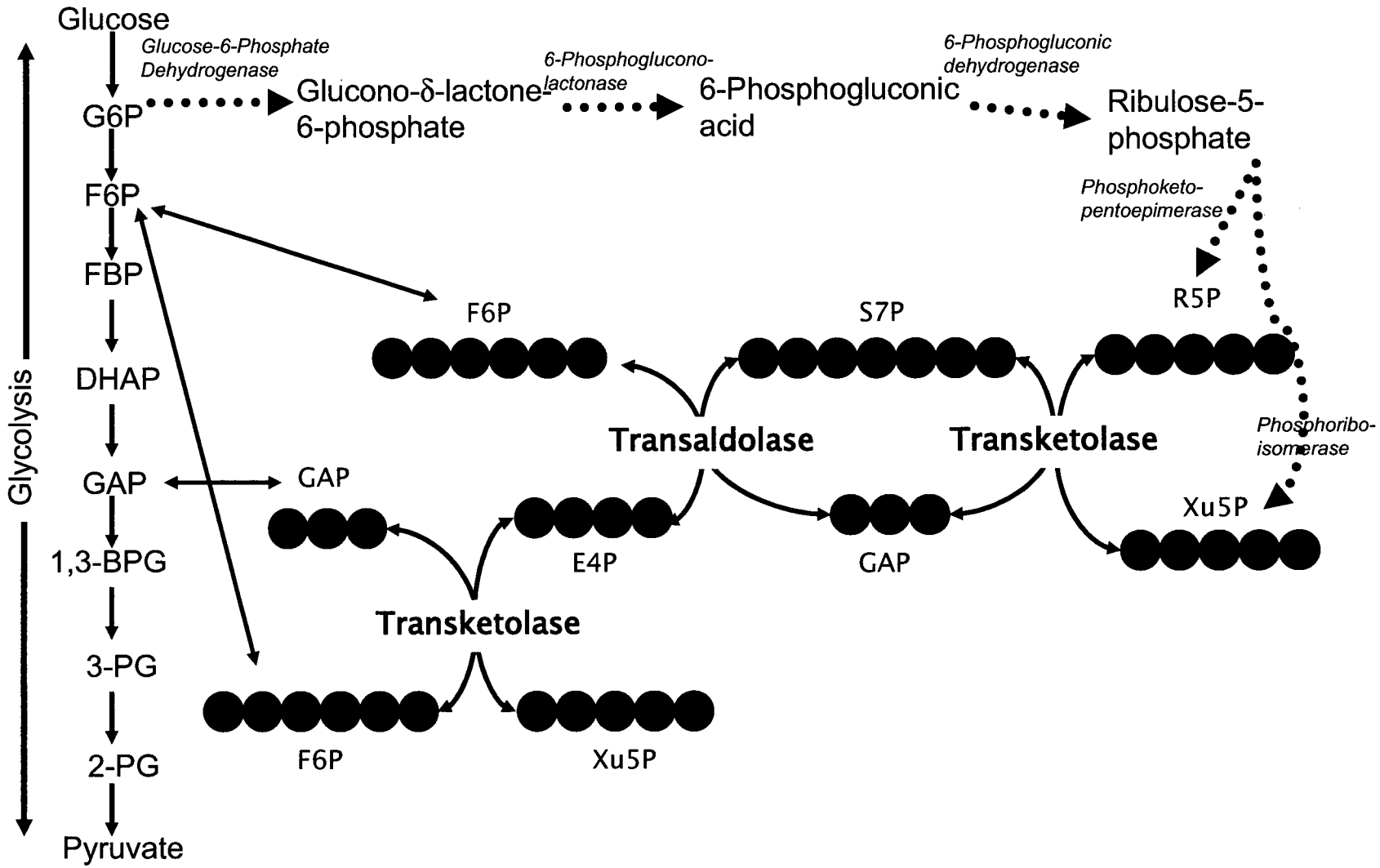


Figure 6: The Oxidative and Non-oxidative Branches of the Pentose Phosphate Pathway (PPP). The Dashed arrows are reactions that consist of the Oxidative PPP. The solid arrows are reactions that consist of the Non-oxidative PPP. Adapted with permission from: Figure 3; Zimmer, Heinz-Gerd (2002) Pentose Phosphate Pathway. In: Encyclopedia of Life Sciences, vol. 14 pp. 14-21. London: Nature Publishing Group. [doi: 10.1038/npg.els.0001365]

activity and expression, the PPP is prominent in the liver because $NADPH_2$ is required for lipid biosynthesis, while PPP activity is extended across many other tissues including cardiac and skeletal muscle (149). Although these tissues and cell types have different metabolic requirements, the $NADPH_2$ produced by this pathway is useful in protection against oxidative stress, an event not all that rare in muscle (145,148,147). To achieve these effects, the PPP consists of two main branches; the oxidative branch and the non-oxidative branch (149). Both branches work collectively in the cytosol, where the oxidative branch of the PPP is linked to glycolysis and nucleotide metabolism, and where the non-oxidative portion of the PPP serves as the reversible link between the oxidative branch of the PPP and glycolysis.

2. Oxidative Portion of the PPP

Glucose-6-phosphate originating from either glucose or glycogen is predominantly catabolized by glycolysis. However, a portion of glucose-6-phosphate may be shunted away from glycolysis to enter the oxidative portion of the PPP so that ribose-5-phosphate and $NADPH_2$ can be generated (145,148,149,147) (Figure 7). The ribose-5-phosphate produced serves as the carbon backbone for nucleic acid synthesis, while $NADPH_2$ is primarily used to protect against oxidative stress by maintaining glutathione in a reduced state (145,148,147). The pivotal reaction that leads to these important functions is catalyzed by Glucose-6-Phosphate Dehydrogenase (G6PDH) and is irreversible (154). G6PDH activity is governed by $NADPH_2$ inhibition, an inhibition that is in competition with $NADP^+$(45). For instance, under normal physiological conditions of $NADP^+$ and free $NADPH_2$, this enzyme is almost completely inhibited.

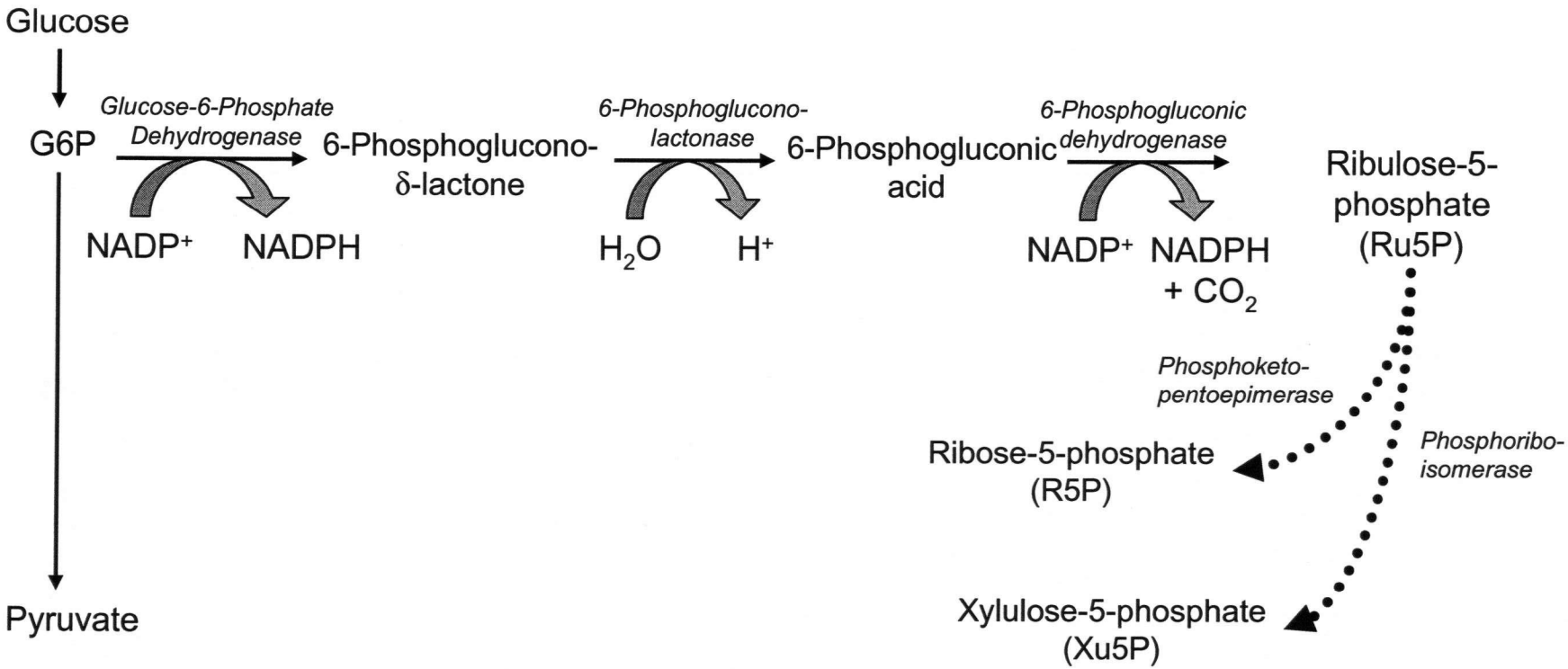


Figure 7: The Oxidative Portion of the Pentose Phosphate Pathway (PPP). Glucose-6-Phosphate Dehydrogenase is the key enzyme of this branch of the PPP. $NADPH_2$ produced will contribute to reducing power for other processes involved in detoxification and biosynthesis. Ribose-5-phosphate (R5P) and Xylulose-5-phosphate (Xu5P) continue on in the non-oxidative PPP.

Therefore, G6PDH activity is only possible by de-inhibition; a simple matter of competition between $NADP^+$ and $NADPH_2$ for binding on G6PDH. The activity of the oxidative PPP is typically of a low capacity in the rat and human heart (126,45,15,153). In fact, the $NADP^+$ pool is normally sufficiently reduced that G6PDH is substantially inhibited and flux through the oxidative portion of the PPP is extremely low in the heart (126,45,15,153).

3. *The Non-Oxidative Portion of the PPP*

The non-oxidative branch of the PPP is involved in the interconversion of 3-, 4-, 5-, 6-, and 7-carbon sugars catalyzed by a series of non-oxidative reactions that occur in cytoplasm (145,148,149,147) (Figure 8). Given that the synthesis of $NADPH_2$ by the oxidative PPP is essential for reductive biosynthetic processes, the non-oxidative portion of the PPP fulfills this demand by cycling and converting excess ribose-5-phosphate generated by the oxidative PPP back into glycolysis. In doing so, the back conversion of the ribose-5-phosphate to triose and hexose glycolytic intermediates allows continual cycling of both portions of the PPP with the aid of the glycolytic pathway, for continual $NADPH_2$ production (Figure 9). Therefore, the non-oxidative branch of the PPP serves to prevent the depletion of the intermediate pools of the oxidative portion of the PPP (145,148,149,147). As a result, a cycling effect is produced (Figure 9) where $NADPH_2$ may be produced without significant loss of carbon.

Overall: 2 xylulose 5-phosphate (5C) + 1 ribose 5-phosphate (5C) ⟶
2 fructose 6-phosphate (6C) + 1 glyeraldehyde 3-phosphate (3C)

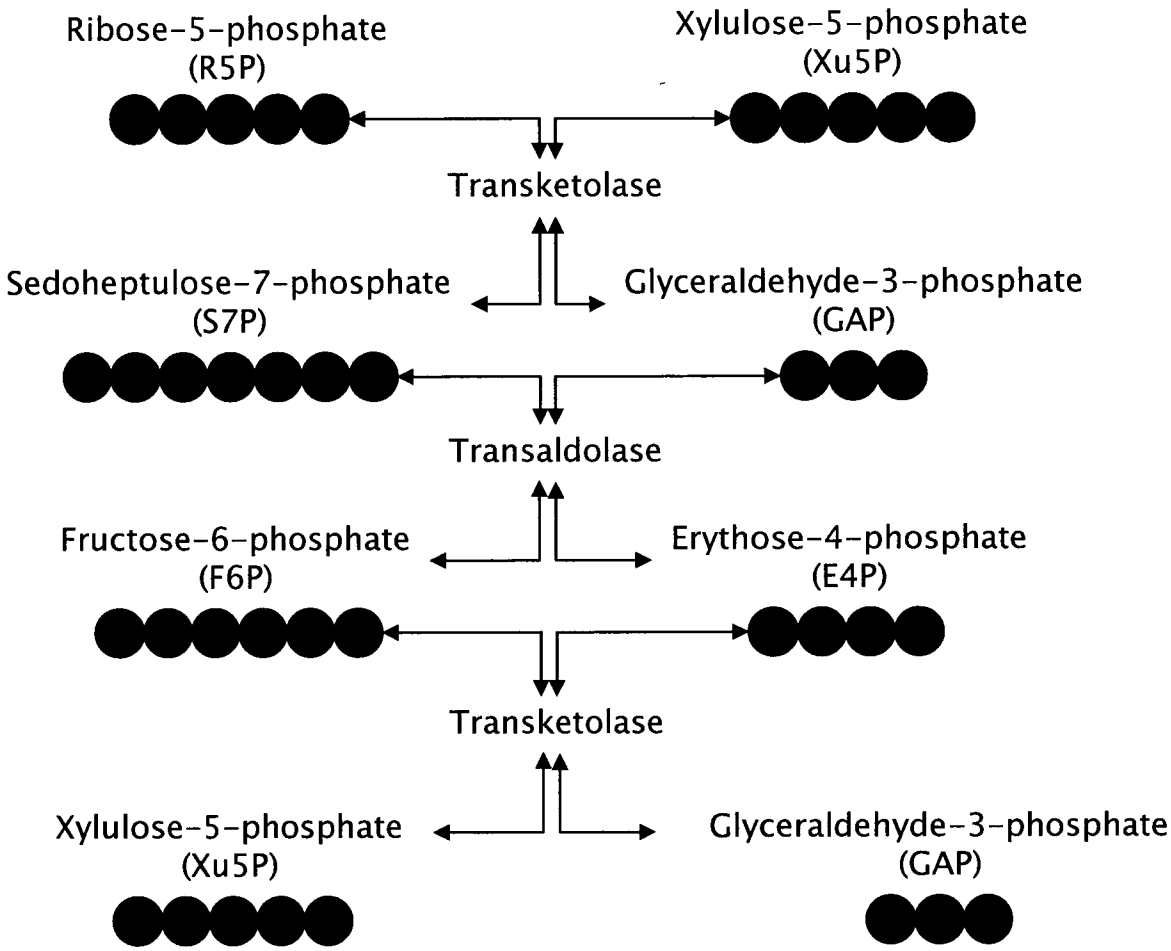


Figure 8: The Non-Oxidative Portion of the Pentose Phosphate Pathway (PPP). Transaldolase is the primary site of control in this branch of the PPP. **Adapted with permission from: Figure 3; Zimmer, Heinz-Gerd (2002) Pentose Phosphate Pathway. In: Encyclopedia of Life Sciences, vol. 14 pp. 14-21. London: Nature Publishing Group. [doi: 10.1038/npg.els.0001365]**

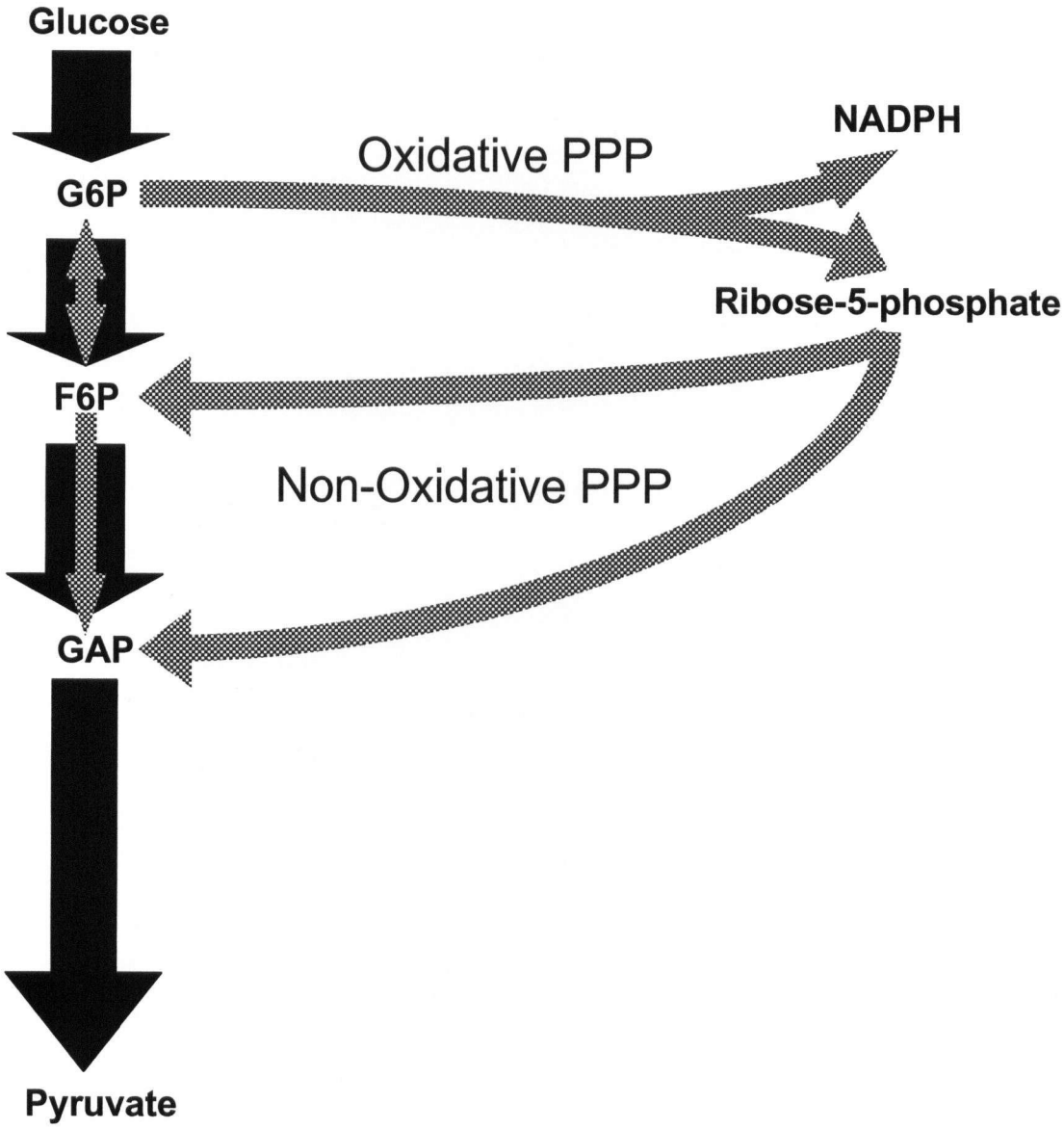


Figure 9: The Cycling of Glycolytic Intermediates by the Pentose Phosphate Pathway as Proposed by *Goodwin et al* (56).

Transketolase and transaldolase are the key enzymes of this pathway (Figure 7), where transketolase catalyzes the conversion of xylulose-5-phosphate (5C) and ribulose-5-phosphate (5C) into glyceraldehyde-3-phosphate (3C) and sedoheptulose-7-phosphate (7C) (145,148,149,147). Transaldolase then catalyzes a conversion between these two end products to synthesize a 4-carbon sugar (E4P) and a 6-carbon sugar (F6P). The glycolytic intermediates fructose-6-phosphate and glyceraldehyde-3-phosphate are also formed at the end of the PPP when transketolase catalyzes a conversion between xylulose-5-phosphate and erythrose 4-phosphate.

This branch of the PPP is controlled by the availability of substrates and because all interconversion reactions are reversible, there is great metabolic flexibility and versatility (45). For example, when ribose 5-phosphate is in higher demand than $NADPH_2$, glucose 6-phosphate will be catabolized by glycolysis to fructose 6-phosphate and glyceraldehyde 3-phosphate. Transaldolase and transketolase will then convert these substrates in a reversible fashion to synthesize ribose 5-phosphate which will be available for purine and pyrimidine nucleotide synthesis (146). Conversely, if the demand for $NADPH_2$ is greater than the need for ribose-5-phosphate, then glucose-6-phosphate is completely catabolized by glycolysis and its intermediates will be shunted to G6PDH and the oxidative PPP. Both the oxidative and non-oxidative branches of the PPP will generate $NADPH_2$ while excess ribose-5-phosphate will be recycled back to its glycolytic intermediate forms by the non-oxidative PPP (146,148,147). Thus, the non-oxidative PPP provides much needed versatility by adjustments according to specific metabolic situations and demands.

D) Metabolic alterations in Cardiac Hypertrophy

1. Fatty Acid Oxidation

Oxidation of fatty acids has been observed to be lower in hypertrophied hearts compared to normal hearts (46,7,120,47), an observation dependent on the degree of hypertrophy, fatty acid levels and workload (8,118). Reduced expression of fatty acid uptake/transport proteins (104,3,135) and reduced carnitine levels (46,7,120,47) is thought to be responsible for low fatty acid oxidation in hypertrophied hearts; carnitine being a structural component of the CPT system for long chain fatty acid translocation, as well as binding to individual fatty acyls-CoA throughout this transport (7,8,29). At the site of the CPT, impaired cooperation between the acyl-CoA dehydrogenase and CPT has also been implicated as being responsible for low fatty acid oxidation rates (29). Finally, it has been shown that the mRNA expression of enzymes necessary for the oxidation of fatty acids is depressed in the hypertrophied heart (115,18). However, the extent to which the down-regulation expression of such enzymes plays a role in reducing fatty acid oxidation has not yet been determined (115,18).

2. Glycolysis and Glucose Oxidation

In the hypertrophied heart, rates of glycolysis have been shown to be accelerated when compared to control hearts in the isolated working mode (5,121,47). In hypertrophied hearts, expression and activities of a number of glycolytic enzymes are increased (53,132), and may even shift towards more fetal, anaerobic isoforms (68) as part of the adaptive response to workload. However, oxidation of pyruvate is not correspondingly increased (Figure 10) (7,121,9,47,138,137,143). In fact, the fraction of

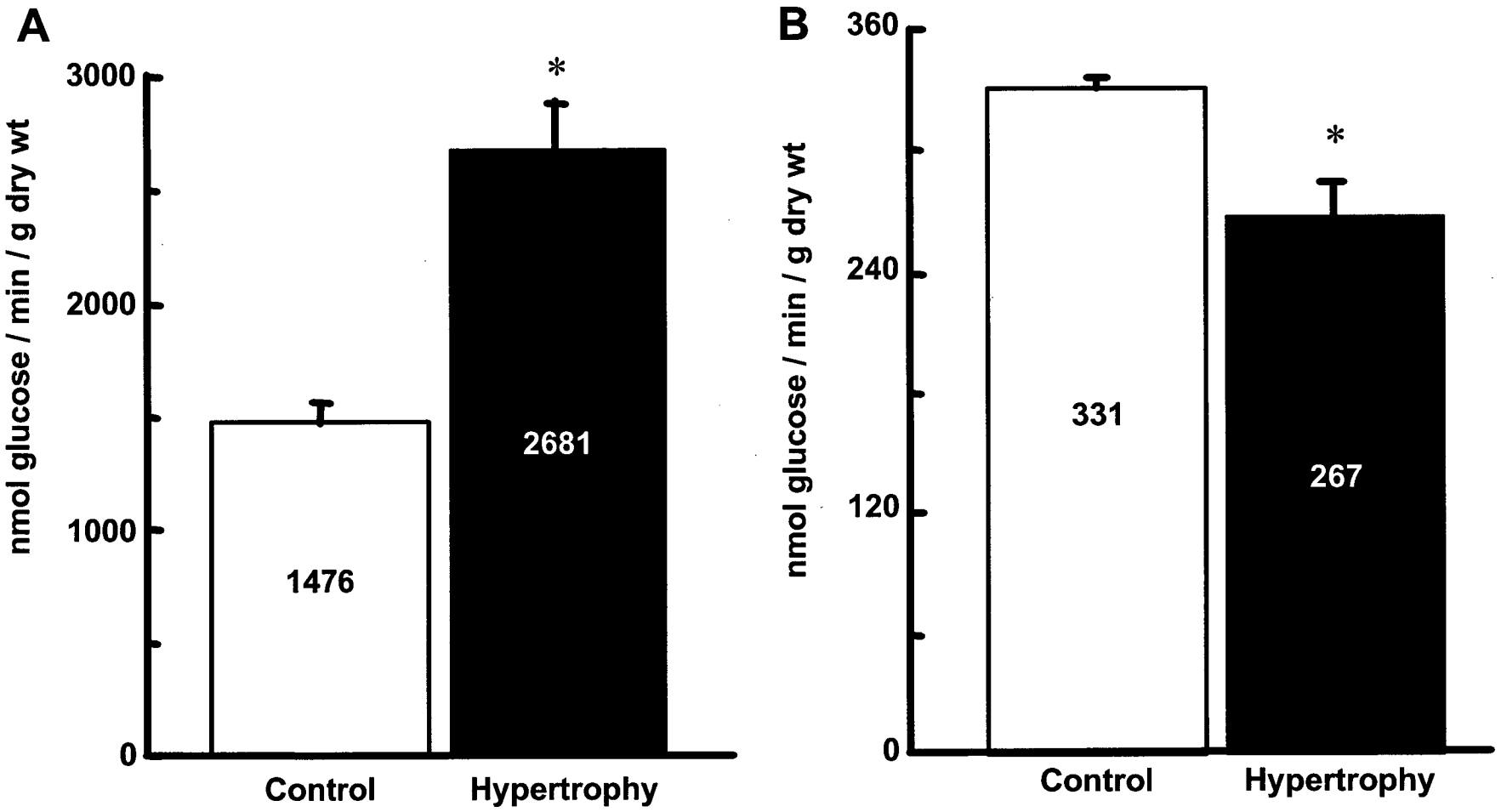


Figure 10: Exemplary glycolysis (A) and glucose oxidation (B) rates in control and hypertrophied hearts post-ischemia. Rates of glycolysis were measured using [5-^{3}H]-glucose while rates of glucose oxidation were measured by U-[^{14}C]-glucose. (*, vs. control, $p<0.05$)
Taken from Allard *et al.* Am J Physiol 267: H742-750, 1994. Data reprinted by permission of American Physiological Society.

pyruvate oxidized (from glycolysis) in hypertrophied hearts is lower than that found in control hearts (5,120,121,9,47,138,10,140,141) because rates of pyruvate/glucose oxidation are comparable or even lower than those in control hearts (46,9,141). These findings are particularly unusual because decreases in fatty acid oxidation in control hearts are typically accompanied by increases in glucose oxidation that result in a higher fraction of pyruvate being completely oxidized (80). This disparity in glucose oxidation between control and hypertrophied hearts has significant functional relevance since low fractional oxidation of glucose has been linked to contractile dysfunction, especially after an ischemic episode.

Normally, decreases in fatty acid oxidation in the normal heart result in low intracellular levels of $NADH_2$ and acetyl-CoA, conditions that induce an activation of PDC and glucose oxidation (112,29). Moreover, accelerated rates of glycolysis in hypertrophied heart produce large quantities of pyruvate that normally maintain a high PDC activation state that also lead to high glucose oxidation rates (112,103,113,109,110). Despite such circumstances, glucose oxidation is still limited in the hypertrophied heart. Hence, the proportion of glycolytically-derived pyruvate that is oxidized is lower in hypertrophied hearts than in normal hearts. It is thought that these alterations in energy metabolism are important factors contributing to the functional changes in the heart and its increased susceptibility to injury during ischemia (14,50,6) (see section *Consequences of Altered Energy Metabolism in Hypertrophied Heart* for details).

The rate of glucose oxidation is mostly dependent on the activation state of the PDC (129), and it is possible that an alteration in its expression or activation is responsible for the low glucose oxidation rates in hypertrophied hearts. Since

intracellular pyruvate and acetyl-CoA levels in the hypertrophied heart are theoretically capable of inducing high glucose oxidation rates, any alteration in PDC regulatory machinery could be responsible for depressed glucose oxidation rates. However, a study by *Lydell et al.* (84) determined that subunit expression, activity, and covalent regulation of PDC machinery were not different between control and hypertrophied hearts. Therefore, the limitation of glucose oxidation in hypertrophied hearts is not due to a reduction or alteration in PDC expression, activity or covalent regulation (84) and that other mechanisms are responsible for this limitation (84).

3. *Lactate Metabolism and Lactate Oxidation*

Upon the degradation/catabolysis of glucose through glycolysis, the major fate of pyruvate is the conversion to lactate by lactate dehydrogenase in the isolated working heart setting (99). This is especially true for pyruvate produced by the hypertrophied myocardium where glycolytic rates are accelerated and rates of glucose oxidation remain relatively stagnant and depressed (21,132). Under such circumstances, lactate production is significantly higher in the hypertrophied heart as proportionally less pyruvate undergoes glucose oxidation compared to normal hearts. In doing so, this increased production of lactate regenerates and stabilizes the cytosolic $NADH_2/NAD^+$ redox state, the stability of which the continuation of glycolysis is dependent on (21). The formation of NAD^+ for every lactate molecule synthesized serves to restore this glycolytic capability and is a major source of NAD^+ in the cytosol.

Enhanced lactate production and release in hypertrophied hearts is believed to be caused by the emergence of a fetal isoform of lactate dehydrogenase in hypertrophied

heart that possesses a higher affinity for pyruvate (132). The emergence of this fetal isoform is associated with the overall alteration in gene expression observed in pressure-overload hypertrophied hearts (21,42). Although this fetal isoform of LDH is characterized as such, the extent to which pyruvate is intentionally converted to lactate is unknown. However, both hypertrophied and normal hearts oxidize lactate (97,7,120), and lactate oxidation in hypertrophied hearts is equivalent or less than that found in normal hearts (7,120). Therefore, although lactate production is enhanced in hypertrophied heart, its oxidation and contribution to ATP pools is not accordingly increased.

4. Consequences of Altered Energy Metabolism in Hypertrophied Heart

Accelerated rates of glycolysis and high physiological levels of lactate are of major concern in the hypertrophied heart due to high H^+ load in the cytosol (14,5). Collectively, high lactate production may compound the problem of proton overload in hypertrophied heart. Surprisingly, it is the dramatic reduction in the oxidative metabolism of glucose that causes a net buildup of H^+, and not the dissociation of lactate which produces H^+(63). When all glycolytically-derived pyruvate undergoes complete oxidation, net H^+ production is zero. However, when there is low fractional pyruvate oxidation, a net 2 H^+ is produced for every glucose molecule passing through glycolysis and not through glucose oxidation (63). Consequent accumulation of protons results when proton release from ATP hydrolysis exceeds proton export capacity of the myocyte. During ischemia, many H^+ transporters are also believed to be inactive and therefore a large intracellular concentration of H^+ is created, often resulting in ion imbalances,

membrane damage and contractile dysfunction. The accumulation of H^+ during ischemia primes the myocardium for Ca^{2+} overload during reperfusion by means of Na^+/H^+ and Na^+/Ca^{2+} exchange (72). Upon reperfusion, a massive export of proton erupts, causing damage to the cell membranes as well as creating an influx of Ca^{2+} into the cell (133,80). Low glucose oxidation rates seen in the hypertrophied heart often compound this predicament by not fully oxidizing pyruvate produced by glycolysis (80), a process which would otherwise cancel out proton release by ATP hydrolysis. Instead, excessive amounts of glycolytic product result in an acidic intracellular environment which increases calcium influx and decreases contractility (48). This enhancement of calcium transport often results in poor contractile activity as the myocyte attempts to deal with the imbalance of ions in the myocyte. However, the amount of energy expended to reach ion homeostasis decreases cardiac efficiency and increases dysfunction.

5. *The Role of the PPP in the Hypertrophied Heart*

There are claims that non-glycolytic detritiation of [5-^{3}H]-glucose may occur at the transaldolase reaction of the Non-Oxidative portion of the PPP (Figure 11). It was observed that glycolytic flux as measured by three independent methods in control hearts was inconsistent and that glycolytic flux as measured by the [5-^{3}H]-glucose method was neither constant nor linear (44). Therefore, a very important corollary was the possibility that rates of glycolysis are overestimated by the [5-^{3}H]-glucose method in the isolated working rat heart. This overestimation may be even more pronounced in the pathologically hypertrophied heart setting because it is possible that the PPP may be elevated or altered due to the heart's increasing requirements for nucleic acids for

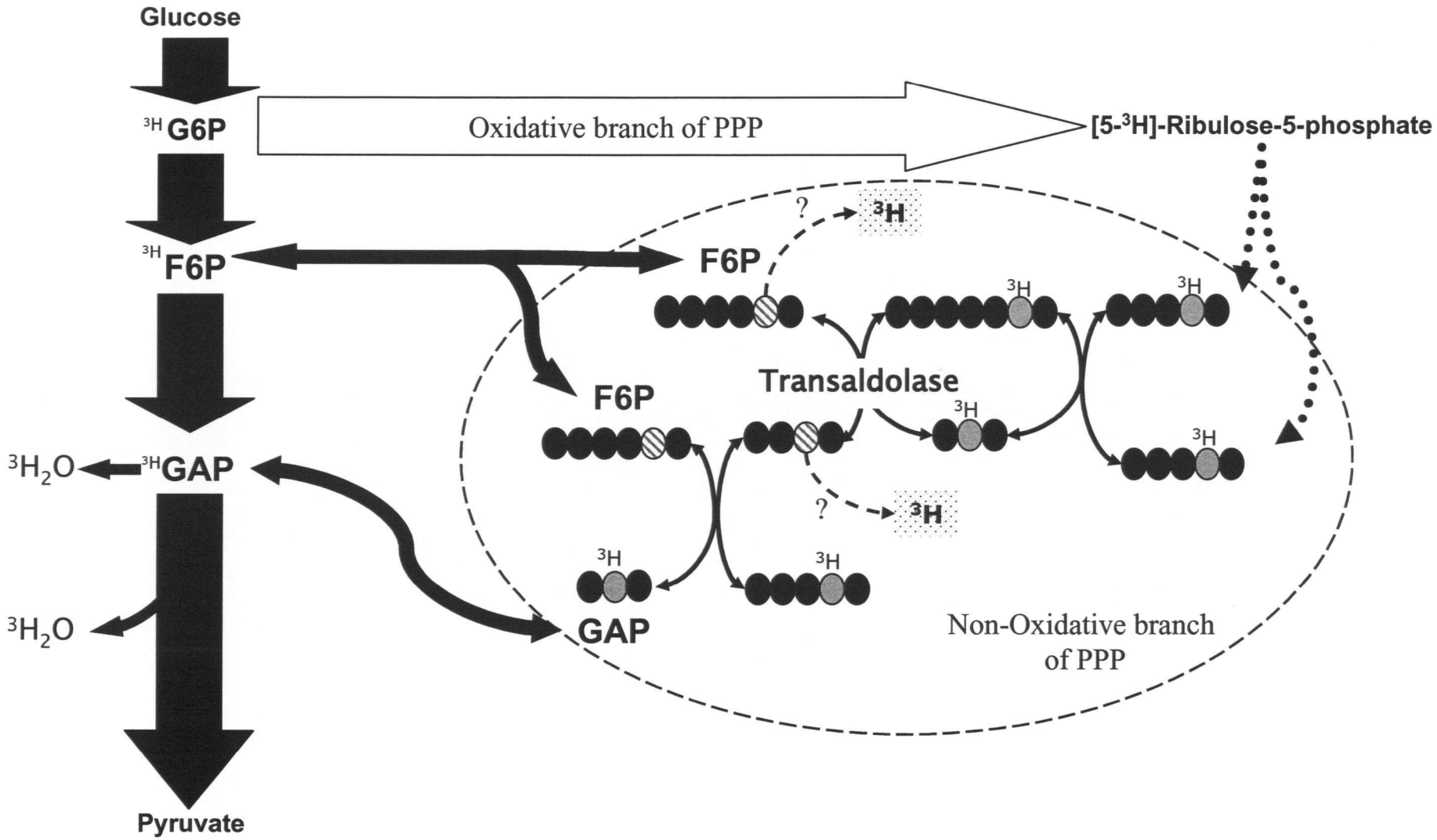


Figure 11: The proposed impact of both branches of the PPP on glycolysis and tritium release from [5-^{3}H]-glucose. Non-glycolytic detritiation may possibly occur at the transaldolase reaction of the non-oxidative PPP, during the interconversion of carbon sugars.

structural enhancement as the heart enlarges, or to protect the myocardium from reactive oxygen species as a consequence of accelerated cellular processes. Therefore, if there is significant PPP activity in the rat heart, the well-documented metabolic profile of the pathologically hypertrophied heart will require further review.

Other studies have also established the presence of a long-term control mechanism regarding G6PDH in which increased activity and expression of G6PDH occurs in skeletal muscle and in rat heart during the development of cardiac hypertrophy and/or by the influence of catecholamines (155,156). Long-term responses to cardiac hypertrophy (by constriction of the abdominal aorta) have also demonstrated varying increases in G6PDH expression and activity in the heart (155,156). Overall, both branches of the PPP may be significant in the hypertrophied isolated working rat heart.

E) Measurement of Glycolysis in the Isolated Working Heart Preparation

In the isolated working heart preparation, tritium-labeled glucose ([5-^{3}H]-glucose or [2-^{3}H]-glucose) is added to the perfusion buffer solution to measure glycolysis (82,37). This method has proven to be a popular method of determining glycolytic rates in the isolated working rat heart because of its commercial availability and specificity. In particular, [5-^{3}H]-glucose is preferentially used because non-glycolytic detritiation of [2-^{3}H]-glucose has been observed during glucose transport (31). Therefore, rates of glycolytic flux are determined by the release of ^3H_2O from [5-^{3}H]-glucose during its passage through glycolysis. It is assumed that [5-^{3}H]-glucose is completely detritriated by the triose phosphate isomerase and enolase reactions of glycolysis (82,37) (Figure 12). The standard measurement of glycolysis by timed collection of perfusate samples and

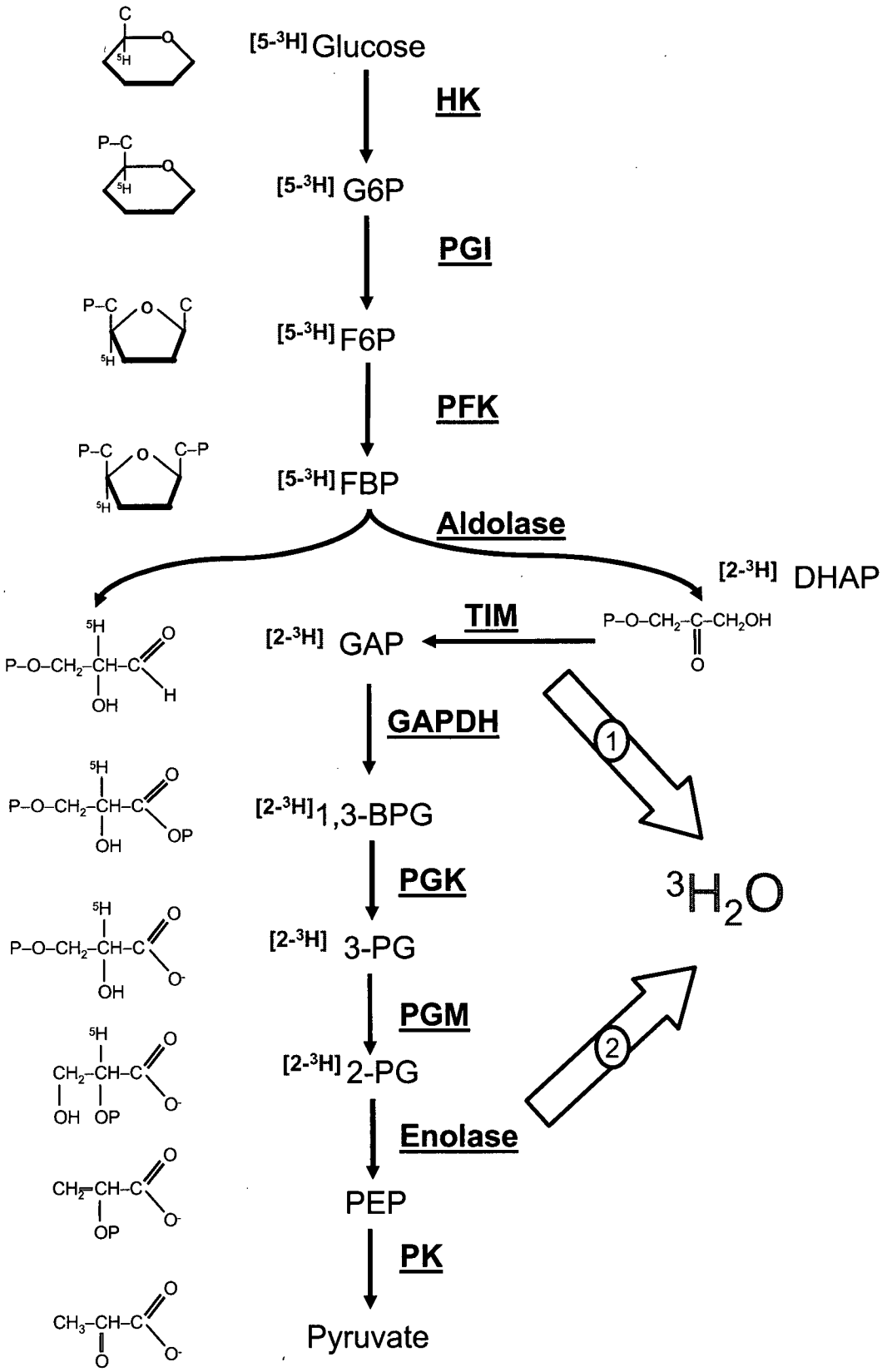


Figure 12: The degradation of glucose via the glycolytic pathway. The tritium label at the fifth carbon of glucose will be released as water at either: 1) the triose isomerase reaction (TIM) or 2) at the enolase reaction. Adapted with permission from: Figure 16-3; Biochemistry 2nd Edition, Voet and Voet, Copyright © (1993). Reprinted by permission of John Wiley & Sons.

separation of ^3H_2O from [5-^{3}H]-glucose, is a well-established means of measuring glycolytic rates in isolated heart preparations (95,82,37).

F) Potential Overestimation of Glycolytic Flux in Isolated Working Rat Hearts

Recently, Goodwin *et al* have suggested that rates of glycolysis obtained by quantitation of ^3H_2O production from [5-^{3}H]-glucose overestimate true rates of glycolysis in isolated working normal rat hearts (56). They propose that non-glycolytic detritiation of [5-^{3}H]-glucose occurs in the non-oxidative portion of the pentose phosphate pathway (PPP) by means of the transaldolase reaction in the heart. In other studies, key enzymes of the oxidative pentose phosphate pathway, such as glucose-6-phosphate dehydrogenase, were found to be elevated in hypertrophied hearts (155,61). If enzymes of the non-oxidative portion of the pentose phosphate pathway are also increased above normal and if substantial non-glycolytic detritiation of [5-^{3}H]-glucose occurs in myocardium, as suggested, it is conceivable that the acceleration of glycolysis observed in isolated working hypertrophied rat hearts is an artifact.

II. HYPOTHESIS AND OBJECTIVES

If there is substantial non-oxidative PPP activity in the normal and hypertrophied heart, in which non-glycolytic detritiation of [5-^{3}H]-glucose occurs, then glycolytic flux measured by this tracer method should be exaggerated when compared to other independent measures of glycolytic flux. If this is the case, then the postulated elevation of the PPP in hypertrophied heart may be responsible for accelerated rates of glycolysis observed when using this technique. However, based upon the long-standing reputation of the method, I hypothesize that ^3H_2O production from [5-^{3}H]-glucose does not overestimate true rates of glycolysis in hypertrophied rat hearts due to the PPP. To test this hypothesis, rates of glycolysis in isolated working hypertrophied and non-hypertrophied hearts were determined by three independent methods. To determine if both oxidative and non-oxidative portions of the pentose phosphate pathway are altered in the hypertrophied heart, I also measured activity or expression of two key enzymes, glucose-6-phosphate dehydrogenase and transaldolase, of the oxidative and non-oxidative portions of the pentose phosphate pathway, respectively.

III. EXPERIMENTAL METHODS

A. Animal Model

Pressure-overload left ventricular hypertrophy was produced in 3-week old male Sprague-Dawley rats (50-75 g) by constriction of the suprarenal abdominal aorta with a metallic clip (0.4mm diameter) (7). In sham-operated control rats, the aorta was isolated but not constricted. Experiments were performed 8 weeks after surgery. Rats, housed in a temperature-controlled (22±1°C) and light-controlled (12:12-h light-dark cycle) room, had free, unlimited access to feed and water. Care of the animals was performed in accordance with guidelines set out by the Canadian Council on Animal Care.

B. Isolated heart preparation and perfusion protocol

Hearts from halothane (2-3%) anesthetized sham-operated and aortic-constricted rats were perfused for 30min with Krebs-Henseleit (KH) solution in the working heart mode at a preload of 11.5 mmHg and an afterload of 80 mmHg, as previously described (7,9,10,141,79). Anesthesia with halothane is expected to have minimal effect on cardiac integrity and metabolism (157). The KH solution contained 1.2 mM or 0.4 mM palmitate prebound to 3% fatty acid-free albumin, 5.5 mM [5-^{3}H/U^{14}C]-glucose, 0.5 mM lactate, 2.5 mM calcium, and 100 mU/L insulin and was continuously circulated through the closed perfusion system. Two different concentrations of palmitate (high fatty acid-1.2 mM and low fatty acid-0.4 mM), that represent the physiological range, were used in these perfusions . To ensure that glucose uptake was not limiting, a high physiologic

concentration of insulin was utilized. The solution was oxygenated with 95% O_2/5% CO_2 and maintained at 37° C throughout the perfusion.

A pressure transducer (Viggo-Spectramed, Oxnard, CA, USA) inserted in the afterload line was used to measure heart rate and peak systolic pressure. Cardiac output and aortic flow was measured via external flow probes (Transonic Systems Inc., Ithaca, NY, USA) on the preload and afterload lines, respectively. Coronary flow was calculated as the difference between cardiac output and aortic flow. Rate-pressure product, the product of heart rate and peak systolic pressure, and hydraulic work, the product of cardiac output and peak systolic pressure, were used to measure external work performed by the heart (7,9,10,141,79). These measures of heart function were assessed every 10 min throughout the working heart perfusion.

At the end of 30 min, hearts were quickly frozen using aluminum tongs cooled to the temperature of liquid nitrogen. Frozen heart tissue was powdered using a mortar and pestle and then stored in cryovials at -70°C until use.

C. Measurement of Glycolysis and Glucose Oxidation

1. Glycolysis

Rates of glycolysis were determined simultaneously in the same hearts by different and independent methods. In one method, glycolysis was calculated as the sum of the rate of lactate and pyruvate accumulation in the perfusate and the rate of glucose oxidation (56). For this calculation, accumulation of total lactate and pyruvate or [^{14}C]-lactate and [^{14}C]-pyruvate in the perfusate was determined (See Appendix I & II for calculation). Total lactate and pyruvate were measured enzymatically using a

commercially available assay (Sigma, St. Louis, MO). Accumulation of radiolabelled lactate and pyruvate was determined by means of a modified version of the two-step assay described by *Lehoux et al* (73). Briefly, the [^{14}C]-lactate in the sample was enzymatically converted to [^{14}C]-pyruvate in the first step. All the [^{14}C]-pyruvate in the sample, including that originating from [^{14}C]-lactate, was then decarboxylated enzymatically to $^{14}CO_2$ in the second step. The $^{14}CO_2$ produced as a gas and from [^{14}C]-bicarbonate in the buffer, released after addition of H_2SO_4, was collected into hyamine hydroxide-soaked filter paper, suspended in a centre well in the reaction vial, and subsequently counted by standard techniques. Recovery rates using this method, which were tested by spiking KH solution with known quantities of [^{14}C]-lactate, range from 85 to 90%. A more detailed description of this assay is provided in Appendix I. Rates of accumulation of lactate and pyruvate (total or ^{14}C-labelled), determined by taking volume of the perfusate, perfusion time, and dry heart weight as well as specific activity of perfusate [^{14}C]-glucose, where appropriate, into account are expressed as glucosyl units/min/g dry heart weight.

In the third method, glycolysis was determined by quantitatively measuring the rate of ^3H_2O liberated from [5-^{3}H]-glucose (7,9,10,141,79). $^3H_20$ was separated from perfusate using columns containing Dowex 1X4 anion exchange resins (200-400 mesh) dissolved in 0.4 M potassium tetraborate. The Dowex resin was extensively washed with dH_2O before use. Duplicate samples (0.2 mL each) were added to the column and eluted into scintillation vials with 0.8 mL dH_2O. Following the addition of scintillation fluid (4 mL) to the tubes containing the eluent, the samples were subjected to double isotope

counting procedures to measure $^{3}H_2O$ and residual [$^{3}H/^{14}C$]-glucose. Rates are expressed as glucosyl units/min/g dry weight.

2. *Glucose Oxidation*

Glucose oxidation rates were measured by quantitative collection of $^{14}CO_2$ from [U-^{14}C]-glucose released as a gas and dissolved in the perfusate as [^{14}C]-bicarbonate, as previously described (7,9,10,141,79). Perfusate and gaseous samples were required to measure glycolysis and glucose oxidation and taken every 10 min of perfusion. Samples for determination of glycolysis and glucose oxidation were ultimately placed in vials containing scintillation cocktail and counted by standard techniques.

D. Immunoblot Analysis of Transaldolase

Expression of transaldolase protein in myocardium was determined by a previously described method (10). Briefly, samples of frozen ventricular tissue homogenate (containing 40 to 50μg total protein) were solubilized by boiling in reducing sample buffer, separated by electrophoresis on 10% SDS-polyacrylamide gels, and transferred by electroblotting to a nitrocellulose membrane. After non-specific blocking, the blots were probed overnight with a primary rabbit anti rat transaldolase antibody (kindly donated by Dr. A. Perl, SUNY). After incubation with anti-rabbit secondary antibody, the signal was detected by an ECL based detection system. Bands were quantified by densitometry. Equivalence of protein loading was confirmed by detection of glyceraldehyde-3-phosphate dehydrogenase.

E. Measurement of Glycogen

Myocardial glycogen content was determined following extraction of frozen, powdered ventricular tissue with 30% KOH, ethanol precipitation, and acid hydrolysis of glycogen (9,79).

F. Measurement of Glucose-6-phosphate dehydrogenase activity

Glucose-6-phosphate dehydrogenase (G6PDH) activity in myocardium was measured by a standard spectrophotometric technique (40) which measures the rate of increase in absorbance at 339 nm. The assay reaction is as follows:

$$\text{Glucose-6-P} + \text{NADP}^+ \xrightleftharpoons{\text{G6PDH}} \text{6-phosphogluconolactone} + \text{NADPH} + \text{H}^+$$

G. Data Analysis

Data are expressed as mean ± standard error of the mean (SEM). Individual group means were compared using t-tests. The Bonferroni procedure was applied to all tests to correct for multiple tests and comparisons. A corrected value of $p<0.05$ was considered significant.

IV. RESULTS

A. Heart and body weight data

a. 0.4 mM palmitate perfused (low fatty acid content group)

The heart and body weight data are summarized in Table 1. Heart weight of aortic-constricted rats (1.85±0.05 g, n=4) was not greater than that of the control rats (1.87±0.02 g, n=4, p=NS). However, the heart weight/body weight ratio was higher in hypertrophied hearts compared to normal hearts, a finding consistent with our previous studies (5,9,10,141), demonstrating that this model produces a mild cardiac hypertrophy. Body weight was not significantly different between aortic-constricted (431±7 g) and control (459±3 g) rats (p=NS).

b. 1.2 mM palmitate perfused (high fatty acid content group)

The heart and body weight data are summarized in Table 1. Heart weight of aortic-constricted rats (1.99±0.05 g, n=6) was ~ 11% greater than that of the control rats (1.78±0.04 g, n=7, $p<0.05$), and consistent with our previous studies (5,9,10,141). Body weight was not significantly different between aortic-constricted (464±12 g) and control (472±10 g) rats.

B. Mechanical Heart Function

Mechanical function in isolated working rat hearts from control and aortic-banded rats is shown in Table 2. Mechanical function was stable throughout the perfusion in both groups under both fatty acid concentrations (Figure 13) while heart rate and rate-pressure product were similar in control and hypertrophied hearts. In high fatty acid

Table 1: Heart and Body Weight Data in High and Low Fatty Acid Perfusions

	Body Weight (g)	Wet Heart Weight (g)	Heart Weight/ Body Weight Ratio Ratio ($\times 10^{-3}$)
1.2 mM Palmitate			
Control (n=7)	464 ± 12	1.78 ± 0.05	3.8 ± 0.1
Hypertrophy (n=6)	472 ± 9	1.99 ± 0.05*	4.2 ± 0.1*
0.4 mM Palmitate			
Control (n=4)	459 ± 3	1.87 ± 0.01	4.0 ± 0.1
Hypertrophy (n=4)	431 ± 7	1.85 ± 0.05	4.28 ± 0.1*

Values are the mean ± SEM

*, vs. Control, $p < 0.05$

Table 2: Mechanical function of control and hypertrophied working rat hearts perfused with 1.2mM and 0.4 mM palmitate.

	1.2 mM Palmitate		0.4 mM Palmitate	
	Control (n=7)	Hypertrophy (n=6)	Control (n=4)	Hypertrophy (n=4)
Heart rate (beats×min^{-1})	265±18	285±16	252±13	223±23
Peak systolic pressure (mmHg)	119±6	113±5	106±1	126±3*
Rate pressure product (mmHg×min^{-1}×10^{-3})	31.1±0.9	31.9±0.6	26.7±1.6	28.1±3.0
Cardiac output (mL×min^{-1})	77.7±5.3	63.0±1.2*	62.5±9.6	65.0±13.1
Hydraulic work (mW)	20.3±2.1	15.7±0.8*	14.8±2.4	18.3±3.8
Coronary flow (mL×min^{-1}/g wet wt)	9.93±0.4	5.86±0.8*	8.1±4.1	5.4±5.3

Values are the mean ± SEM
*, vs. Control, $p < 0.05$

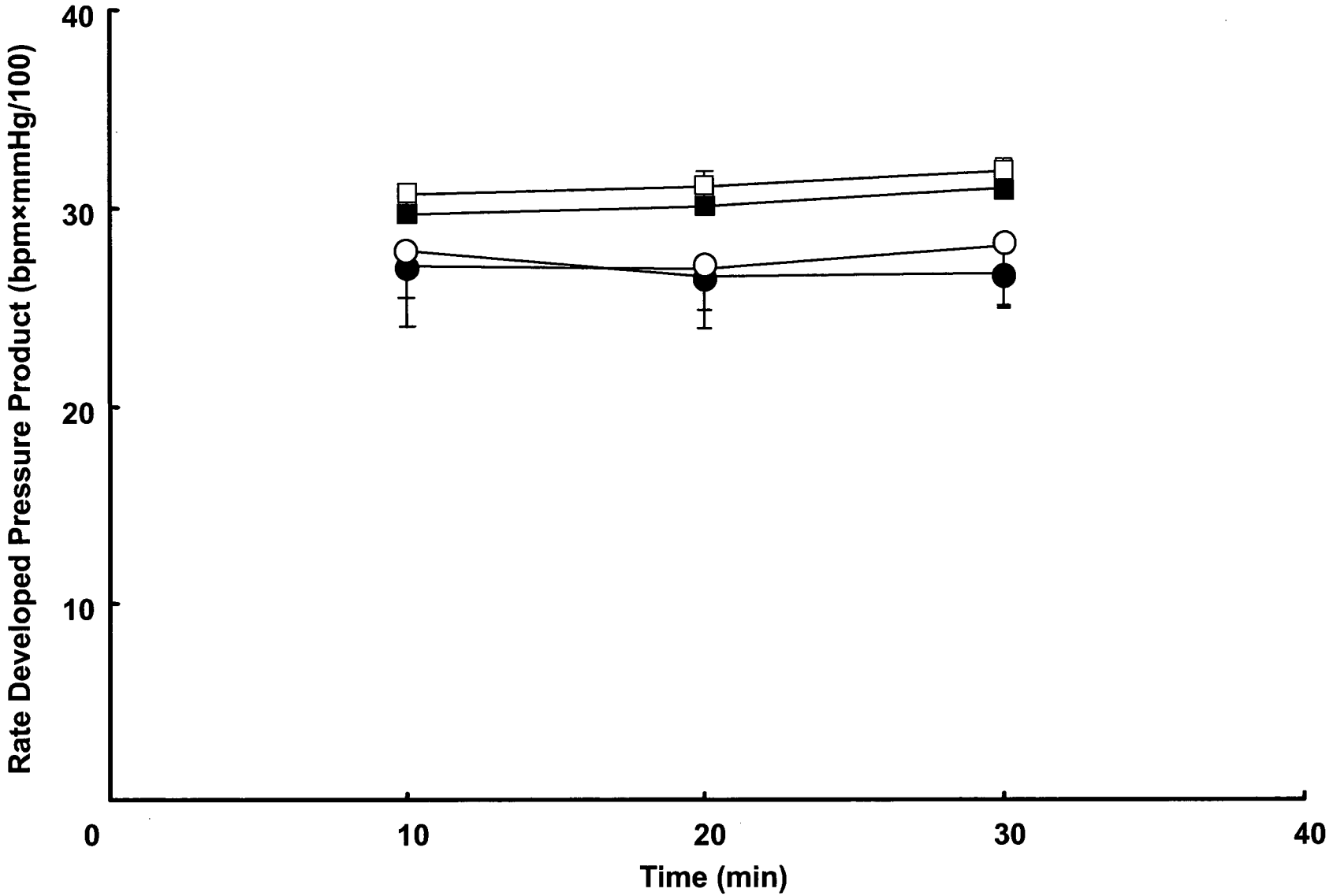


Figure 13: Mechanical function in Control (open shapes) and Hypertrophied (solid shapes) hearts in high fatty acid conditions (square shapes) and low fatty acid conditions (circle shapes).

conditions, other parameters of heart function were lower in hypertrophied hearts than in control hearts, with statistically significant differences in cardiac output, hydraulic work and coronary flow. In low fatty acid content perfusions, only peak systolic pressure was significantly higher in hypertrophied hearts compared to control hearts. All other parameters of heart function were not statistically significant between hypertrophied and control hearts.

C. Rates of Glycolysis

1. *Glycolysis from Total Lactate plus Pyruvate and Glucose Oxidation*

a. *High Fatty Acid Conditions*

Over the duration of the perfusion under high fatty acid conditions, accumulation of lactate and pyruvate was linear in both hypertrophied and control hearts (Figure 14). Lactate and pyruvate were released into the perfusate at significantly higher rates by hypertrophied hearts than by control hearts which lead to a greater overall accumulation of lactate and pyruvate in hypertrophied hearts (Table 3). Under high fatty acid (1.2 mM palmitate) conditions, rates of accumulation of lactate (Hypertrophy, 2948 ± 375 vs. Control, 1095 ± 288 nmol glucose equivalents/min/g dry wt, n=7, $p<0.05$) and pyruvate (Hypertrophy, 647 ± 99 vs. Control, 423 ± 54 nmol glucose equivalents/min/g dry wt, $p<0.05$) were significantly greater in hypertrophied hearts than in control hearts. In addition, glucose oxidation rates (measured directly by $^{14}CO_2$ production from [U-^{14}C]-glucose) were not significantly different between hypertrophied hearts (619 ± 48 nmol/min/g dry wt) and control hearts (475 ± 70 nmol/min/g dry wt, p=NS). Overall,

glycolysis calculated from these rates of lactate and pyruvate accumulation and glucose oxidation were higher in hypertrophied hearts compared to control hearts (Figure 15).

b. *Low Fatty Acid Conditions*

Perfusions performed under low fatty acid conditions, resulted in accumulation of lactate and pyruvate that was also linear in both hypertrophied and control hearts (Figure 16). Although lactate was released into the perfusate at similar rates in hypertrophied and control hearts, there was a greater overall accumulation of lactate in hypertrophied hearts than in control hearts (Table 3). Pyruvate accumulation was higher in hypertrophied hearts than in control hearts over the 30 min, but this was not statistically significant. Thus, rates of accumulation of lactate (Hypertrophy, 2453 ± 273 vs. Control, 1364 ± 238 nmol glucose equivalents/min/g dry wt, $p<0.05$) were significantly greater in hypertrophied hearts than in control hearts under these conditions. Meanwhile, rates of accumulation of pyruvate (Hypertrophy, 423 ± 128 vs. Control, 317 ± 35 nmol glucose equivalents/min/g dry wt, p=NS) were not significantly different. Glucose oxidation rates (measured directly by $^{14}CO_2$ production from [U-^{14}C]-glucose) did not differ significantly between hypertrophied hearts (1472 ± 198 nmol/min/g dry wt) and control hearts (1336 ± 270 nmol/min/g dry wt, p=NS).

Under low fatty acid conditions, rates of glycolysis in hypertrophied hearts were higher compared to control hearts (Figure 17), but this difference was not statistically significant. The low physiological level of fatty acids (0.4 mM palmitate) resulted in an enhancement of glucose oxidation in both hypertrophied and control hearts (as compared to 1.2 mM palmitate). In doing so, a greater degree of glucose passing through glycolysis was completely oxidized in hearts perfused with low fatty acid perfusate compared to

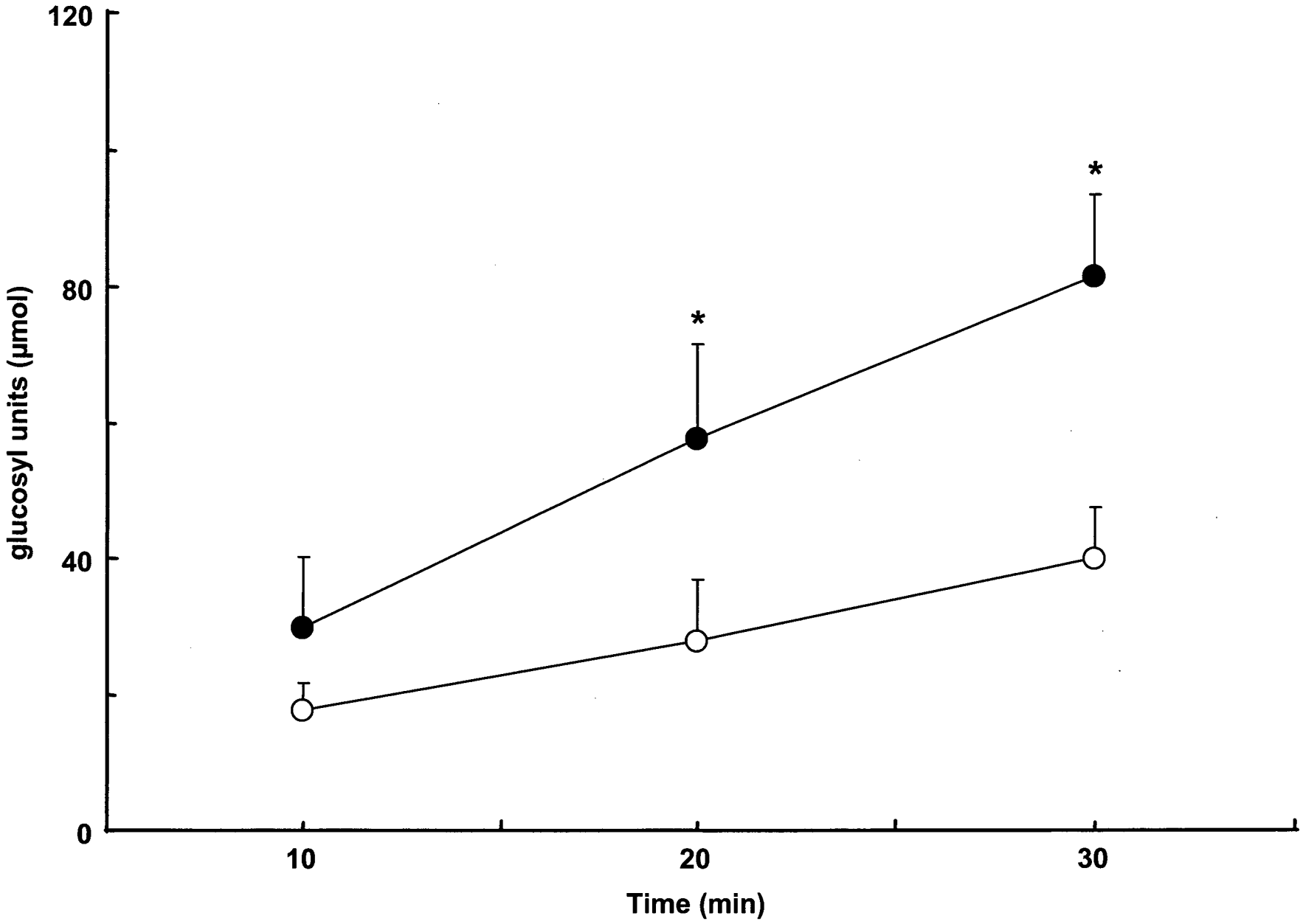


Figure 14: Accumulation of Lactate and Pyruvate in Control (empty circles) and Hypertrophied (dark circles) hearts in high fatty acid conditions. *, vs. control, $p < 0.05$.

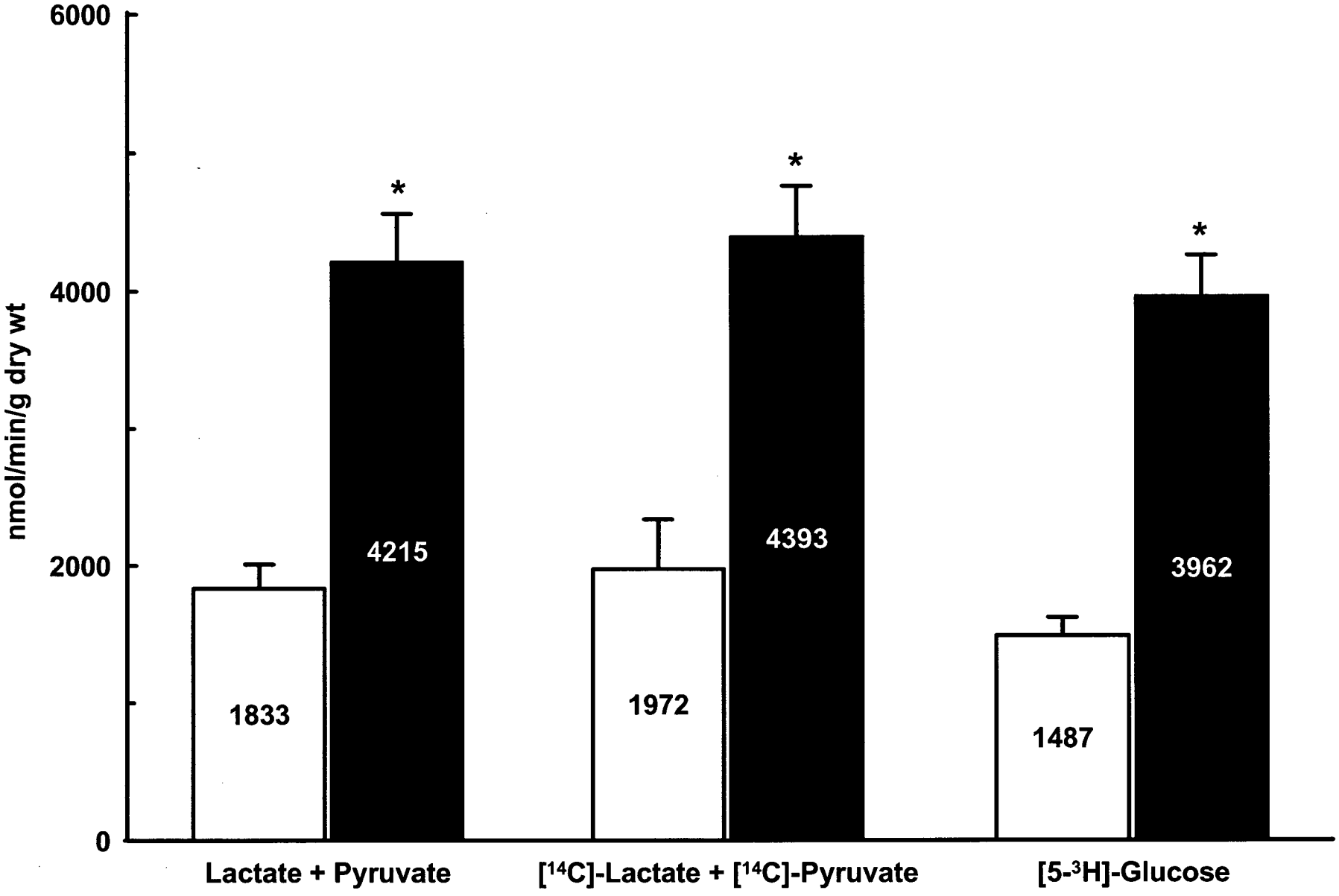


Figure 15: Comparison of Three Methods of determining Glycolytic Flux in Control and Hypertrophied Heart in High Fatty Acid Conditions. *, vs. control, $p < 0.05$.

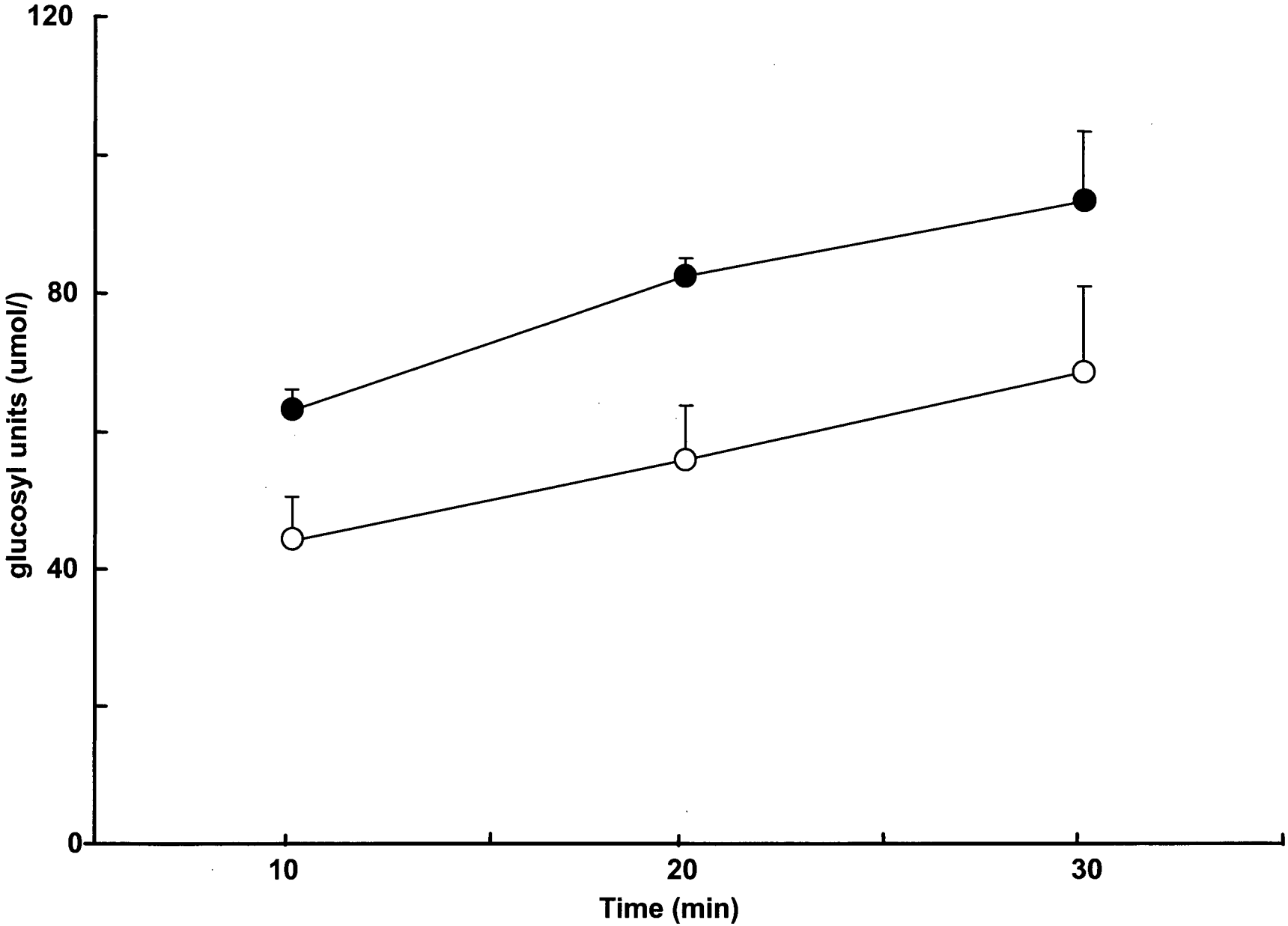


Figure 16: Accumulation of Lactate and Pyruvate in Control (empty circles) and Hypertrophied (solid circles) hearts in low fatty acid conditions.

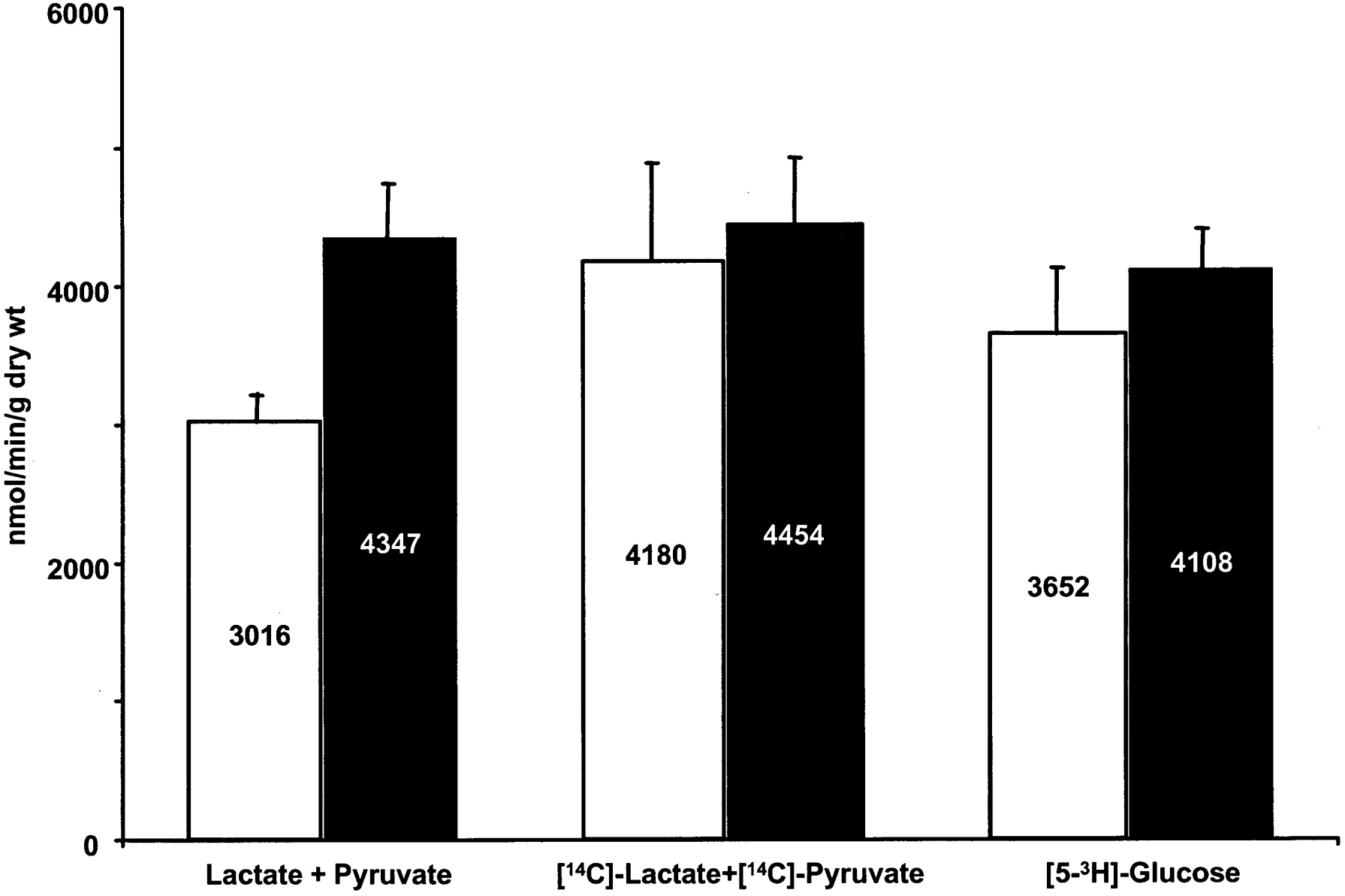


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Figure 17: Comparison of Three Methods of determing Glycolytic Flux in Control and Hypertrophied Heart in Low Fatty Acid Conditions.

hearts perfused with high fatty acid perfusate (36% vs. 16% of glucose oxidized respective to fatty acid concentration)

2. *Glycolysis from [^{14}C]-Lactate Production &[^{14}C]-Pyruvate Production & Glucose Oxidation*

Similar results were obtained when glycolysis was calculated from [^{14}C]-lactate and [^{14}C]-pyruvate in the perfusate (Figures 15 & 17). Rates of glycolytic flux as measured by this method were higher in hypertrophied hearts compared to control hearts under high fatty acid conditions (4393 ± 369 nmol/min/g dry wt vs. 1972 ± 363 nmol/min/g dry wt in high fatty acid perfusions and 4454 ± 487 nmol/min/g dry wt vs. 4181 ± 693 nmol/min/g dry wt in low fatty acid perfusions; hypertrophy and control hearts respectively). These values are not different from those measured by the aforementioned method of accumulation of lactate + pyruvate + glucose oxidation (Figure 23). Linearity of rates of glycolytic flux determined by this radiolabel assay was not assessed because the assay was only performed on perfusate collected at time point t=30 min. Data collection at earlier time points (10 min and 20 min) was not possible because of the low accumulation of [^{14}C]-lactate and [^{14}C]-pyruvate and because sensitivity of the assay was not considered optimal at such low counts/activity.

3. *Glycolysis from Detritiation of [5-^{3}H]-Glucose*

Cumulative rates of $^{3}H_2O$ production from [5-^{3}H]-glucose were linear in both control and hypertrophied hearts at both fatty acid levels (Figure 18 & 19). As with the other methods of determining glycolysis, rates of glycolysis determined by this

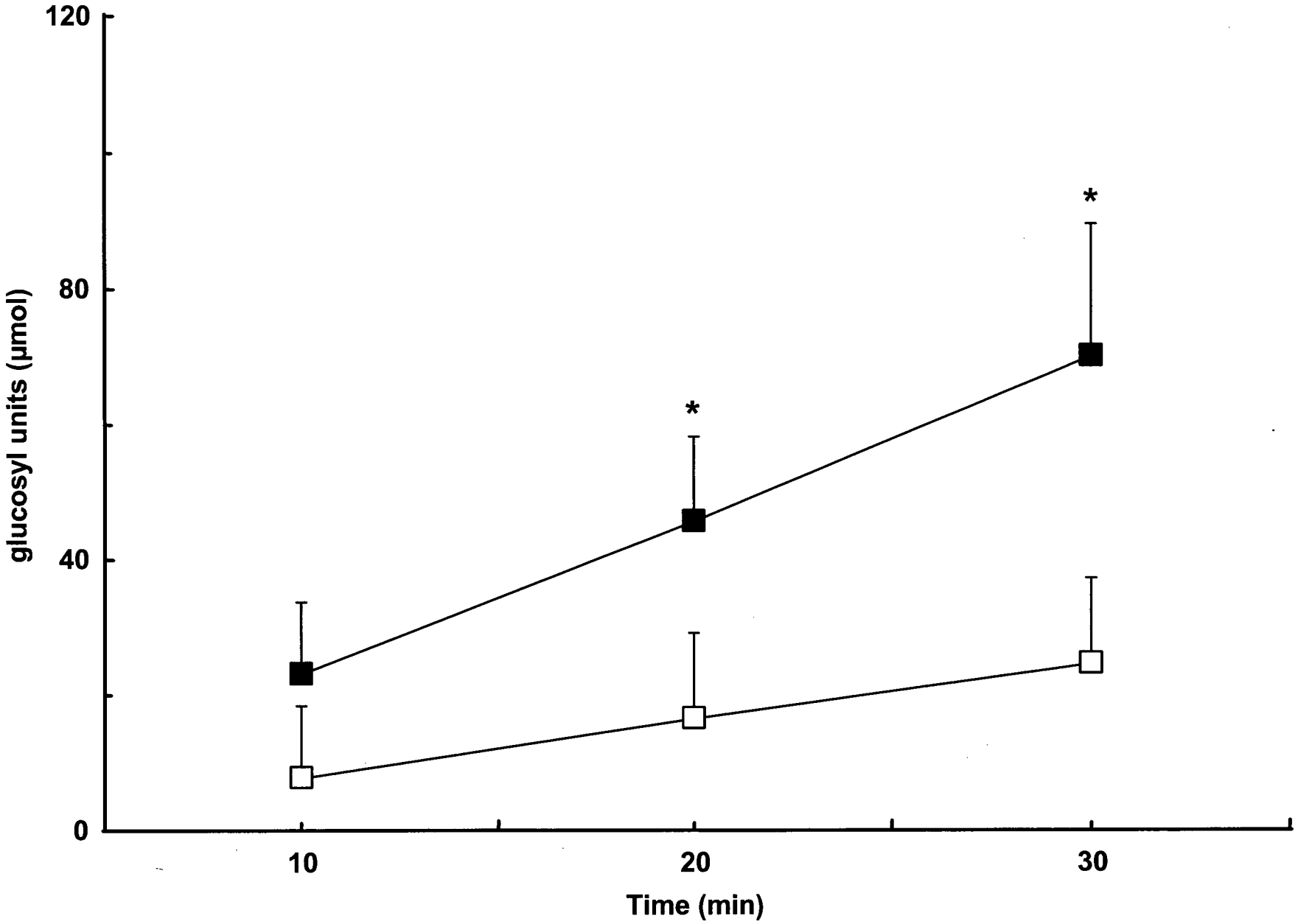

Figure 18: ^3H_2O production in Control (empty squares) and Hypertrophied (solid squares) hearts in high fatty acid conditions. *, vs. control, p = 0.05.

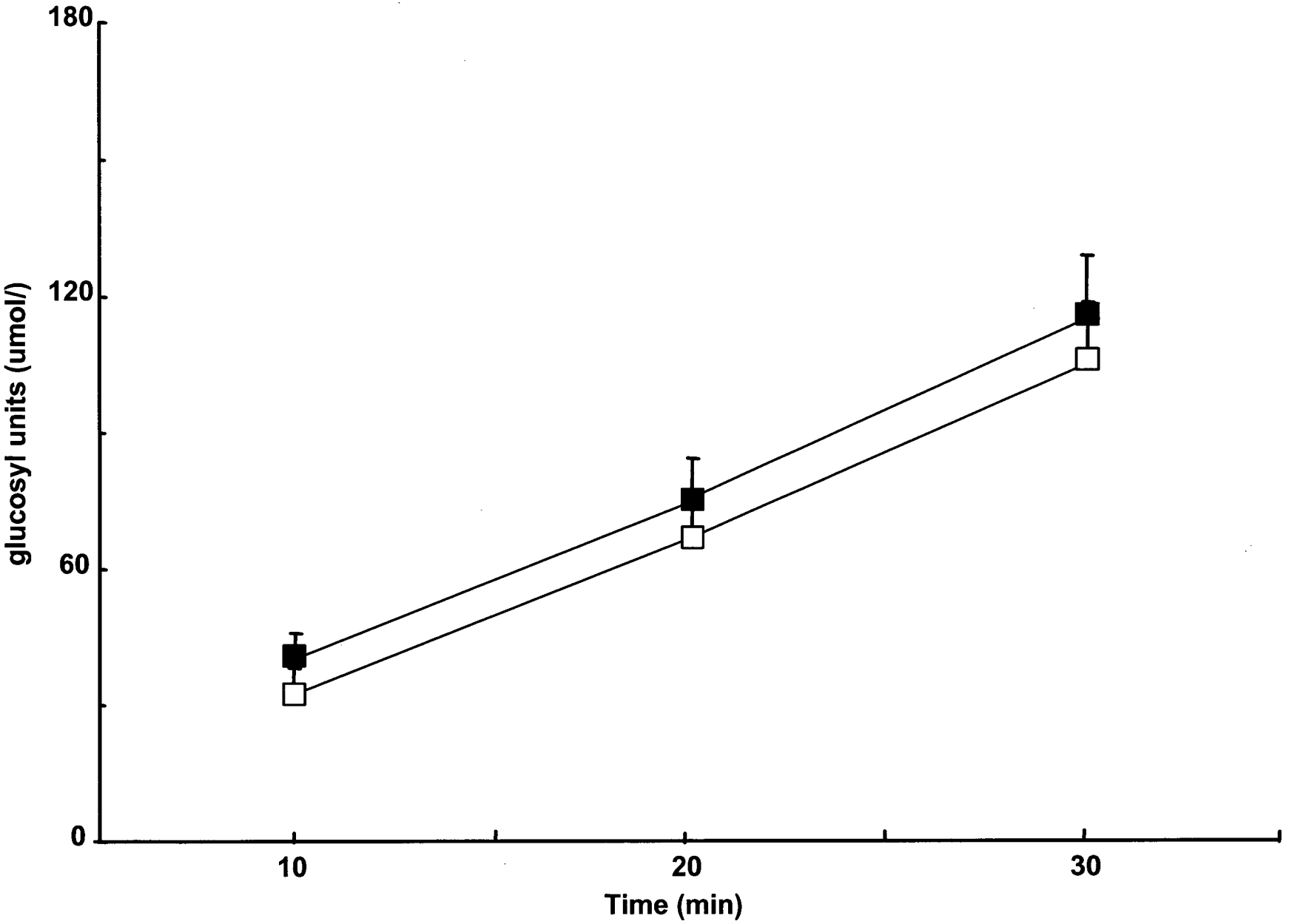

Figure 19: ^3H_2O production in Control (empty squares) and Hypertrophied (solid squares) hearts in low fatty acid conditions.

methodology were greater in hypertrophied hearts than in control hearts under high fatty acid conditions but not significantly different under low fatty acid conditions (1487±132 nmol/min/g dry wt vs. 3962±298 nmol/min/g dry wt in high fatty acid perfusions and 4108±298 nmol/min/g dry wt vs. 3652±477 nmol/min/g dry wt in low fatty acid perfusions; hypertrophy and control hearts respectively; Figures 15 & 17). Glycolytic rates obtained in this way did not differ significantly from rates calculated on the basis of accumulation of total or [^{14}C]-labeled lactate and pyruvate in either hypertrophied or non-hypertrophied hearts. Thus, there was excellent concordance between all three methods of determining glycolytic flux in isolated working rat hearts (Figure 15 & 17).

4. *Comparison of Three Methodologies of Determining Glycolytic Flux in Isolated Working Heart Preparations*

As evidenced by Figures 15 and 17, glycolytic flux is not different between the three techniques over differing fatty acid concentrations. The excellent concordance observed in these figures demonstrates no overestimation of glycolytic flux by the [5-^{3}H]-glucose method. Overall, this method is equivalent to two other independent methods.

D. Myocardial Glycogen Content

At the end of the 30 minute aerobic perfusion under high fatty acid conditions, control hearts had a glycogen level of 113.9±12.6 μmol/g dry wt, whereas hypertrophied hearts had a glycogen level of 107.5±12.6 μmol/g dry. Therefore, glycogen content at the end of the perfusion was not significantly different between hypertrophied and control hearts. Under low fatty acid conditions, control hearts had a glycogen level of 50.6.2±4.4

μmol/g dry (n=4), whereas glycogen in hypertrophied hearts was significantly higher at a level of 71.4.7±5.4 μmol/g dry (n=4, $p<0.05$).

E. Enzymes of the Pentose Phosphate Pathway

Expression of transaldolase (based on protein abundance, Figure 20), and of glucose-6-phospate dehydrogenase (based on enzyme activity, Figure 21) did not differ between hypertrophied and control hearts. Expression of transaldolase in heart was higher than muscle but lower than liver (Figure 22).

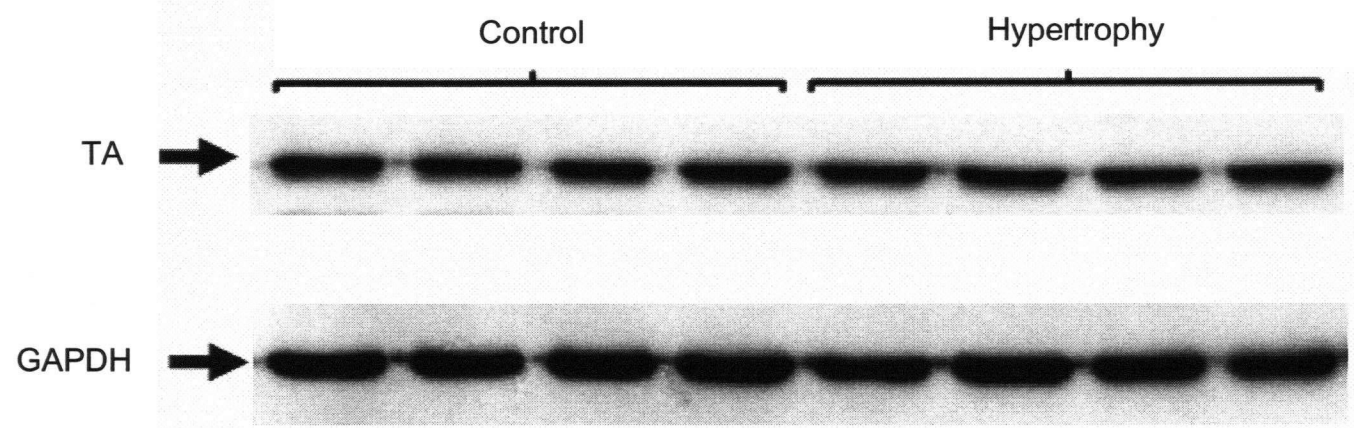

Figure 20: Representative immunoblots of transaldolase and glyceraldehyde-3-phosphate dehydrogensase in control (Control) and hypertrophied (Hypertrophy) hearts. Each lane represents protein isolated from a single heart. TA, transaldolase. GAPDH, glyceraldehyde-3-phosphate dehydrogensase.

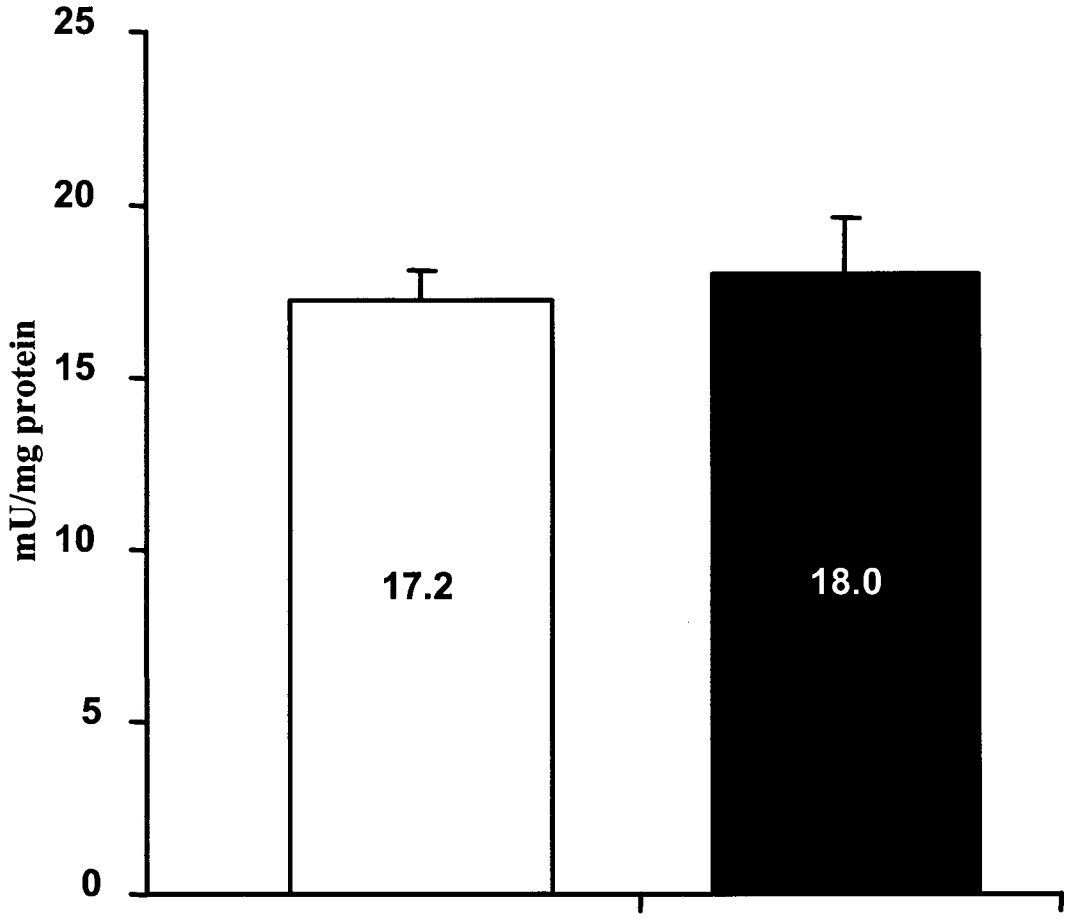

Figure 21: G6PDH Assay on Hypertrophied and Control Hearts. Values represent Mean ± SEM. Measurements were made in hearts frozen at the end of the 30 min perfusion period. [1], Units of glucose-6-phosphate dehydrogenase (G6PDH) are μmol/min. N = 7 per group.

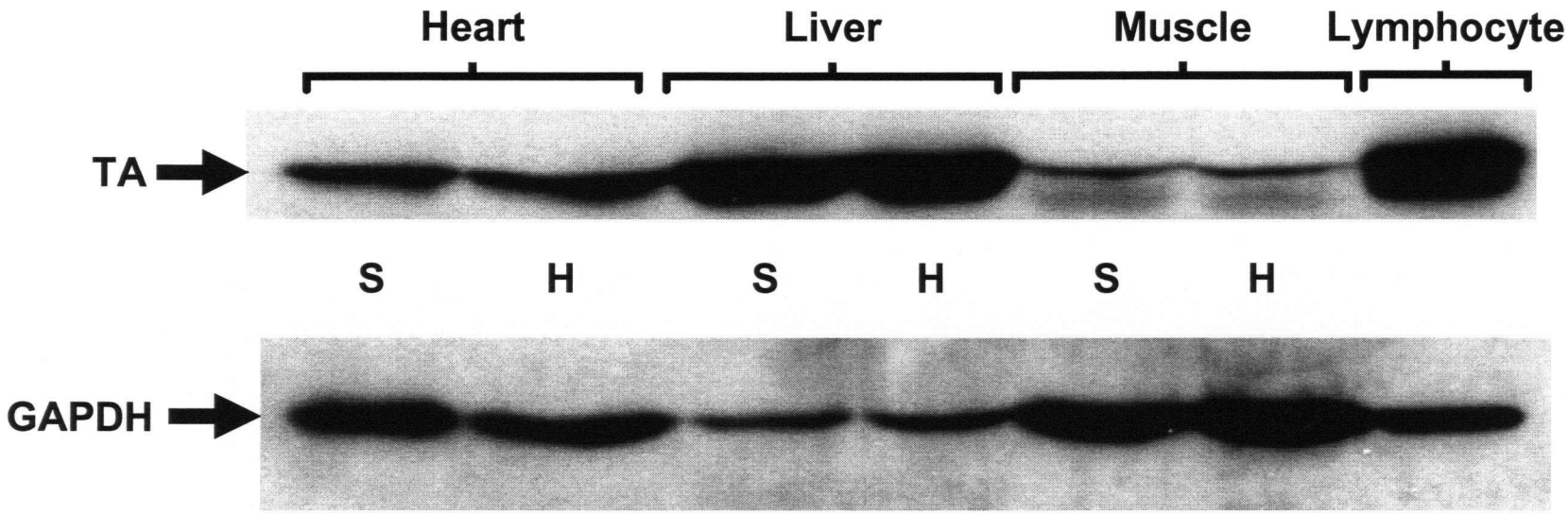

Figure 22: Representative immunoblots of transaldolase and glyceraldehyde-3-phosphate dehydrogensase in organs of control (Control) and hypertrophied (Hypertrophy). "S" denotes sham-operated (control) and "H" denotes aortic-constricted (hypertrophy) animal. Each lane represents protein isolated from that organ. TA, transaldolase. GAPDH, glyceraldehyde-3-phosphate dehydrogensase. Lymphocyte is positive control.

IV. DISCUSSION

The use of [5-^{3}H]-glucose as a tracer to determine glycolytic rates in the heart is a well-established method that quantitatively measures glycolytic rates by collection of ^3H_2O released from [5-^{3}H]-glucose to the perfusate (95,82,38). [5-^{3}H]-glucose, during its catabolism in the glycolytic pathway, is assumed to be completely detritriated by the triose phosphate isomerase and enolase reactions (95,82,38). However, *Goodwin et al.* claim that ^3H_2O production from [5-^{3}H]-glucose overestimates "true" rates of glycolysis in rat hearts due to non-glycolytic detritiation in the non-oxidative PPP (56). This aberration, if it exists, may be even more severe in the pathologically hypertrophied heart, raising the possibility that accelerated rates of glycolytic flux (measured using [5-^{3}H]-glucose) may be artifactual.

A. Summary of Findings

In this study, I confirm that glycolysis is accelerated in isolated working hypertrophied rat hearts as assessed with three different methods. I also demonstrate that non-glycolytic loss of ^3H_2O from [5-^{3}H]-glucose is insignificant in isolated working hypertrophied and non-hypertrophied rat hearts. Most importantly, my data conclusively show that glycolytic rates determined by measuring rates of ^3H_2O production from [5-^{3}H]-glucose do <u>not</u> overestimate true rates of glycolysis and, thereby, confirm that this method is an accurate means to measure glycolysis in this setting. Lastly, I provide evidence that the pentose phosphate pathway is not increased in rat hearts hypertrophied by constriction of the abdominal aorta.

B. Glycolysis in Hypertrophied and Control Hearts

To investigate if glycolytic flux is overestimated by use of [5-^{3}H]-glucose, three independent methods of determining glycolysis were used: detritiation of [5-^{3}H]-glucose; the accumulation of lactate and pyruvate combined with rates of glucose oxidation; and the accumulation of [^{14}C]-lactate and [^{14}C]-pyruvate combined with rates of glucose oxidation. When hearts were perfused under high fatty acid conditions (1.2 mM palmitate), excellent concordance was found between these three different and independent methods. Glycolytic rates were significantly higher in hypertrophied hearts than in control hearts, and this was consistent across all three methodologies. As well, glycolytic production was linear and glycolytic rates in both hypertrophied hearts and control hearts are typical of previous results in other experiments with similar perfusion conditions (at 1.2 mM palmitate) (7,8,121,122,139,10,141) demonstrating a linear trend of glycolytic flux.

Hypertrophied and control hearts perfused under low fatty acid conditions (0.4 mM palmitate) also exhibited similar values of glycolytic flux amongst the three methodologies used. In hypertrophied hearts, rates of glycolytic flux were equivalent in all three methodologies. In control hearts exposed to 0.4 mM palmitate, the glycolytic flux measured by lactate and pyruvate accumulation and glucose oxidation was lower than values of glycolytic flux determined by the other two independent methods, but this difference was not statistically significant. Moreover, the glycolytic flux determined by the accumulation of [^{14}C]-lactate and [^{14}C]-pyruvate and glucose oxidation remained equivalent to the [5-^{3}H]-glucose method, thus confirming the validity of all three methodologies as being equivalent techniques of measuring glycolytic flux.

At high fatty acid levels (1.2 mM), glycolytic flux is accelerated in hypertrophied hearts compared to control hearts, presumably a result of low fatty acid oxidation rates and enhanced activity and expression of glycolytic enzymes. Under low fatty acid levels (0.4 mM palmitate), both hypertrophied and control hearts exhibited similar values of glycolytic flux. The abolishment of disparity in glycolytic flux between both groups can be mainly attributed to the availability and presence of glucose over fatty acid in the perfusate. This situation results in very high rates of glycolysis and glucose oxidation in both groups of hearts.

Although the three independent methods of determining glycolytic flux are equivalent and accurate, there is some concern regarding the depletion of glycogen in hearts exposed to low fatty acid conditions and its possible contribution to lactate and pyruvate production. Control and hypertrophied hearts perfused with low fatty acids typically demonstrate glycogen depletion, with the added benefit that such endogenous forms of glucose are preferentially oxidized (9,139,79). However, excessive recruitment of glycogen as a source of unlabelled glucose into glycolysis could dilute the exogenous glucose pool and overestimate lactate and pyruvate accumulation levels upon glycolysis. If so, then glycolytic flux (as measured by accumulation of lactate & pyruvate + glucose oxidation) is overestimating. However, endogenous glucose is typically preferentially oxidized over exogenous sources of glucose (54,55,9). Therefore, this endogenous glucose likely does not contribute to lactate and pyruvate production and measured glycolytic rates because glycogen-derived glucose is catabolized by glycolysis and pre-destined for oxidation in the mitochondria. All in all, a more suitable explanation for the

low accumulation of lactate, pyruvate, and glucose oxidation in this group may simply be due to error in handling or analysis of the perfusate samples.

Our finding that rates of glycolysis are accelerated in hypertrophied hearts exposed to 1.2 mM palmitate, regardless of the method used, is in keeping with previous *in vivo* and *in vitro* observations based on a variety of independent parameters. Specifically, increased accumulation of 2-deoxyglucose-6-phosphate has been observed in hypertrophied dog (150) and rat (66) hearts *in vivo* . Furthermore, activity of a number of glycolytic enzymes is greater in hearts exposed to a pressure overload than in normal hearts (132) and isoenzymes of lactate dehydrogenase (21) and enolase (68) shift toward more anaerobic, fetal forms in hypertrophied hearts. The acceleration of glycolysis in hypertrophied hearts has been proposed as a compensatory response to low fatty acid oxidation rates in these hearts (5,7,47). As a result, rates of glycolysis may be near maximal in hypertrophied hearts despite any changes in fatty acid content.

C. Pentose Phosphate Pathway Flux in Hypertrophied and Control Hearts

In the event that loss of ^3H from [5-^3H]-glucose does occur in the heart by way of the non-oxidative PPP, the extent of flux through the PPP can be calculated by comparison of fluxes determined by the [5-^3H]-glucose method and the accumulation of lactate & pyruvate. A comparison of both these methods could determine non-glycolytic detritiation of [5-^3H]-glucose, a detritiation assumed to be mainly caused by the PPP. However, in this study, glycolytic flux as measured by the accumulation of lactate, pyruvate and glucose oxidation is consistently higher than or equal to rates of glycolytic flux as measured by the [5-^3H]-glucose method, but this difference was not statistically different. Although flux

through the PPP cannot be accurately assessed through this method, it appears that flux through the PPP is minimal.

Therefore, in light of these crude calculations, significant or detectable production of ^3H_2O from [5-^{3}H]-glucose by detritiation in the PPP is unlikely. This view is consistent with evidence that the capacity of the oxidative pentose phosphate pathway in heart is very low compared to liver and other tissues. Maximal cardiac oxidative PPP flux is generally less than 50 nmol/min/g dry wt, a value far lower than that of glycolysis (~2500 nmol/min/g dry wt) (126,27,124,106,147). Furthermore, non-oxidative pentose phosphate pathway flux between the hexose and ribose pools in the heart is likely even lower, probably not exceeding 2 nmol/min/g dry wt (151,114,43).

Expression analysis of transaldolase and G6PDH in control and hypertrophied cardiac muscle provides proof that both branches of the PPP are not elevated in hypertrophied heart (Figure 20 & 22)). As well, expression of transaldolase in both control and hypertrophied cardiac muscle was significantly lower than that found in liver. These two pieces of data provide further evidence that activity of PPP is low in the heart and supports the view that the PPP does not cause any non-glycolytic detritiation of any magnitude. This conclusion is also consistent with previous studies on the PPP in cardiac hypertrophy, in which changes in PPP enzyme activities, when observed, were transient and reversed with time after aortic constriction (89,30,152).

D. Detritiation of [5-^{3}H]-Glucose as a Measure of Glycolytic Flux

Recently, Goodwin *et al* suggested that use of [5-^{3}H]-glucose to measure glycolysis leads to an overestimation of "true" rates of glycolysis in isolated working rat hearts because

of non-glycolytic loss of ^3H_2O from [5-^{3}H]-glucose (56). This suggestion was based upon their finding that rates of glycolysis determined by measuring ^3H_2O production from [5-^{3}H]-glucose were significantly higher than rates of glycolysis determined as the sum of lactate and pyruvate released to the perfusate and glucose oxidation rates measured using [^{14}C]-glucose. They proposed that glucose utilization by the non-oxidative PPP, specifically, the enzyme transaldolase, was responsible for any non-glycolytic detritiation of [5-^{3}H]-glucose.

In contrast to Goodwin *et al* (56), we found an excellent concordance between rates of glycolysis determined by different and independent methods (Figure 15 &17) and confirmed that glycolysis is accelerated in hypertrophied hearts. Our data indicate the non-glycolytic loss of ^3H_2O from [5-^{3}H]-glucose is insignificant, relative to loss in the glycolytic pathway, in both hypertrophied and non-hypertrophied hearts under both high fat and low fat conditions used in our experiments. The concordance of glycolytic rates determined by the different methods also indicates that glycogen did not contribute significantly to total lactate and pyruvate accumulation in the perfusate. This is in keeping with the finding the glycogen content did not differ in hearts exposed to high fatty acids, and that the observed loss of glycogen levels in hearts exposed to low fatty acid conditions cannot easily account for the differences observed.

We attribute the discrepancy between the two studies primarily to two unexpected observations by Goodwin et al. The first is the exceedingly low glucose oxidation rates reported by Goodwin *et al* (~100nmol/min/g dry wt), especially when considered in relation to rates of glycolysis (56). In their study, glycolytic rates are, at least, 26 fold higher than glucose oxidation rates, which contrasts dramatically with other studies with isolated working hearts perfused with Krebs-Henseleit solution containing a similarly low (i.e.,

0.4 mM) concentration of fatty acid (67). In Kantor et al.'s study with similar perfusion conditions (67), glycolytic rates are only approximately 2 fold higher than glucose oxidation rates (~1900nmol/min/g dry wt). The relatively high rates of glucose oxidation observed in these studies are to be expected in the presence of low concentrations of fatty acid because of the well-established reciprocal relationship between catabolism of fatty acids and glucose in the heart (95,112,129). Moreover, if Kantor's rates of glucose oxidation are substituted for Goodwin's rates of glucose oxidation, the methodological differences observed by Goodwin are eliminated, with all three methodologies being equivalent. Use of [5-^{3}H]-glucose, is therefore, an accurate means to determine glycolytic rates in the isolated working rat heart.

The explanation for the dramatically lower glucose oxidation rates obtained in the study by Goodwin *et al* (56) is unclear but could be methodological. They used a modified working heart preparation in which the coronary flow was not recirculated (56) (56), as it is in the more traditional preparation (82). Thus, rather than calculating rates of glucose oxidation from the accumulation of $^{14}CO_2$ over the duration of the perfusion, perfusate containing $^{14}CO_2$ released in a single pass through the myocardium is collected into open pre-weighed vials over short time periods (i.e., 5min). An aliquot (10ml) of this perfusate is subsequently used to measure the $^{14}CO_2$ produced. Given the fact that ^{14}C-labelled lactate and pyruvate accumulate in the recirculating working heart preparation and likely contribute to overall $^{14}CO_2$ production, it may be argued that the low glucose oxidation rates reported with the use of a non-recirculating perfusion by Goodwin *et al* are a better representation of true glucose oxidation rates. However, review of elegant studies in which flux through the pyruvate dehydrogenase reaction (i.e., equivalent to

glucose oxidation) was determined by isotopomer analysis in non-recirculating isolated rat hearts perfused with 0.4mM oleate shows that this is not the case (32,136). In these isotopomer studies, glucose oxidation rates in isolated normal hearts were at least ~1.1μmol/min/g dry wt or about 10-fold higher than those reported by Goodwin *et al.* and comparable to values obtained in a recirculating working heart preparation under similar conditions (67).

The second unexpected observation by Goodwin et al. is the lack of linearity of ^3H_2O production over a 30-min time period. At the outset of perfusion, glycolytic rates determined from all three methodologies were relatively similar; however, over time, ^3H_2O production rates increased, whereas lactate and pyruvate production rates did not. This increase, which is the primary basis for their conclusions with respect to detritiation of [5-^{3}H]-glucose by the PPP, is inconsistent with the findings of others, including those in the present study and previous investigations originating in Goodwin's laboratory (96,7,22,82). As summarized in Figures 17 and 18, we do not observe a time-dependent increase in ^3H_2O production rates, a finding consistent with the published literature (95,82,10). Of additional importance is the fact that the recent conclusions by Goodwin *et al.* (56) contradict their own data from a previous study (22) in which they concluded that detritiation of [5-^{3}H]-glucose traces glycolytic flux from exogenous glucose (22) where they found that ^3H_2O production from [5-^{3}H]-glucose did not differ from ^3H_2O production from [2-^{3}H]-glucose. Other studies have also confirmed that the use of either [5-^{3}H]-glucose and [2-^{3}H]-glucose is equivalent (80,117,85). This is important because detritiation of [2-^{3}H]-glucose, which occurs at the hexose-6-phosphate isomerase

reaction, is not affected by the reactions in the pentose phosphate pathway implicated as being responsible for non-glycolytic loss of ^{3}H.

E. Methodological Considerations

As the majority of radiolabelled by-products will be accounted for by the production of lactate, pyruvate and glucose oxidation, some radiolabelled intermediates may be lost or unaccounted for, as in the case of [^{14}C]-alanine via the "pyruvate-alanine cycle". [^{14}C]-alanine, an essential amino acid, may be reincorporated into the myocardium and thus hidden away from major metabolic processes. However, flux through this particular pathway is typically small (~50 nmol/min/g dry wt) and is generally considered negligible. However, to confirm flux through this pathway, direct measurement of [^{14}C]-Alanine release from [^{14}C]-glucose will be required to determine if this is the case.

F. Importance of this Study

The results of our study also have great general relevance to investigators in the field. Specifically, a very important corollary of the data obtained is that measurement of the rate of $^{3}H_2O$ production from [5-^{3}H]-glucose is an accurate means to determine rates of glycolysis in isolated working normal and pathologic rat hearts. Glycolysis and $^{3}H_2O$ production in isolated working rat hearts are also linear and constant. This method can be applied in many different experimental settings, ranging from cell culture to isolated working rat heart preparations. Moreover, the data obtained alleviate any doubts about conclusions from many studies over the years that have used this methodology to

measure glycolysis in isolated hearts and is an ideal technique for measuring glycolytic flux in many experimental settings.

Appendix I – Modified [^{14}C]-Lactate+[^{14}C]-Pyruvate Radiolabel Assay

The quantification of lactate and pyruvate concentrations in perfusate can be used as a method of determining myocardial glycolytic rates, assuming glycogen degradation is minimal. In the event of significant contribution of glycogen to total lactate and pyruvate accumulation, this assay was developed to determine only [^{14}C]-lactate and [^{14}C]-pyruvate accumulation, a method that only measures exogenous glycolysis. In general, the amount of lactate and pyruvate produced combined with the amount of glucose oxidized in a given amount of time equals glycolytic flux as shown in Figure 23. Moreover, the use of U-[^{14}C]-glucose generates [^{14}C]-lactate and [^{14}C]-pyruvate, end products of glycolysis which can be used to calculate glycolytic flux in conjunction with glucose oxidation rates. The assay developed for this study converts all [^{14}C]-lactate into [^{14}C]-pyruvate, and then decarboxylates all [^{14}C]-lactate-derived [^{14}C]-pyruvate and [^{14}C]-pyruvate from glycolysis, producing $^{14}CO_2$ gas, which can be collected to calculate glycolytic flux. The main advantage of using this method will confirm the idea that all lactate produced and released into the perfusate is glycolytically derived. Because the glucose is also U-[^{14}C] labeled, concordance between both methodologies of determining glycolytic flux should confirm that all lactate produced is from glycolysis.

This assay was initially described by *Lehoux et al.* (73), and is a convenient and accurate method that can determine the specific activities of [^{14}C]-lactate and [^{14}C]-pyruvate in perfusate. In order to determine accumulations for both metabolite species simultaneously, a modification was made to this assay as will be described in this section (and in Figure 24). This two-point assay initially enzymatically decarboxylzes [^{14}C]-lactate to [^{14}C]-pyruvate catalyzed by lactate oxidase in an enclosed environment. The

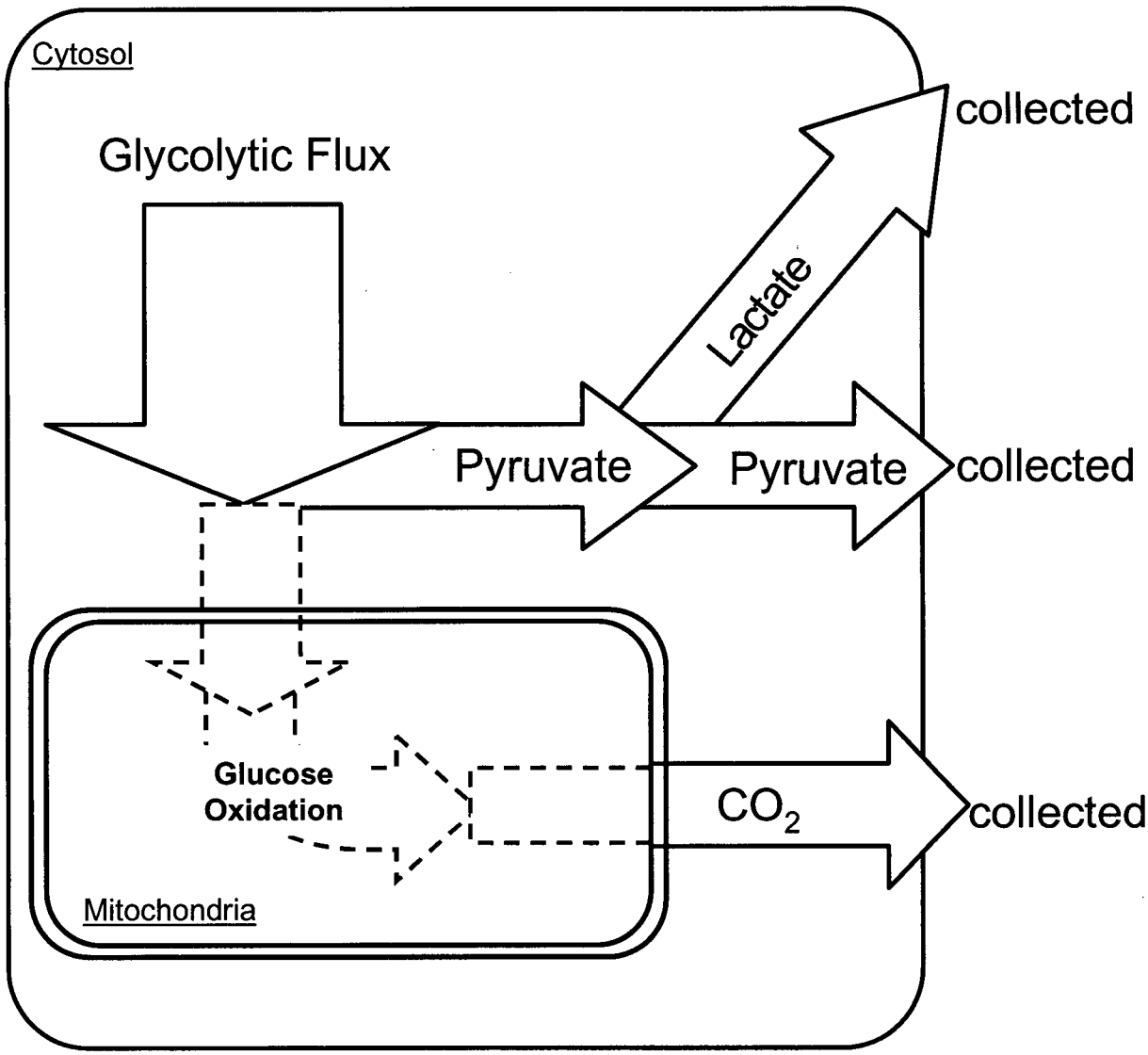

Figure 23: Accumulation of Lactate and Pyruvate Combined with Rates of Glucose Oxidation are Equivalent to Glycolytic Flux.

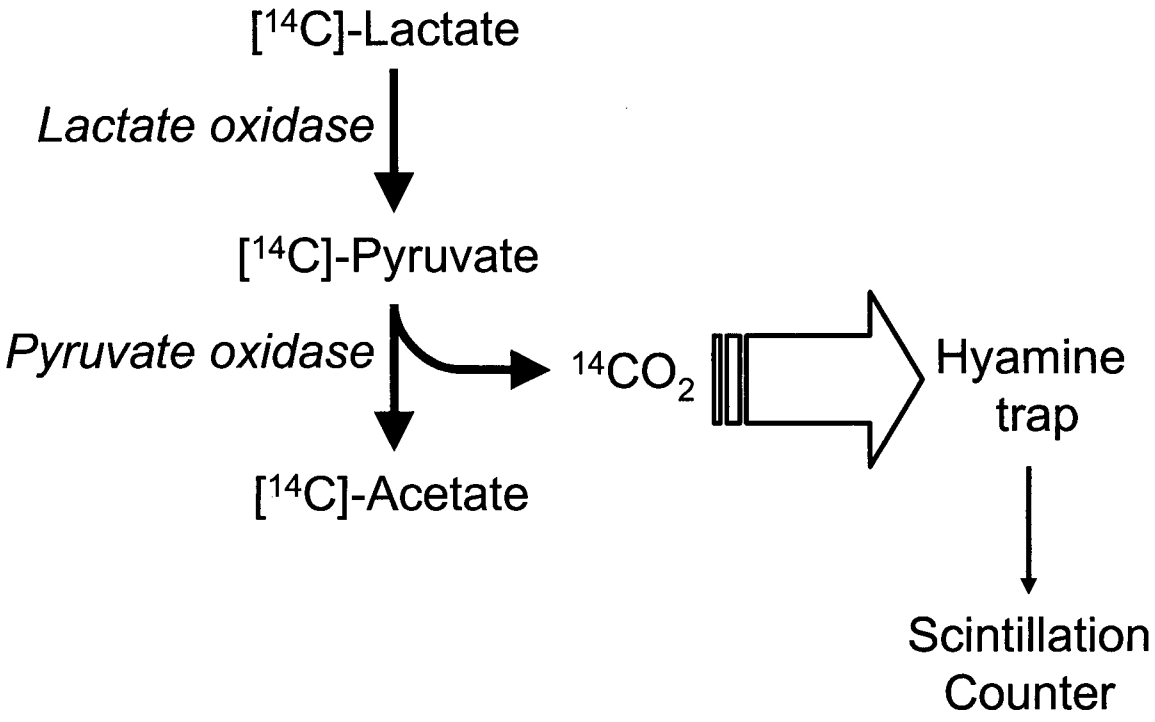

Figure 24: Modified Two Step [^{14}C]-Lactate & [^{14}C]-Pyruvate Assay.

second half of the assay enzymatically decarboxylzes [^{14}C]-pyruvate to [^{14}C]-acetate by pyruvate oxidase. This half of the reaction releases one $^{14}CO_2$ per [^{14}C]-pyruvate molecule, which is then collected by the hyamine trap. The detailed protocol is described as follows:

[^{14}C]-Lactate+[^{14}C]-Pyruvate Radiolabel Assay Protocol

1. Place filter paper into scintillation tube and soak with 300 µL hyamine hydroxide.
2. Reaction Mixture (for one reaction)
   - -125 µL dH_2O
   - -40 µL KH_2PO_4-NaOH buffer
   - -25 µL 2% BSA soln
   - -20 µL 10 mM $MgCl_2$ soln
   - -5 units cocarboxylase
   - -2 µL FAD soln
   - -2 units catalase
   - -1 unit Lactate Oxidase
   - -2 units Pyruvate Oxidase
   - -50 µL Sample
3. Incubate at 37°C for 90 minutes with constant gentle shaking.
4. With a 10cc syringe, inject 0.5 mL of 9N H_2SO_4 into the reaction mixture via the rubber adaptor.
5. Incubate at 37°C for 90 minutes with constant gentle shaking.
6. Remove scintillation vial and add 4 mL of scintillation fluid.

Accumulation of [^{14}C]-lactate and [^{14}C]-pyruvate was determined by the use of a two-step assay system that, 1) oxidizes all [^{14}C]-lactate in a given sample to [^{14}C]-pyruvate and then, 2) all remaining [^{14}C]-pyruvate is decarboxylated by pyruvate oxidase to [^{14}C]-acetate releasing $^{14}CO_2$ gas. All $^{14}CO_2$ gas is collected by hyamine soaked filter papers in an enclosed vessel. To ensure specificity and consistency of this two-step assay, mock trials of this assay were done with three different lactate standards with varying specific activities. The three standards consisted of lactate spiked with [^{14}C]-lactate at: 10mM (3000dpm); 5mM (1000dpm); and 1 mM (500dpm). Upon proper examination of the assay protocol, [^{14}C]-lactate recovery

efficiencies ranged from ~75% to ~100%, regardless of the standard used or the day of assay (Table 4). Consistently high recoveries were found regardless of the day of experiment or the standard used. Additionally, these results do not differ whether or not the substrates ([^{14}C]-Lactate and [^{14}C]-Pyruvate) were dissolved in distilled water, or dissolved in 3% BSA, or when dissolved in KH buffer complexed with varying palmitate concentrations (Table 5). Positive control standards were used to test for inter- (between days) and intra-test (duplicates of standards/samples) variability and assay consistency. The three standards tested were: 10mM (3000 dpm), 5mM (1000 dpm) and 1 mM (500 dpm), all dissolved in dH_2O. U-[^{14}C]-lactate (0.5μCi/mL) was used for the construction of the standards. Due to the uniform ^{14}C labeling on all three carbons of the tracer, by way of enzymatic decarboxylation under optimal conditions, one third of the original specific activity of lactate should theoretically be released as $^{14}CO_2$ and collected by the hyamine sink trap. Efficiency of recovery by the assay was calculated as follows, where "n" denotes number of samples:

$$\left(\frac{\text{Counts recovered by Sample} \times 3}{\text{Counts in Standard}}\right) \times n \times 100 = \text{Assay Efficiency}$$

Statistically, intra- test variability was low (coefficient of variability= 8.1, 19.8, 9.3 on three separate occasions), approximating a 10.0% range of variability, while inter-test variability across all three experiment occasions was also low (coefficient of variability = 9.5) and also approximating a 10% range of variability. Therefore, quenching or complexing of [^{14}C]-lactate and [^{14}C]-pyruvate in perfusate is not significant and does not affect the efficacy of this radiolabel assay.

Table 3: Perfusate lactate and pyruvate production in control (Control) and hypertrophied (Hypertrophy) working rat hearts.

	1.2 mM Palmitate		0.4 mM Palmitate	
	Control (n=7)	Hypertrophy (n=7)	Control (n=4)	Hypertrophy (n=4)
Lactate (umol/gdry/30min)	66.4±17.3	198.2±22.2*	81.8±14.3	147.2±16.4*
Pyruvate (umol/gdry/30min)	19.0±1.4	31.5±3.5*	19.0±2.1	25.4±8.3

Values represent Mean ± SEM. 1, values for lactate and pyruvate represent total accumulation in perfusate over the duration of 30min perfusion. Expressed as glucosyl units. *, vs. Control, $p < 0.05$.

Table 4: Inter- and Intra- test variability in [^{14}C]-Lactate and [^{14}C]-Pyruvate Assay

	12/10/2001 Standards			12/12/2001 Standards			12/13/2001 Standards		
Vessel	3000 dpm	1000 dpm	500 dpm	3000 dpm	1000 dpm	500 dpm	3000 dpm	1000 dpm	500 dpm
1	811.9	274.9	146.4	1078.8	271.3	245.7	1745.6	296.8	173.2
2	700.7	266.2	162.2	1288.5	256.7	190.1	1951.6	281.4	183.6
3	738.4	256.4	158.2	947.9	278.1	169.0	844.9	286.3	184.1
4	816.2	267.3	135.9	897.8	222.3	194.7	951.2	345.4	184.3
Average (dpm)	766.8	266.2	150.7	1053.2	257.1	199.9	915.6	302.5	181.3
Total (dpm)	3084.1	995.0	516.2	3084.1	995.0	516.2	3084.1	995.0	516.2
%Recovery	74.6	80.3	87.6	102.5	77.5	116.2	89.1	91.2	105.4

Table 5: Inter- and Intra- test variability in [^{14}C]-Lactate and [^{14}C]-Pyruvate Assay in various conditions

		Replicates (dpm)										
	Sample Condition	1	2	3	4	5	6	7	8	Average (dpm)	Before Reaction	Efficiency (%)
A	dH_2O	1020.35	941.97	967.41	1140.88	1063.47	1118.65	1122.03	1089.40	1058.02	4266.03	74.40
B	Krebs+BSA	905.02	877.19	873.98	1007.73	1086.39	1204.00	1216.11	1110.00	1035.12	3806.71	81.58
C	Krebs	793.49	794.89	823.37	984.18	893.47	786.70	805.79	977.48	857.42	3236.64	79.47

Sample legend:

A- 0.75 mM lactate standard dissolved in dH_2O

B- 0.75 mM lactate standard dissolved in 1X Kreb's with 3% BSA complexed with 1.2 mM palmitate and 5.5 mM glucose

C- 0.75 mM lactate standard dissolved in 1X Kreb's

Appendix II - Calculation of Glycolytic Flux from Specific Activity of [^{14}C]-Lactate and [^{14}C]-Pyruvate

Glycolytic flux is determined from the amount of lactate and pyruvate produced combined with the amount of glucose oxidation that occurs in the 30 minute isolated working heart perfusion period. The counts recovered from the radiolabel assay consist of $^{14}CO_2$ gas released from the enzymatic decarboxylation of [^{14}C]-Lactate and [^{14}C]-Pyruvate. These $^{14}CO_2$ counts are converted to glucosyl equivalents/min/gram dry weight by extrapolating the counts registered by the 50 μL aliquot sample to the counts in 100 mL of perfusate. Then the total amount of ^{14}C dpm counts is divided by the initial specific activity of glucose in the perfusate prior to the isolated working heart mode. The amount of glucose equivalents catabolized is then divided by the time (min) and dry wt of the heart (g dry wt). The formula is on Figure 25:

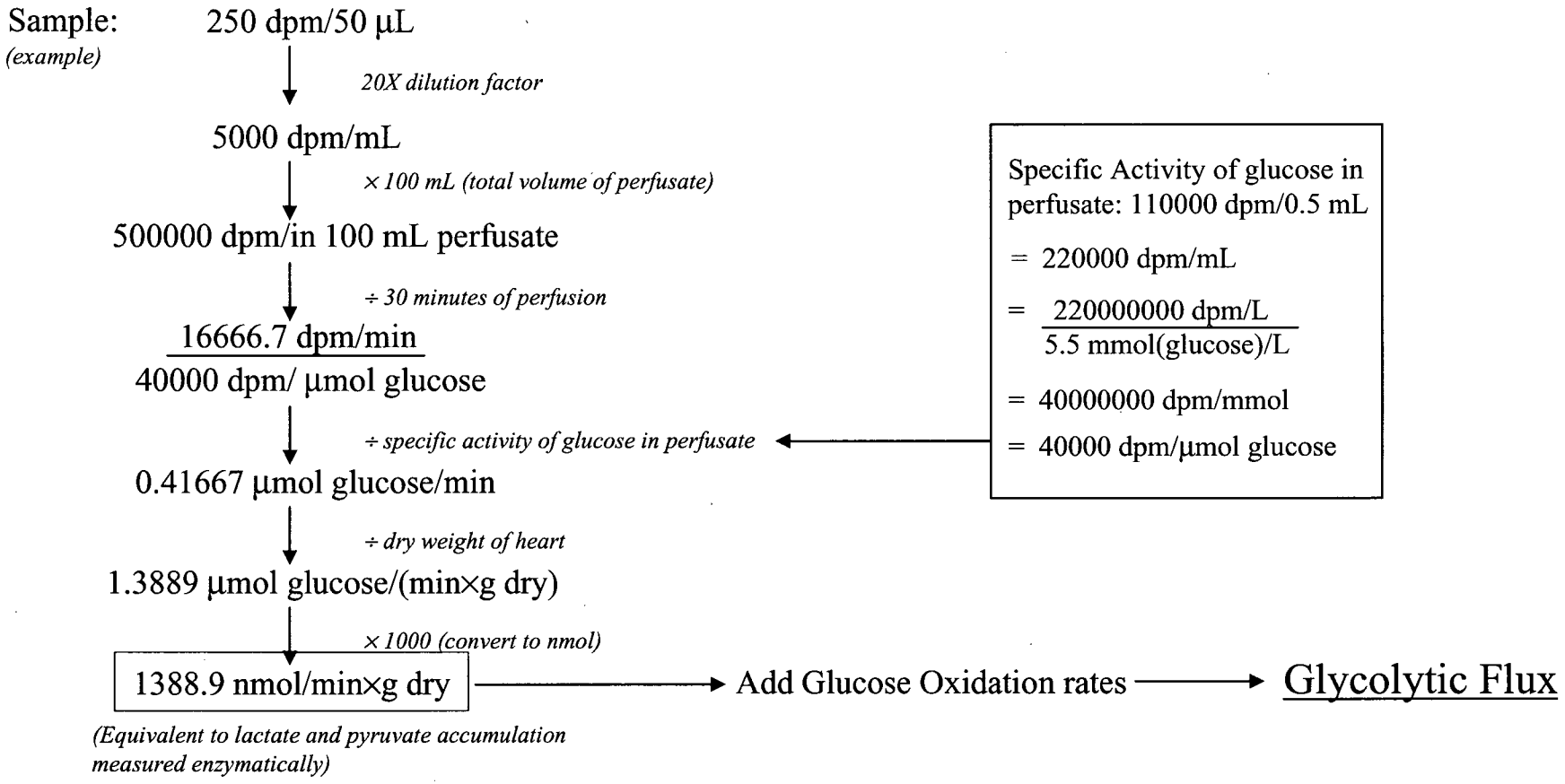

Figure 25: Calculation of Glycolytic Flux from Specific Activity of [^{14}C]-Lactate and [^{14}C]-Pyruvate

Appendix III – Calculation of Glycolytic Flux by Accumulation of Lactate and Pyruvate

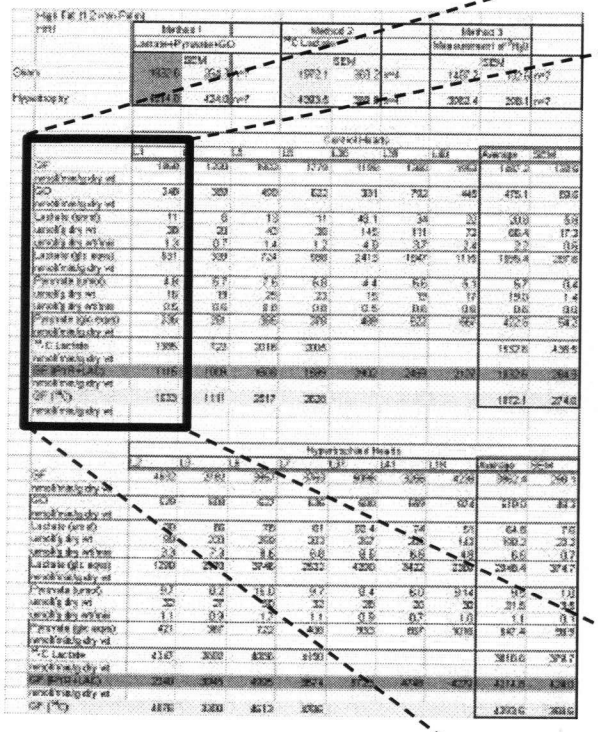

Table 1-6: Summary of Metabolic Data

		L1	
	GF	1068	◄ GF = Glycolytic Flux as measured by ^3H_2O release
	nmol/min/g dry wt		
3	GO	248	◄ GO = Glucose Oxidation as measured by $^{14}CO_2$ release
	nmol/min/g dry wt		
	Lactate (umol)	11	◄ Total nmol Lactate Produced
	umol/g dry wt	38	◄ ÷ Dry heart weight (g)
	umol/g dry wt/min	1.3	◄ ÷ Time of perfusion (30 min)
1	Lactate (glc eqvs)	631	◄ ÷ 2 nmol lactate/nmol glucose
	nmol/min/g dry wt		
	Pyruvate (umol)	4.8	◄ Total nmol Pyruvate Produced
	umol/g dry wt	16	◄ ÷ Dry heart weight (g)
	umol/g dry wt/min	0.5	◄ ÷ Time of perfusion (30 min)
2	Pyruvate (glc eqvs)	236	◄ ÷ 2 nmol pyruvate/nmol glucose
	nmol/min/g dry wt		
	^{14}-C Lactate	1385	◄ Determined by [^{14}C]-Lactate & [^{14}C]-Pyruvate Assay
	nmol/min/g dry wt		
4	GF (PYR+LAC)	1115	◄ Determined by adding 1+ 2 + 3
	nmol/min/g dry wt		
	GF (^{14}C)	1633	◄ Determined by adding 3 + 4
	nmol/min/g dry wt		

◄ L_ - Heart Number

Excerpt of L1 Metabolic Data

Details of Calculations of L1 Metabolic Data

Above is a schematic of the summary of metabolic data, in calculations based on g dry wt and min. In descending order is a logical sequence of how each parameter is assessed.

Appendix IV: Summary of Metabolic Data for Hearts Perfused with High Fatty Acid

High Fat (1.2 mm Palm)										
HH1	Method 1			Method 2			Method 3			
	Lactate+Pyruvate+GO			^{14}C Lactate			Measurement of $^{3}H_2O$			
		SEM			SEM			SEM		
Sham	1832.6	264.3	n=7	1972.1	363.2	n=4	1487.2	132.6	n=7	
Hypertrophy	4214.8	424.0	n=7	4393.5	368.6	n=4	3962.4	298.1	n=7	

	Control Hearts								
	L1	L4	L5	L8	L36	L39	L40	Average	SEM
GF	1068	1230	1803	1779	1198	1380	1953	1487.2	132.6
nmol/min/g dry wt									
GO	248	389	499	622	331	792	445	475.1	69.6
nmol/min/g dry wt									
Lactate (umol)	11	6	13	11	48.1	34	22	20.8	5.8
umol/g dry wt	38	20	43	36	145	111	72	66.4	17.3
umol/g dry wt/min	1.3	0.7	1.4	1.2	4.8	3.7	2.4	2.2	0.6
Lactate (glc eqvs)	631	339	724	598	2413	1847	1115	1095.4	287.6
nmol/min/g dry wt									
Pyruvate (umol)	4.8	5.7	7.5	6.8	4.4	5.6	5.1	5.7	0.4
umol/g dry wt	16	19	25	23	15	19	17	19.0	1.4
umol/g dry wt/min	0.5	0.6	0.8	0.8	0.5	0.6	0.6	0.6	0.0
Pyruvate (glc eqvs)	236	281	385	378	489	622	567	422.5	54.2
nmol/min/g dry wt									
^{14}-C Lactate	1385	722	2018	2005				1532.6	435.5
nmol/min/g dry wt									
GF (PYR+LAC)	1115	1009	1608	1599	2902	2469	2127	1832.6	264.3
nmol/min/g dry wt									
GF (^{14}C)	1633	1111	2517	2628				1972.1	274.6
nmol/min/g dry wt									

	Hypertrophied Hearts								
	L2	L3	L6	L7	L37	L41	L18	Average	SEM
GF	4632	2782	3957	3763	5098	3266	4238	3962.4	298.1
nmol/min/g dry wt									
GO	529	608	527	636	500	659	874	619.0	48.3
nmol/min/g dry wt									
Lactate (umol)	30	66	78	61	92.4	74	51	64.6	7.6
umol/g dry wt	99	220	259	203	257	205	143	198.2	22.2
umol/g dry wt/min	3.3	7.3	8.6	6.8	8.6	6.8	4.8	6.6	0.7
Lactate (glc eqvs)	1290	2970	3746	2532	4290	3422	2389	2948.4	374.7
nmol/min/g dry wt									
Pyruvate (umol)	9.7	8.2	15.0	9.7	8.4	6.0	9.14	9.5	1.0
umol/g dry wt	32	27	50	32	28	20	30	31.5	3.5
umol/g dry wt/min	1.1	0.9	1.7	1.1	0.9	0.7	1.0	1.1	0.1
Pyruvate (glc eqvs)	421	367	722	406	933	667	1016	647.4	98.9
nmol/min/g dry wt									
^{14}-C Lactate	4347	2692	4086	4150				3818.6	379.7
nmol/min/g dry wt									
GF (PYR+LAC)	2240	3945	4995	3674	5723	4748	4279	4214.8	424.0
nmol/min/g dry wt									
GF (^{14}C)	4876	3300	4613	4786				4393.5	368.6

Appendix V: Summary of Metabolic Data for Hearts Perfused with Low Fatty Acid

Low Fat (0.4 mm Palm)

	Method 1 Lactate+Pyruvate+GO			Method 2 14-C Lactate+Pyruvate			Method 3 Measurement of 3H20		
		SEM			SEM			SEM	
Sham	3016.6	198.1	n=4	4180.9	693.3	n=4	3652.8	476.6	n=4
Hypertrophy	4347.9	402.8	n=4	4454.4	487.2	n=4	4108.0	297.7	n=4

	Control Hearts						Hypertrophied Hearts					
	L42	L45	L46	L49	Average	SEM	L43	L44	L47	L48	Average	SEM
GF nmol/min/g dry wt	4094	2388	4609	3520	3653	477	3974	4908	4078	3472	4108	298
GO nmol/min/g dry wt	1664	536	1671	1471	1336	270	1231	1964	1610	1083	1472	198
Lactate (umol)	28	35	23	15	25	4.3	57	42	50	32	45	5
umol/g dry wt	92	115	74	47	82	14.3	189	136	153	111	147	16
umol/g dry wt/min	3.1	3.8	2.5	1.6	2.7	0.5	6.3	4.5	5.1	3.7	4.9	0.5
Lactate nmol/min/g dry wt	1531	1911	1228	785	1364	238	3146	2273	2551	1843	2453	273
Pyruvate (umol)	6.0	6.9	4.0	6.8	5.9	0.7	15.1	4.9	5.0	5.9	7.7	2.5
umol/g dry wt	20	22	13	21	19	2.1	50	16	15	20	25	8.3
umol/g dry wt/min	0.7	0.7	0.4	0.7	0.6	0.1	1.7	0.5	0.5	0.7	0.8	0.3
Pyruvate nmol/min/g dry wt	328	372	216	354	317	35	832	269	253	337	423	138
14-C Lactate nmol/min/g dry wt	3980	1777	2881	2744	2845	451	2775	3893	2703	2558	2982	307
GF (PYR+LAC) nmol/min/g dry wt	3523	2820	3114	2609	3017	198	5208	4506	4414	3263	4348	403
GF (14C) nmol/min/g dry wt	5644	2313	4552	4215	4181	693	4006	5857	4313	3641	4454	487

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