UNIVERSITY OF ALBERTA

SKELETAL MUSCLE MITOCHONDRIAL PROTEIN SYNTHESIS

BY

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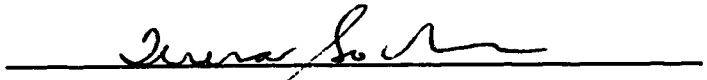

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DEDICATION

To my husband Tom Potter, for his love and support, and to our daughter Stephanie who made this dissertation more meaningful.

ABSTRACT

The objective of this research was to study *in vitro* (attributed to the mitochondrial genetic system) and *in vivo* (attributed to the combined involvement of the nuclear-cytoplasmic and mitochondrial genetic systems) protein synthesis in two subpopulations of skeletal muscle mitochondria. Subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondrial fractions were isolated from rat skeletal muscle. Measures of respiration, and *in vitro* and *in vivo* rates of amino acid incorporation were compared. State III respiration was 1.5 fold greater in IMF than SS mitochondria ($p<0.05$). *In vitro* L-[^{35}S]methionine incorporation was 2.1 fold greater in IMF (3.84 ± 0.41 pmol methionine·30 min^{-1}·mg $protein^{-1}$) than in SS (1.81 ± 0.29 pmol methionine·30 min^{-1}·mg $protein^{-1}$) mitochondria ($p<0.05$). In contrast, *in vivo* fractional rates of protein synthesis, determined by a flooding dose injection of L-[2,6-^{3}H]Phenylalanine, were 8.0 ± 0.4%·day^{-1} and 4.0 ± 0.2%·day^{-1} for SS and IMF mitochondrial fractions, respectively ($p<0.05$). Furthermore, the acute effect of a 1.5% concentration of ethanol on *in vitro* protein synthesis resulted in a 20% ($p>0.05$) and 40% ($p<0.05$) reduction in the rate of amino acid incorporation into SS and IMF mitochondrial fractions, respectively. The distinct rates of both *in vivo* and *in vitro* protein synthesis which were similar in magnitude yet diametrically opposed may be a

result of the different genetic origins studied. The *in vitro* results may partially be explained by the characteristic 1.5-2.0 greater rate of respiration in IMF than in SS mitochondria, since the proteins encoded by the mitochondrial genome are all associated with oxidative phosphorylation. The *in vivo* results correspond with skeletal muscle mitochondrial adaptations in response to physiological and pathological stresses in each of the subpopulations of mitochondria, supporting the hypothesis of different functional roles.

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CHAPTER 1

INTRODUCTION

A. GENERAL INTRODUCTION

Introduced in 1898 by Benda, the name 'mitochondria' was given to the organelles located in the cytoplasm of mammalian cells which specialize in oxidative phosphorylation, providing adenosine triphosphate (ATP) and its equivalents as a universal source of metabolic energy (Tzagoloff, 1982). While their primary role is oxidative phosphorylation, mitochondria also contribute to ionic homeostasis, thermoregulation, and to the biosynthesis of amino acids, nucleotides, pyrimidines, phospholipids, cholesterol, folate coenzymes, haem, urea, and many other metabolites (Attardi and Schatz, 1988; Bereiter-Hahn, 1990). In metabolically active tissues, mitochondria are the most abundant organelles, representing 10-20% of all intracellular protein (Glover and Lindsay, 1992).

It is well documented that cardiac (Duan and Karmazyn, 1989; Hoppel et al., 1982; McMillin-Wood et al., 1980; Palmer et al., 1977; Weinstein et al., 1986) and skeletal (Farrar et al., 1981, 1982; Federico et al., 1987; Joffe et al., 1981, 1983; Martin, 1987) muscle tissue is comprised of two distinct subpopulations of mitochondria: subsarcolemmal (SS), located directly beneath the sarcolemma, and intermyofibrillar (IMF), found dispersed between the

myofibrils. While it remains to be proven, it has been proposed that the two mitochondrial subpopulations have functionally distinct roles in muscle (Kreiger et al., 1980). Subsarcolemmal mitochondria are suggested to supply the energy needs for the following processes: the active transport of ions (Sjodin and Beaugé, 1973) and metabolites (Müller, 1976) across the sarcolemma; the phosphorylation of substrates and sarcolemmal proteins (Korbl et al., 1977; Walaas et al., 1977); and, protein synthesis in the nuclei (Morton and Rowe, 1974). In contrast, IMF mitochondria are suggested to maintain the contraction-relaxation processes (Palade, 1956) and aid in the regulation of the concentration of the sarcoplasmic Ca^{2+} during the contraction-relaxation cycle (Carafoli, 1975).

Mitochondria are highly dynamic structures whose number, composition, and morphology can be altered in response to various physiological and pathological stresses imposed on the organelle. Sufficient morphological and biochemical evidence exists which demonstrates that the two mitochondrial subpopulations respond distinctly to various perturbations. Subsarcolemmal mitochondria from skeletal muscle adapt more readily and to a greater extent to chronic use (Eisenberg and Salmons, 1981; Farrar et al., 1981, 1982; Hoppeler et al., 1973, 1985; Howald et al., 1985; Kreiger et al., 1980; Martin, 1987). Conversely, SS mitochondria experience greater reductions in content and biochemical

properties with muscle disuse compared to the IMF mitochondria (Bell et al., 1992; Desplanches et al., 1990; Kreiger et al., 1980; Taillandier et al., 1993). The fact that the two mitochondrial subpopulations display distinct adaptability supports the hypothesis that they have functionally different roles in skeletal muscle. It has been speculated that the underlying principle for functionally discrete mitochondrial subpopulations may be differing capacities for protein synthesis (Hood, 1992).

Protein turnover can be regarded as a substrate cycle between protein and the respective amino acid pool, whereby under steady state conditions, the rate of protein synthesis equals the rate of protein degradation. The greatest sensitivity in metabolic regulation is achieved when proteins exhibit high turnover rates. The concentration of such proteins can therefore be rapidly altered by modifications in either the rate of synthesis or degradation (Newsholme & Leech, 1983, p395, 673). Thus, the observed greater sensitivity of SS mitochondria to adapt in response to various physiological and pathological stresses imposed on the organelle may be a result of higher rates of protein turnover in SS than in IMF mitochondria. Under steady state conditions, this may be viewed as a greater rate of protein synthesis which corresponds to the weighted averages of the turnover rates of individual proteins.

At the present time, relatively little information is available on the regulation of protein synthesis within the two mitochondrial subpopulations. Mayer et al. (1981) demonstrated a 1.5 fold greater rate of amino acid incorporation into SS than into IMF mitochondria in cardiac tissue. Conversely, in skeletal muscle, Cogswell et al. (1993) reported a 1.8 fold higher rate of amino acid incorporation into IMF than into SS mitochondria. The discrepancy between results may be a result of different tissue types and/or methodology. The *in vitro* method used by Cogswell et al. (1993) estimates protein synthesis attributed to the mitochondrial genome whereas the *in vivo* technique used by Mayer et al. (1981) measures the combined involvement of the nuclear and mitochondrial genomes.

Mitochondrial protein synthesis requires the concerted effort of two genetic systems: the nuclear-cytoplasmic and the mitochondrial systems. The majority of the proteins present in the mitochondrion are encoded by cellular nuclear genes, translated on cytoplasmic ribosomes and subsequently imported from the cytoplasm into the organelle for assembly. However, 5% of all mitochondrial proteins are encoded by mitochondrial DNA and translated on mitochondrial ribosomes (Anderson et al., 1981). Hence, to obtain a complete representation of the capacities for protein synthesis in SS and IMF mitochondrial subpopulations, both genetic systems must be studied.

This dissertation explores both genetic systems in verifying the hypothesis that SS and IMF mitochondrial subpopulations exhibit differing rates of protein synthesis. Furthermore, an overview of the proposed biological functions of the two mitochondrial subpopulations and their morphological and biochemical differences, and some of the methodologies used to examine protein synthesis are presented.

B. MORPHOLOGICAL PROPERTIES OF MAMMALIAN SKELETAL MUSCLE MITOCHONDRIA

Striking variations are reported in the number, size, shape, and intracellular localization of skeletal muscle mitochondria. Skeletal muscle mitochondria have generally been depicted as discrete spherical or ovoid organelles, resulting from the examination of isolated mitochondria which form spherical vesicles in vitro (Tzagoloff, 1982). They are presently considered by researchers to be somewhat complex in configuration (Bakeeva et al., 1978, 1981; Kayar et al., 1988; Kirkwood et al., 1986, 1987; Ogata and Yamasaki, 1985).

Analyses of skeletal muscle mitochondria utilizing serial tissue section reconstruction (Bakeeva et al., 1978, 1981; Kayar et al., 1988), high-voltage electron microscopy (Kirkwood et al., 1986, 1987) or scanning electron microscopy (Ogata and Yamasaki, 1985) display a rather

complex configuration. While Ogata and Yamasaki (1985) support the notion of two distinct mitochondrial subpopulations, the work of Bakeeva et al. (1978, 1981) and Kirkwood et al. (1986, 1987) suggest that mitochondria are interlinked into a single intricate reticular structure. Kayar et al. (1988) proposed that a combination of both situations may exist. Three-dimensional computer reconstructions using electron micrographs of serial sections obtained from equine skeletal muscle depicted the appearance of oval structures in addition to mitochondria resembling a network of interconnected cylindrical segments. In particular, it was found that the degree of connectivity appeared to be associated with mitochondrial volume density, and the distinction between mitochondrial subpopulations became less apparent in fibres with an elevated mitochondrial volume density (Kayar et al., 1988).

Recent attention has been focused on the intrafibre distribution of succinate dehydrogenase (SDH) activity using quantitative histochemical analysis as an indirect measure of mitochondrial distribution in skeletal muscle fibres. Radial gradients in SDH activity from the sarcolemma to the fibre centre are described as linear, a 5th order polynomial, and a 3rd order polynomial in muscle fibres from the cat tibialis anterior (Martin and Edgerton, 1992), rat soleus (Bell et al., 1992), and the human vastus lateralis (Socha et al., 1993), respectively. While all three studies

reported greater SDH activity in the SS region of skeletal muscle fibres, the differing patterns of distribution between the studies suggest muscle and/or species specificity. These reports corroborate with earlier studies by Swatland (1984a, 1984b, 1985). Based on the aforementioned ultrastructural and quantitative histochemical studies, it is likely that the complex organization of mitochondria within a muscle cell is optimal for supplying oxygen and substrates to mitochondria and for moving high energy phosphates such as adenosine triphosphate (ATP) from mitochondria for use by the myofibrils (Kayar and Banchero, 1987).

C. BIOCHEMICAL PROPERTIES OF SS AND IMF MITOCHONDRIAL SUBPOPULATIONS

Tissue homogenization and subsequent purification by differential centrifugation with the use of nagarse, a bacterial protease, enables distinct separation of the SS and IMF mitochondria. Mild fragmentation using a polytron homogenizer preferentially releases SS mitochondria, while protease digestion of the myofibrils in the residual pellet using nagarse releases intermyofibrillar mitochondria (Palmer et al., 1977). While it has been demonstrated that nagarse inactivates acyl-coenzyme A synthetase, a long chain fatty acid activating enzyme, as observed by the inability of the IMF mitochondria to oxidize free palmitic acid

(Pande and Blanchaer, 1971), nagarse exposure does not appear to damage the IMF mitochondria in any other manner.

A number of biochemical studies have demonstrated that isolated SS and IMF mitochondria possess enzymatic and respiratory properties which distinguish them from one another. In cardiac muscle, the IMF mitochondrial rates of oxidative metabolism of various lipid and non-lipid substrates entering the electron transport chain at Complex I (NADH-coenzyme Q reductase), II (succinate-coenzyme Q reductase), or III (Coenzyme Q-cytochrome c reductase) are reported to be 1.4-1.9 times higher than those of SS mitochondria (Duan and Karmazyn, 1989; Mayer et al., 1981; McMillin-Wood et al., 1980; Palmer et al., 1977, 1985). However, these differences are not shown to persist when reducing equivalents enter the mitochondrial electron transport chain at Complex IV (cytochrome oxidase) (Palmer et al., 1985). Also, relative specific activities of both citrate synthase (McMillin-Wood et al., 1980; Palmer et al., 1977) and succinate dehydrogenase (Mayer et al., 1981; Palmer et al., 1977) are shown to be 1.5 times greater in IMF than in SS mitochondria. It has been argued that the oxidative differences reported in the SS and IMF mitochondria are an artifact generated by the experimental procedures used during mitochondrial isolation (Matlib et al., 1981). However, care has been taken to establish that the apparent differences observed between the two

mitochondrial subpopulations are not a result of experimental manipulation but represent actual biochemical differences (Bell et al., 1992; Palmer et al., 1985). Additionally, the variation observed in the reported magnitude of the biochemical differences between the two mitochondrial subpopulations may represent differences in the amount of cross-contamination of these mitochondrial types between studies.

Numerous studies report comparable conclusions in skeletal muscle mitochondria using slightly modified versions of the isolation method of Palmer et al. (1977) (Farrar et al., 1981,1982; Joffe et al., 1981,1983; Kreiger et al., 1980; Martin, 1987). Intermyofibrillar mitochondrial rates of oxidative metabolism using substrates entering the electron transport chain at Complex I are demonstrated to be 1.6-2.6 fold greater than for SS mitochondria (Farrar et al., 1981, 1982; Joffe et al., 1983; Kreiger et al., 1980; Martin, 1987). Similarly, reducing equivalents entering the electron transport chain at Complex IV resulted in a 1.6 fold higher rate of oxidative metabolism in IMF than SS mitochondria (Martin, 1987). This difference was not observed in cardiac tissue (Palmer et al., 1985). Relative specific activities of succinate dehydrogenase are shown to be 1.7 times greater in IMF than in SS mitochondria (Kreiger et al., 1980). The IMF mitochondria also display a greater rate and capacity for

Ca^{+} uptake than the SS fraction (Joffe et al., 1981). The aforementioned data provide support for the hypothesis that two biochemically distinct classes of mitochondria exist in both skeletal and cardiac muscle.

D. METHODOLOGY

The investigation of mitochondrial protein synthesis requires the use of *in vivo* and *in vitro* experimentation. As previously stated, the *in vitro* method estimates protein synthesis attributed to the mitochondrial genome while the *in vivo* technique measures the combined involvement of the nuclear-cytoplasmic and mitochondrial genomes. The methods currently used for studying protein synthesis *in vivo* and *in vitro* rely on the use of isotopically-labelled amino acids (Hasselgren et al., 1988). The administration of an isotopically-labelled amino acid by injection into a whole animal or into an incubation medium results in its rapid distribution into the free amino acid pools, from which it can be incorporated into protein. The precursor-product relationship of amino acid incorporation into protein is the basis for the measurement of protein synthesis rates (Barnes et al., 1992). Assuming that the specific activity (SA) of the amino acid pool measured represents the SA of the immediate precursor pool and that the SA remains constant over the time of incorporation, protein synthesis can be calculated using the following equation:

$$\text{Protein synthesis} = \frac{\text{protein-bound SA}}{\text{free amino acid SA} \times \text{time}}$$

where time represents the time of incorporation (Garlick et al., 1980; McNurlan et al., 1979).

The two predominant methods of choice for determining protein synthesis *in vivo* are the 'constant infusion' and 'flooding dose' techniques. See Garlick et al. (1994) for a detailed critical analysis of these two contrasting methods. The constant tracer infusion method consists of the intravenous administration of a trace amount of isotope at a constant rate and amount so that the plasma amino acid pool attains isotopic equilibrium. This technique is often preceded by a priming bolus injection to reduce the length of time needed to reach the equilibrium phase. Difficulty in identifying the true precursor pool for the calculation of protein synthesis has been a major criticism of the constant tracer infusion method (Waterlow et al., 1978).

The immediate precursor for protein synthesis is the aminoacyl-tRNA pool, which is seldom used for measurement due to its extremely small concentration in muscle, its short half-life, and the associated difficulty in its isolation (Garlick et al., 1994; Zak et al., 1979). Therefore, measures of intracellular and extracellular amino acid SA are more commonly used. However, the administration of trace amounts of isotope produces an aminoacyl-tRNA pool SA that lies between those of the intracellular and extracellular amino acid pools (Airhart et al., 1974;

Khairallah and Mortimor, 1976; McKee et al., 1978). In an effort to overcome this discrepancy in SA between amino acid pools, Garlick et al. (1980) and McNurlan et al. (1979) adopted and subsequently modified the flooding dose technique used by Henshaw et al (1971), first introduced by Lotfield and colleagues in the 1950's (Keller et al., 1954; Lotfield and Eigner, 1958).

The flooding dose technique involves the rapid administration of a large bolus of unlabelled amino acid, equivalent to a several fold increase in the endogenous free amino acid pool, along with the isotopically labelled amino acid. In theory, this procedure increases the intracellular and extracellular amino acid pools and equilibrates the SA between the tRNA, intracellular and extracellular amino acid pools. This theory was based on the premise that amino acids acylated to tRNA are derived solely from the free amino acid pools and not directly from intracellular protein degradation - the suggested source for cultured cells (Barnes et al., 1992; Sugden & Fuller, 1991). The evidence from studies using isolated perfused liver (Khairallah & Mortimore, 1976), heart (McKee et al., 1978), and muscle (Bylund-Fellenius et al., 1984) suggests that flooding in all three pools can be achieved with all three amino acid pools having similar SA. Additionally, plasma and tissue SA remain almost constant throughout the period of incorporation up to a duration of 30 min, thus facilitating

the measurement of its time course (Garlick et al., 1980; Reeds et al., 1982; Taillandier et al., 1993). The short labelling period minimizes the recycling of tracer in rapidly turning over proteins, and any dilution effect of nonradioactive amino acid derived from proteolysis on the intracellular SA is negligible as a result of the highly elevated concentration of unlabelled amino acid.

The validity of the method is also contingent upon the bolus injection of a large concentration of amino acid not influencing the rate of protein synthesis. For this reason, phenylalanine (Phe) is frequently used as there is little indication that it does alter protein synthesis (Garlick et al., 1994). Also, Phe is relatively soluble enabling the administration of a large dose in order to flood the free Phe pool which is small and easily flooded. An additional assumption made by the flooding dose technique is that of linear incorporation of label into protein. Linearity has been demonstrated in muscle, liver, gut, and whole body using animal experiments (Attaix et al., 1988; McNurlan et al., 1979; Obled et al., 1989).

There are several advantages to the flooding dose technique some of which have been alluded to earlier. One practical advantage of the method is the short duration in which the rate of protein synthesis is measured enabling the examination of more acute changes in tissue protein synthesis (Garlick et al., 1994). In contrast, the short

duration of incorporation is also the main drawback of this method. The measured rate of protein synthesis may not be representative of the daily overall rate of protein synthesis resulting from preferential weighting in favour of proteins with high turnover rates.

The flooding dose technique for *in vitro* estimations of protein synthesis rates share the same theoretical base as for *in vivo* experimentation. The addition of a high concentration of the precursor to the incubation medium results in rapid equilibration of SA in the different amino acid pools. The amount of amino acid incorporated is therefore calculated using the extracellular SA. Additional advantages to *in vitro* experimentation include ease with which conditions can be standardized, the ability of maintaining strict control of substrate and hormone concentrations, and the elimination of complicating interactions with other tissues (Hasselgren et al., 1988). However, one concern with *in vitro* preparations is that they tend to be in a net catabolic state resulting in lower rates of protein synthesis than those estimated *in vivo* (Waterlow, 1984).

E. OBJECTIVES

The aim of this dissertation was to estimate the rate of incorporation of radioactive label into mitochondrial proteins in two distinct subpopulations of skeletal muscle mitochondria (SS and IMF) 1) *in vitro* and 2) *in vivo* using the flooding dose technique in order to establish whether SS and IMF mitochondria possess different rates of protein synthesis.

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CHAPTER 2*

MEASUREMENT OF IN VITRO AND IN VIVO AMINO ACID INCORPORATION IN TWO SUBPOPULATIONS OF SKELETAL MUSCLE MITOCHONDRIA

A. INTRODUCTION

Skeletal muscle mitochondria undergo significant modifications induced by numerous physiological and pathological stresses (Hoppeler and Desplanches, 1992; Lomax and Robertson, 1992; Takahashi and Hood, 1993). For instance, a characteristic metabolic response to endurance training is an increase in mitochondrial content and related enzyme activities (Holloszy and Coyle, 1984). There is growing evidence that these adaptations occur differentially in the two subpopulations of skeletal muscle mitochondria: subsarcolemmal (SS), located beneath the sarcolemma, and intermyofibrillar (IMF), found dispersed between the myofibrils (Farrar et al., 1981; Joffe et al., 1983; Kreiger et al., 1980; Martin, 1987). Many of these alterations could possibly be related to, or accompanied by, distinct capacities for mitochondrial protein synthesis. Although differences in morphological properties and biochemical characteristics between SS and IMF subpopulations of skeletal muscle mitochondria have been clearly defined (Farrar et al., 1981; Kreiger et al., 1980; Ogata and

*In vitro pilot work was presented at the 1992 Canadian Association of Sport Sciences conference in Saskatoon, Saskatchewan, and was awarded the Canadian Association of Sport Sciences Graduate Student Award.

Yamasaki, 1985), only one study has sought to define their abilities to synthesize protein (Cogswell et al., 1993).

The biogenesis of mitochondria requires the complex cooperation between the nucleo-cytoplasmic protein synthesizing machinery, and a second genetic system which is located within the mitochondria. Mitochondrial DNA (mtDNA) encode 13 structural proteins which are subunits of respiratory chain complexes, in addition to the 22 transfer RNA and 2 ribosomal RNA molecules required for intra-mitochondrial protein synthesis (Anderson et al., 1981). The 13 polypeptides are translated within the organelle on ribosomes which are bound to the inner surface of the inner membrane and represent approximately 5% of all mitochondrial proteins. The vast majority of mitochondrial proteins are encoded by nuclear DNA, synthesized in the cytoplasm and imported into the organelle for assembly. Thus, it is essential that both genetic systems be studied in order to fully assess the capacities of SS and IMF mitochondria for protein synthesis. The research by Cogswell et al. (1993) utilized a modified technique of Mockel and Beattie (1975) to estimate *in vitro* amino acid incorporation by isolated SS and IMF skeletal muscle mitochondrial preparations. While the results indicated an 80% greater rate of protein synthesis in IMF than in SS mitochondria, it is important to emphasize that the technique fails to examine a major portion of mitochondrial proteins, that of nuclear origin.

At the present time, *in vivo* protein synthesis research on two subpopulations of mitochondria has only been performed using cardiac tissue (Mayer et al., 1981; Siddiq et al., 1993). Both studies reported a 1.5 fold greater rate of amino acid incorporation in SS than in IMF mitochondria which is in direct contrast to the results of Cogswell et al. (1993). The discrepancy between results may be a result of different tissue types, genetic origins, and/or methodology. Therefore, this study investigated the estimation of *in vitro* and *in vivo* rates of protein synthesis in two subpopulations of skeletal muscle mitochondria.

B. METHODS

Experimental animals

Studies were carried out in accordance with the guidelines of the Canadian Council on Animal Care. Twenty-five female Sprague-Dawley rats (University of Alberta) weighing 188-229 g (202 ± 2.1g) were utilised for the various studies. The animals were maintained on a 12 h light/dark cycle (lights on 0800 h), and were given free access to water and food until time of experimentation. The diet consisted of 20% protein (Samuels and Baracos, 1992). Animals were weighed and killed by cervical dislocation following brief carbon dioxide anaesthesia.

Mitochondrial isolation procedure

Isolation of SS and IMF mitochondria was achieved through differential centrifugation utilizing a slight modification of the methodology outlined by Kreiger et al. (1980). Refer to Appendix B for a detailed description of the isolation procedure. Briefly, this procedure used PT-20 Polytron homogenization (SS mitochondria) and proteolytic enzyme (Nagarse) digestion (IMF mitochondria) of the myofibrils to separate the two subpopulations of mitochondria. All procedures were performed on ice. Mitochondrial protein content was estimated by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as the standard. Mitochondrial purity of the SS and IMF mitochondria was assessed in part by measuring F_1-adenosinetriphosphatase (ATPase) activity in the presence and absence of 1 μg oligomycin (Kreiger et al., 1980).

Respiratory measurements

Mitochondrial respiration was measured polarographically as described by Davies et al., (1981) with a Clark oxygen electrode (Yellow Springs Instrument, OH). Briefly, 0.2 mg of mitochondrial protein suspended in Buffer II was added to the O_2 electrode buffer consisting of 15 mM KCl, 0.4 mM NAD^+, 45 mM sucrose, 12 mM mannitol, 5 mM $MgCl_2$, 7 mM EDTA, 0.2% BSA, 20 mM glucose, 30 mM KH_2PO_4, and 25 mM Tris, (pH 7.4). Substrates added to the medium included

10 mM pyruvate and 2.5 mM malate, and oxidation was initiated by the addition of ADP. The final assay volume was 1.5 mL. Following depletion of endogenous mitochondrial substrates, state 3 respiration at 30°C was initiated by the addition of 200 nmol ADP (Sigma).

Respiratory control ratios (RCR) were quantified from polarographic tracings by standard procedures according to Chance and Williams (1955), and Estabrook (1967). Briefly, RCR, an index of intactness and coupling between electron transport and oxidative phosphorylation, was calculated as the state III rate [respiration rate in the presence of added substrate and phosphate acceptor (ADP)] divided by the state IV rate (rate after consumption of ADP).

Measurement of in vitro protein synthesis

The rate of mitochondrial protein synthesis in skeletal muscle was estimated by the incorporation of L-[^{35}S]methionine into trichloroacetic acid-precipitable protein using a modification of the procedures of McKee et al. (1990a,b). Refer to Appendix C for a detailed protocol. Solutions used for mitochondrial isolation and *in vitro* protein synthesis were made with glass-distilled water and filtered through 0.2 μm filters before use. Mitochondrial fractions were incubated in open 25 mL polycarbonate test tubes at 30 °C in a shaking water bath. The incubation medium (pH 7.4) contained 90 mM KCl, 4 mM $MgSO_4$, 2.5 mM

KH_2PO_4, 25 mM MOPS, 20 μM methionine, 0.1 mM amino acids, 20 mM pyruvate, 0.5 mM malate, 2 mM ADP, 1.0 $mg \cdot mL^{-1}$ BSA and 0.1 $mg \cdot mL^{-1}$ cycloheximide. The mitochondrial fractions were incubated at a protein concentration of 4 $mg \cdot mL^{-1}$ with approximately 0.22 $mCi \cdot mL^{-1}$ L-[^{35}S]methionine (1000 $Ci \cdot mmol^{-1}$, ICN Biomedicals). At various times, 10 μL aliquots were removed in triplicate from each incubation medium and incorporation of L-[^{35}S]methionine was measured by the filter disc method of Mans and Novelli (1961) as modified by McKee et al. (1984). It has been reported that less than 2% of the protein originally present is lost during the steps of the aforementioned protocol using proteins of known specific radioactivity (McKee et al., 1984). Samples were counted for 20 min or until 40 000 counts were accumulated using a Beckman liquid scintillation spectrophotometer. The time zero incorporation of label [disintegration $\cdot min^{-1}$ (dpm)] was subtracted from the rest of the results, subsequently divided by the specific radioactivity, and expressed as pmol methionine $\cdot 30\ min^{-1} \cdot mg\ protein^{-1}$.

Measurement of in vivo protein synthesis

Fractional rates of protein synthesis were estimated using the flooding dose technique of Garlick et al. (1980). Refer to Appendix C for a detailed description of the experimental protocol. On separate experimental days, each rat received an intraperitoneal injection of 150 μmoles of

phenylalanine (Phe) per 100 g body weight, containing 150 μCi L-[2,6-^{3}H]Phe (Amersham, U.S.A.) per 100 g body weight in 2.5 mL of sterile phosphate buffered saline. After precisely 20 min, the rats were euthanized by cervical dislocation and immersed in ice-cold water for one minute during which time the skin of both hindlimbs was immediately removed. Subsequently, the gastrocnemius of one hindlimb was quickly removed and frozen in liquid nitrogen. The elapsed time from cervical dislocation to muscle freezing was approximately 2.5 min. The muscle was stored at -70°C until analysed. In addition, the entire contralateral hindlimb was rapidly excised and placed in ice-cold mitochondrial isolation buffer (Buffer I). The elapsed time from cervical dislocation to hindlimb removal was approximately 2.25 min. Muscles were subsequently removed in preparation for the isolation of SS and IMF mitochondria.

Analyses

The specific radioactivity of free (S_A) and mitochondrial protein bound (S_B) Phe was measured following conversion to ß-phenylethylamine as described in detail by Baracos et al. (1991). A 150 mg sample of the gastrocnemius was homogenized on ice with approximately 2 mL ice-cold 2% perchloric acid ($HClO_4$) using a motorised ground glass tissue homogenizer and subsequently centrifuged in preparation for the determination of free specific

radioactivity. The mitochondrial fractions were washed twice in 4 ice-cold 2% $HClO_4$, centrifuged and hydrolysed in 6 N HCl in preparation for the measurement of protein bound specific radioactivity. All analyses were performed at least in duplicate.

The fractional rate of protein synthesis (k_{syn}) expressed as % synthesized per day was calculated from the following equation (Garlick et al., 1983):
$k_{syn} = S_B \div (S_A \times t) \times 100$ where t is the time of incorporation in days.

Statistical analyses

All data are presented as mean ± SE. Differences between two means were assessed using the Student's independent *t* test. Variability of data is expressed as the coefficient of variability (S.D. ÷ X). In vitro rates of protein synthesis were analysed using multivariate analysis of variance (MANOVA) with planned polynomial comparisons (SPSSx). Significance was established at $p<0.05$ for all comparisons.

C. RESULTS

Mitochondrial preparations

The isolation of SS and IMF mitochondrial fractions produced protein yields which were not significantly different. The RCR is a critical index of mitochondrial

function and intactness; higher values generally indicate a better quality preparation. The RCRs from the present study indicate that our mitochondrial preparations have coupled electron transport and oxidative phosphorylation, and have functionally intact inner membranes (Table 2-1). While RCRs provide evidence for the functional integrity of each subpopulation of mitochondria, they do not establish their purity.

Mitochondrial purity was assessed by establishing the degree of contamination with non-mitochondrial components (i.e., myofibrils, sarcolemma and sarcoplasmic reticulum) by measuring ATPase activity in the presence and absence of oligomycin. Both fractions demonstrated minor contamination as evidenced by a 4.33 ± 0.17 and 3.08 ± 0.08 % oligomycin-insensitive ATPase activity in the SS and IMF mitochondrial subpopulations, respectively.

The IMF mitochondria exhibited higher state III respiratory rates than did the SS mitochondria (Table 2-1) which is in accordance with previous findings (Farrar et al., 1981, 1982; Joffe et al., 1983; Kreiger et al., 1980; Martin, 1987; Cogswell et al., 1993) and provides biochemical support for the attainment of distinct mitochondrial fractions through sequential isolation.

In vitro mitochondrial protein synthesis

Figure 2-1 illustrates that the kinetics of methionine incorporation into SS and IMF mitochondrial protein was relatively linear during 30 min of incubation. Additional data presented in chapter 3 also show linear incorporation between 15 and 60 min with correlation coefficients of $r=0.999$ and $r=0.998$ for SS ($n=3$) and IMF ($n=3$) mitochondrial fractions, respectively (Figure 3-1). Although the correlation coefficients in Figure 2-1 are similar to those observed previously (Cogswell et al., 1993) for skeletal muscle mitochondria, the statistical analysis revealed a significant quadratic function in addition to a significant linear function ($p<0.05$). The linear function was greater in the IMF mitochondria ($p<0.05$), yet there was no significant difference between the two mitochondrial subpopulations with respect to the quadratic function. Both mitochondrial subpopulations exhibited a decreased rate of incorporation at approximately 15 min. These data corroborate with those previously obtained with skeletal muscle (Rinehart et al., 1982) and liver mitochondria (Coleman and Cunningham, 1990; Marcus et al., 1982). Absolute rates were 1.81 ± 0.29 and 3.84 ± 0.41 pmol methionine·30 min^{-1}·mg $protein^{-1}$ in SS and IMF mitochondria, respectively, which indicate a 2.1 fold greater rate of L-[^{35}S]methionine incorporation in IMF mitochondria.

Background incorporation was determined from samples removed in triplicate at time zero. The washing procedure resulted in the removal of 99.94 ± 0.007% (n=10) of the added label, which agrees with the results reported by Bollum (1959). The residual label represents a background incorporation of approximately 2900 disintegrations·min^{-1}. The magnitude of labelling at time zero varied directly with the amount of radioisotope added (r=0.977). It is believed that the 10-15 s time lag between the addition of the label and removal of the time zero sample was not sufficient to account for a significant amount of the radioactivity observed in the time zero sample. Therefore, the time zero sample was interpreted as a measure of the extent of contamination of the washed sample with unincorporated labelled amino acid. Data were routinely corrected by subtracting the counts detected in the time zero sample. Background incorporation was additionally monitored over time by measuring the amount of incorporation observed in samples lacking only the addition of mitochondria. The increase in disintegrations·min^{-1} over time represented a linear (r=0.999) increase of approximately 0.015 pmol methionine·30 min ($p>0.05$) (data presented in Chapter 3). This increase may be a result of the washing procedure used for the filter discs, or less likely, due to bacterial contamination.

In vivo mitochondrial protein synthesis

In contrast to the *in vitro* rates of protein synthesis, the *in vivo* fractional rate of protein synthesis in the SS mitochondrial fraction was two fold greater than in the IMF mitochondrial fraction ($p<.05$). The fractional rates of protein synthesis for SS and IMF mitochondria were $8.0 \pm 0.4\% \cdot day^{-1}$ and $4.0 \pm 0.2\% \cdot day^{-1}$, respectively.

D. DISCUSSION

The aim of the present study was to estimate the synthesis rate of mitochondrial proteins in two subpopulations (SS and IMF) of skeletal muscle mitochondria using both *in vitro* and *in vivo* methodology in order to encompass both nuclear and mitochondrial genomes. The study of mitochondrial biogenesis requires that the methods be reliable and valid. One requirement for satisfying the criteria for reliability and validity is that the isolation procedure yield relatively pure, highly intact and distinct mitochondrial fractions.

Several methods have been reported for the isolation of mitochondria. Compared to soft tissue such as the heart and liver, skeletal muscle is more rigid due to a larger quantity of collagen. The greater shearing force required to homogenize skeletal muscle mitochondria is achieved by a brief exposure of the minced tissue to the Polytron, which disrupts the muscle membrane releasing mitochondria that are

predominantly of subsarcolemmal origin. Intermyofibrillar mitochondria are released from the myofibrils with the assistance of a relaxing buffer by brief exposure to the proteolytic enzyme Nagarse. Protein yields for SS and IMF skeletal muscle mitochondrial fractions reported in the literature range between 0.76-2.0 mg·g wet wt^{-1} and 1.92-2.74 mg·g wet wt^{-1}, respectively (Cogswell et al., 1993; Farrar et al., 1981,1982; Kreiger et al., 1980; Martin, 1987). The SS protein yield in this experiment falls within the above range; however the IMF protein yield is somewhat lower. This may be due to the extra care taken to minimize contamination of the IMF fraction at the expense of reducing the protein yield (appendix A). In support of this explanation, the percent contamination in the IMF fraction as measured by the ATPase activity not inhibited by oligomycin is lower than the 5-8% contamination reported by others (Cogswell et al., 1993; Kreiger et al., 1980).

The assessment of purity is very important since it is well documented that fractionation procedures yield a reduced amount of mitochondria originally present in the intact muscle. This form may be contaminated to varying degrees with other cellular components (De Duve, 1967). The degree of contamination in both SS and IMF mitochondrial fractions in the present study were lower than the 5-6% and 5-8% contamination, respectively, reported in the studies of Cogswell et al. (1993) and Kreiger et al. (1980). These

results along with RCR values of 7.5-8.6 and state III respiration rates that are 1.5 fold greater in IMF than in SS mitochondrial fractions confirm the capability of the isolation procedure to produce highly purified, intact and distinct SS and IMF mitochondrial fractions.

In addition to mitochondrial purity and integrity, precautions should be taken to prevent bacterial growth during *in vitro* experimentation by ensuring sterile incubations. Bacteria are unicellular prokaryotic microorganisms that are not unlike the mitochondrion (Yang et al., 1985). Bacterial protein synthesis is not inhibited by cycloheximide, an inhibitor of cytoplasmic translation activities, and can lead to inflated rates of amino acid incorporation. An insignificant amount of amino acid was incorporated over a duration of 60 minutes in incubated samples lacking only mitochondria (Chapter 3). This increase may be a result of the washing procedure rather than bacterial contamination, although it is not possible to differentiate between the two.

The major finding of the present study is that SS and IMF mitochondria demonstrate independent capacities for protein synthesis irrespective of genomic origin. It is important to emphasize that the *in vitro* method utilised in the present study estimates protein synthesis attributed to the mitochondrial genome whereas the *in vivo* technique measures the combined involvement of the nuclear and

mitochondrial genomes, although the proteins synthesized are predominantly (≈95%) of nuclear origin. *In vitro* experimentation has previously revealed a greater rate of isotopically labelled leucine incorporation into skeletal muscle IMF compared to SS mitochondrial proteins (Cogswell et al., 1993). Although difficult to compare due to the use of different radioactive labels, the protein synthesis rates in the present investigation are approximately 2 fold greater in both IMF and SS mitochondrial fractions than those recently reported by Cogswell et al. (1993). However, the magnitude of difference between the two mitochondrial subpopulations are similar in both studies. One possible explanation for the lower rates of protein synthesis observed in the study of Cogswell et al. (1993) is that although the kinetics of amino acid incorporation into SS and IMF mitochondrial protein were linear during 90 minutes of incubation, they did not intercept at time 0, but at approximately 15 minutes. Rates of amino acid incorporation reported by others for a single population of mitochondria isolated from rat skeletal muscle (Rinehart et al., 1982) and human skeletal muscle (Marzuki et al., 1988) are 8 and 12 pmol methionine·mg protein^{-1}·hr^{-1}, respectively, which are in agreement with the IMF results obtained from the present study. Isolation techniques resulting in a single mitochondrial fraction is comprised predominantly of IMF mitochondria since SS mitochondria represent only 14-30% of

total mitochondrial volume (Puntschart et al., 1995). In addition, SS mitochondria are likely to be damaged following the lengthy duration typically used for tissue homogenization (Ernster and Nordenbrand, 1967; McKee et al., 1990).

The question that arises is whether the differences in rates of amino acid incorporation between SS and IMF mitochondria are a result of the varying activities of mitochondrial ATPase (Table 2-1). Although this possible phenomenon was not investigated in the present study, it has been previously documented that differences in rates of protein synthesis between the two mitochondrial subpopulations are retained in the presence of an exogenous ATP-regenerating system (Cogswell et al., 1993).

At the present time, experimentation on protein synthesis in two subpopulations of mitochondria encompassing both genetic systems has been performed solely on cardiac tissue (Mayer et al., 1981; Siddiq et al., 1993). Both studies revealed a 1.5 fold greater rate of protein synthesis in the SS than in the IMF mitochondria which is similar to the 2 fold greater fractional rate of protein synthesis in the SS compared to the IMF mitochondria observed in the present study. The fractional rates of protein synthesis (SS:8%·day^{-1}; IMF:4%·day^{-1}) in this experiment agree with the 4.5-6.0 %·day^{-1} fractional synthesis rates of cytochrome c in red quadriceps muscle

reported by Booth (1991), and Morrison et al. (1987).

The vast majority of the proteins present in the mitochondrion are encoded by nuclear DNA, and are subsequently imported into the organelle for assembly. However, mitochondria contain their own unique protein synthesizing system which encodes 13 proteins representing 5-10% of all mitochondrial proteins. These proteins are subunits of enzyme complexes of the inner membrane that are associated with respiration and oxidative phosphorylation: subunits I, II, and III of cytochrome oxidase; apocytochrome b of the bc_1 complex; subunits 6 and 8 of the F_oF_1-ATPase; subunits 1-6 and 4L of the NADH dehydrogenase (Attardi and Schatz, 1988). This may partially explain the higher *in vitro* rate of amino acid incorporation observed in the IMF mitochondrial fraction, since they are characterised as having a 1.5-2.0 greater rate of respiration than the SS mitochondrial fraction (Farrar et al., 1981,1982; Joffe et al., 1983; Kreiger et al., 1980), which is also confirmed in the present study.

The intent of this study was to use protein synthesis as an indirect marker of protein turnover, as valid estimates of protein degradation are difficult to obtain. The major assumption made in doing so is that the rate of protein synthesis equals the rate of protein degradation, which is theoretically correct under steady state conditions. Therefore, the regional disparity in *in vivo*

fractional rates of protein synthesis observed in the present study potentially represents differences in rates of protein turnover, which may in part explain the distinct adaptations observed in skeletal muscle mitochondria in response to increased or decreased muscle use. It is not possible to reach a conclusion as to the functional significance of the differential rates of protein synthesis found in skeletal muscle mitochondria in various regions of the cell, but it may reflect the functional diversity of these two areas as postulated by other researchers. Subsarcolemmal mitochondria are suggested to supply ATP for the active transport of Na^+ and K^+ across the sarcolemma, the phosphorylation of substrates and sarcolemmal proteins, and protein synthesis in the nuclei. In contrast, IMF mitochondria are suggested to maintain the contraction-relaxation processes and aid in the regulation of sarcoplasmic Ca^{2+} concentrations during the contraction-relaxation cycle (Kreiger et al., 1980; Martin, 1987).

Although plausible interpretations for varying rates of protein synthesis have been addressed, the underlying mechanisms producing distinct and contrasting *in vitro* and *in vivo* rates of protein synthesis in two subpopulations of skeletal muscle mitochondria remain to be elucidated. It is generally agreed that mitochondrial gene expression is controlled by nuclear genes, yet little is understood about its regulation (Attardi and Schatz, 1988). It is possible

that the lower rate of *in vitro* protein synthesis in the SS mitochondrial preparation may be rapidly activated during increased mitochondrial biogenesis to support the elevated *in vivo* fractional rate of protein synthesis in this mitochondrial subpopulation. Mitochondrial biogenesis requires the integration of numerous processes. Potential control mechanisms at the level of nuclear gene action and interaction, protein synthesis, transmembrane transport, and protein assembly may partially explain the distinct *in vivo* rates of amino acid incorporation observed in the present study.

E. SUMMARY

In summary, isolation of two biochemically distinct subpopulations of skeletal muscle mitochondria was achieved and shown to have different rates of both *in vivo* and *in vitro* protein synthesis which were similar in magnitude yet diametrically opposed. Further research is required to determine the underlying mechanisms responsible for this observed diversity.

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Table 2-1. Respiratory capacity and enzymatic activity of SS and IMF mitochondria

	SS mitochondria	IMF mitochondria	IMF/SS
mg·g wet wt muscle^{-1}	1.39 ± 0.04 (25)	1.44 ± 0.09 (25)	1.0
State III, natom O·min^{-1}·mg^{-1}	270 ± 8 (18)	414 ± 17 (18)	1.5*
RCR	7.5 ± 0.5 (18)	8.6 ± 0.6 (18)	1.1
ATPase nmol ADP/mg protein·min^{-1}	1 610 ± 150 (4)	3 070 ± 80 (4)	1.9*

Number of mitochondrial isolations are given in parentheses. RCR, respiratory control ratios. NS = not significantly different. * = significantly different ($p<0.05$).

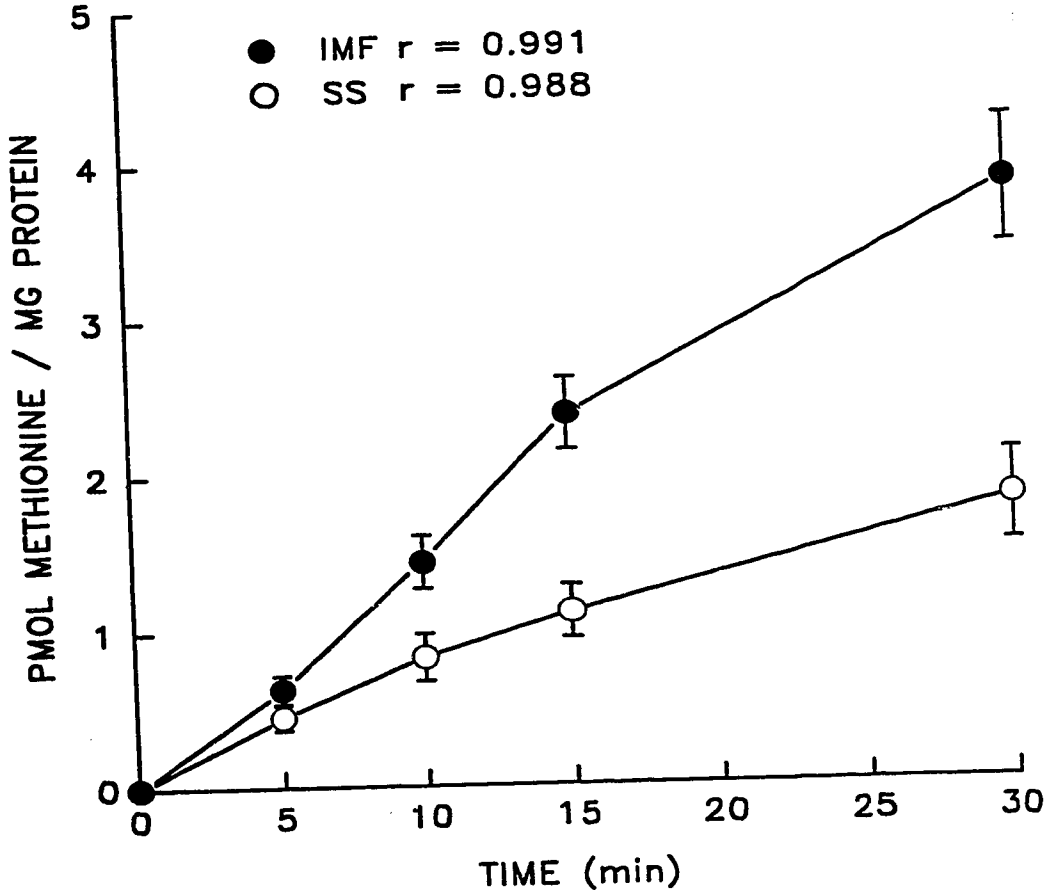

Figure 2-1. *In vitro* time course of L-[^{35}S]methionine incorporation in SS (n=6) and IMF (n=6) mitochondrial fractions

CHAPTER 3

THE STUDY OF AMINO ACID INCORPORATION IN VITRO IN TWO SUBPOPULATIONS OF SKELETAL MUSCLE MITOCHONDRIA: METHODOLOGICAL CONSIDERATIONS

A. INTRODUCTION

Antibiotics are commonly used in studies which examine the role of mitochondrial protein synthesis in the biogenesis of mitochondria (for review see Kroon and De Jong, 1979). Chloramphenicol (CAP) is the most widely used antibiotic in cell biology for the inhibition of mitochondrial protein synthesis. It is commonly used to establish an experimental blank which is subsequently subtracted from amino acid incorporation results (McKee et al., 1990), or as a means of validating the system as a measure of membrane biogenesis by demonstrating up to a 90-95% inhibition of mitochondrial protein synthetic rate (Burke and Rubin, 1979; Marzuki et al., 1988; Rinehart et al., 1982). Additionally, cytoplasmic protein synthesis remains unimpaired. The inhibition results from CAP binding to the 50S ribosomal subunit in a manner similar to aminoacyl-tRNA thus preventing mitochondrial translation activities (Caskey, 1973). While it is commonly and effectively used at high concentrations (4-7 $mg \cdot mL\ medium^{-1}$) in the *in vitro* study of mitochondrial biogenesis (Chow and Rajbhandary, 1989; Huang et al., 1966; McKee et al., 1990),

it is poorly soluble in water (4.4 $mg \cdot mL^{-1}$) (Weiss et al., 1957). Therefore, to obtain such high CAP concentrations as reported in the literature, solubility is attained with the addition of ethanol. This can result in final ethanol concentrations as high as 2-4% in the incubation medium.

With the exception of Chow and Rajbhandary (1989), methods used by researchers to dissolve CAP are often not reported. If ethanol is used as the preferred means of solubilising CAP, it is critical that the same final concentration of ethanol be used in each control and experimental incubation mediums. In the application of mitochondrial inhibitors *in vitro*, it has been recommended that final concentrations of ethanol be kept as low as possible, preferably not exceeding 1-2% in order to minimize the effects of ethanol on mitochondrial protein synthesis (Kroon and De Jong, 1979).

The adverse effects of ethanol on protein synthesis are well documented. *In vivo* studies which examined chronic and acute ethanol consumption have shown to inhibit protein synthesis in rat liver (Preedy et al., 1988), cardiac muscle (Preedy and Peters, 1990) and skeletal muscle (Preedy et al., 1988; Preedy and Peters, 1988a,b). Similarly, several studies have demonstrated inhibition of mitochondrial protein synthesis by acute ethanol administration *in vitro* in hepatic (Burke and Rubin, 1979; Marcus et al., 1982; Rubin et al., 1970) and *in vivo* in cardiac mitochondria

(Siddiq et al., 1993). However, the inhibitory effect of ethanol on two subpopulations of skeletal muscle mitochondria has not been studied. Therefore, the purpose of this preliminary research was to examine the effect of a 1.5% ethanol concentration on *in vitro* protein synthesis in two subpopulations of skeletal muscle mitochondria, in order to determine whether the use of CAP at concentrations greater than 4 mg·mL medium^{-1} is a viable measure of background amino acid incorporation. It was also of interest to examine potential alternatives for measuring background incorporation.

B. METHODS

Experimental animals

Studies were carried out in accordance with the guidelines of the Canadian Council on Animal Care. Female Sprague-Dawley rats (Biosciences Animal Services, University of Alberta) weighing 200-208 g were utilized for the various studies. The animals were maintained on a 12 h light/dark cycle (lights on 0800 h). The rats were given free access to water and food, the diet consisting of 20% protein (Samuels and Baracos, 1992).

Experimental procedures

Detailed procedures for the isolation of two subpopulations of skeletal muscle mitochondria, as well as the methods to measure *in vitro* mitochondrial protein

synthesis, are presented in appendices A and B, respectively. Deviations from the methods described in the appendices are presented in the results section of this study.

Statistical Analyses

A repeated measures analysis of variance (ANOVA) was used to analyse the effect of time on background L-[^{35}S]methionine incorporation. Regression analysis was utilized to determine the linear slopes for SS and IMF mitochondrial incubations, and to establish their respective correlation coefficients (r). The effect of ethanol on SS and IMF mitochondria was analysed using Student's *t* test for independent samples on the linear slopes obtained from the regression analyses. Significance was established at $p<0.05$ for all comparisons.

C. RESULTS

Figure 3-1 illustrates the time course of L-[^{35}S]methionine incorporation in SS and IMF mitochondrial fractions incubated with and without the presence of ethanol. The addition of ethanol at a final concentration of 1.5% resulted in a 20% ($p>0.05$) and 40% ($p<0.05$) reduction in the rate of amino acid incorporation into SS and IMF mitochondrial fractions, respectively. Methionine incorporation into SS and IMF mitochondrial fractions was linear between 15 and 60 min for both the control and

ethanol incubations. Correlation coefficients for the control incubations were r=0.999 and r=0.998 for SS and IMF mitochondrial fractions, respectively; and, correlations coefficients for the ethanol incubations were r=0.970 and r=0.999 for SS and IMF mitochondrial fractions, respectively. Time zero blanks were not measured therefore precluding the determination of absolute rates of amino acid incorporation. Rates of L-[^{35}S]methionine incorporation were 2.1 fold greater in IMF than in SS mitochondria, which confirms the results of the previous study (Chapter 2).

Background incorporation was monitored over time by measuring the amount of incorporation observed in samples lacking only the addition of mitochondria. The increase in disintegrations·min^{-1} over time corresponded to a linear rate of 0.015 pmol methionine·30 min^{-1} (Figure 3-2). This increase may be a result of the washing procedure used for the filter discs, or less likely, due to bacterial contamination.

Sodium azide (NaN_3) was also examined as a potential method for monitoring background incorporation since azide (N_3^-) inhibits cytochrome oxidase (Mathews and van Holde, 1990) (Figure 3-3). Because a single sample was used, the results are presented descriptively. Sodium azide (15 mM) inhibited the rate of amino acid incorporation into SS and IMF mitochondrial fractions over a 15 min period by 100% and 56%, respectively.

C. DISCUSSION

Relatively little is known about the effect of ethanol on mitochondrial biogenesis in two distinct subpopulations of skeletal muscle mitochondria. Studies which have examined ethanol's effect on mitochondrial protein synthesis have generally analysed a mixture of the two types of mitochondria, predominantly of hepatic and cardiac origin (Burd and Rubin, 1979; Coleman and Cunningham, 1990; Marcus et al., 1982). The aim of the present study was to 1) determine the acute effect of a 1.5% concentration of ethanol on *in vitro* amino acid incorporation in two distinct subpopulations of skeletal muscle mitochondria; and 2) to investigate other potential measures of background incorporation.

The results of the present study reveal that the addition of ethanol at a final concentration of 1.5% to a mitochondrial protein synthesizing medium induces distinct reductions in the rate of amino acid incorporation into SS and IMF fractions of skeletal muscle mitochondria, albeit significant reductions were only observed in the IMF fraction. It should be noted that a small sample size (n=3) was used in the experiment, therefore resulting in little power to detect statistical significance. Further experimentation with a larger sample size is required to ascertain the inhibitory effect of ethanol on the SS mitochondrial population, since a 20% inhibition appears to

be biochemically significant (Rubin et al., 1972; Burke and Rubin, 1979). The IMF finding agrees with previous reports demonstrating deleterious effects of acute ethanol on protein synthesis by a single population of liver mitochondria (Burke and Rubin, 1979; Rubin et al., 1970). Burke and Rubin (1979) showed an increasing inhibitory effect on [^{3}H]leucine incorporation *in vitro* into protein of hepatic mitochondria with increasing concentrations of ethanol. Following 30 min of incubation, a concentration range of 25 to 100 mM significantly depressed the rate of mitochondrial protein synthesis 9 to 22%. Similarly, Marcus et al. (1982) demonstrated a 21.9% inhibition of mitochondrial protein synthesis in liver following a 30 min incubation with 150 mM of ethanol. Analogous to the aforementioned findings are the *in vitro* results of Coleman and Cunningham (1990) which demonstrated depressed translation activity in hepatic mitochondria in the order of 35-40% following chronic ethanol consumption.

Inhibition of *in vitro* mitochondrial protein synthesis by ethanol results from a direct action of ethanol on mitochondria (Rubin et al., 1970). Specifically, evidence exist to suggest that the acute effects of ethanol involve the interaction of ethanol with membrane processes, although the precise mechanisms of membrane-mediated action remain unidentified. Barry and Gawrisch (1994) studied the interaction of ethanol with phospholipid bilayers and found

that ethanol interacts with the phospholipid bilayers at the lipid-water interface. These results suggest that ethanol disrupts the outer membrane of mitochondria which, in turn, may affect protein function. Thus, ethanol may potentially alter the transport of amino acids into mitochondria via disruption of membrane transporters, thereby reducing rates of protein synthesis.

Studies measuring amino acid uptake and transporter number and activity in SS and IMF mitochondria may shed some light on why the two subpopulations of mitochondria isolated from skeletal muscle respond differently to the same concentration of ethanol *in vitro*. The distinct protein synthesis inhibition by ethanol may be a result of initial differences in transporter number and/or activity between the two subpopulations of mitochondria rather than varying levels of disruption of membrane transporters.

In contrast to the results of this study, *in vivo* experimentation has revealed similar reductions in the synthesis of ventricular mitochondrial proteins into SS (23%) and IMF (26%) fractions following acute ethanol administration (Siddiq et al., 1993). It is important to note that ethanol was administered and protein synthesis was measured *in vivo* which encompasses a variety of hormonal and metabolic responses which may have influenced the observed results. In addition, *in vivo* experimentation examines the combined influence of both the mitochondrial and nuclear-

cytoplasmic genetic systems. Therefore, the discrepancy between results may be an outcome of different genetic origins, methodology and/or tissue types used in the two studies.

Due to the disparate inhibitory effect of ethanol on SS and IMF mitochondria, other measures of background incorporation were examined. Background measures monitored over time in samples lacking only the addition of mitochondria averaged <2% of total incorporation. While this method accounts for bacterial contamination and washing procedure combined, it does not encompass bacterial contamination that may have originated from the mitochondrial isolation procedure. However, the presence of up to 40,000 bacteria in the incubation medium results in no further increases in rate of amino acid incorporation over a 60 min duration (Cogswell et al., 1993).

Little can be concluded from the sodium azide data and therefore cannot be recommended as a viable measure for background incorporation. Of interest, however, is the 50% reduction in amino acid incorporation observed in hepatic mitochondria by Rubin et al. (1970) following the addition of 0.1 mM of sodium azide to the incubation medium. This finding supports the result obtained in the present study with IMF mitochondria. However, it is not known why complete inhibition was achieved in the SS mitochondrial fraction, while amino acid incorporation was reduced by only

56% in the IMF mitochondria. This remains to be determined.

Mitochondrial protein synthesis studies which have used CAP at concentrations not exceeding 1 $mg \cdot mL^{-1}$ have met with varying success. These studies have shown to inhibit protein synthesis by 30-35% in heart (Hamberger et al., 1969), 80-87% in liver (Burke and Rubin, 1979; Coleman and Cunningham, 1990), and 80-87% in skeletal muscle (Mockel and Beattie, 1975) mitochondria. Each of the aforementioned studies ruled out contaminating extramitochondrial ribosomes as a potential factor for the reduced level of CAP inhibition by adding cycloheximide and other potent inhibitors of ribosomal protein synthesis to the incubation medium.

In summary, the use of CAP in the study of mitochondrial protein synthesis in quantities requiring the use of ethanol at concentrations greater or equal to 1.5% is not advocated due to ethanol's distinct inhibitory effect on amino acid incorporation in SS and IMF mitochondria. Elucidation of the mechanism of the independent effect of ethanol on two subpopulations of mitochondria isolated from skeletal muscle requires further study. Measures of amino acid incorporation in samples lacking only the addition of mitochondria is a viable background measure, although periodic assessment of bacterial contamination is recommended.

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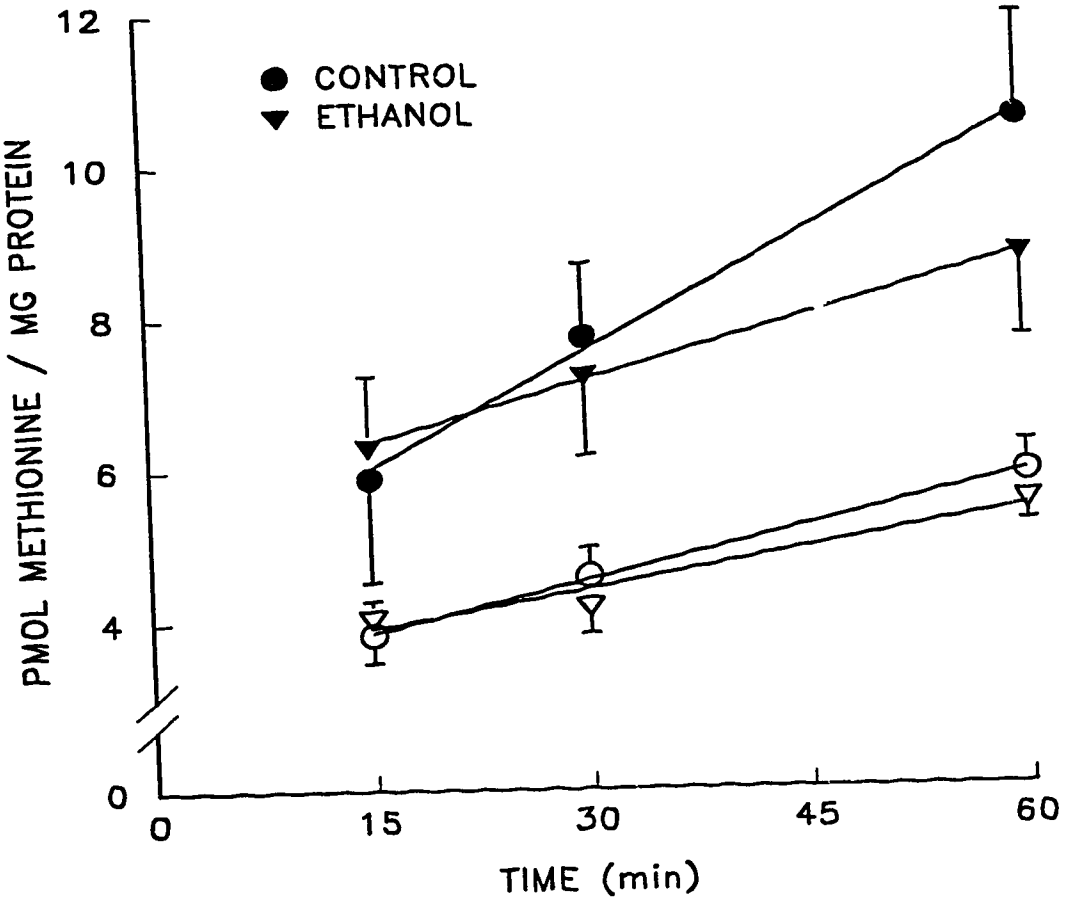

Figure 3-1. In vitro time course of L-[^{35}S]methionine incorporation in SS (n=3) and IMF (n=3) mitochondrial fractions in the presence and absence of ethanol at a final concentration of 1.5%. Closed symbols represent IMF and open symbols represent SS mitochondria.

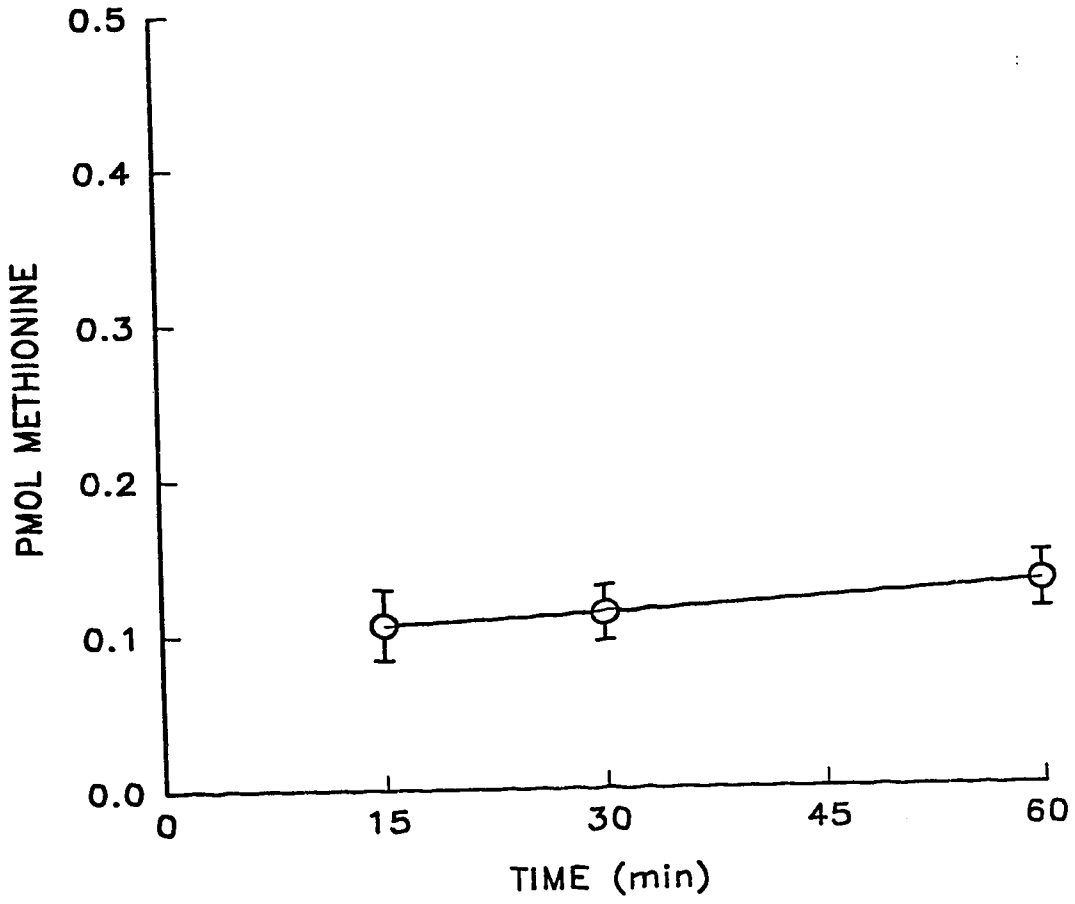

Figure 3-2. In vitro time course of L-[^{35}S]methionine incorporation in samples lacking only the addition of mitochondria (n=4). The regression equation (r=0.998) was $y = 0.097 + (5 \cdot 10^{-4})x$. A repeated measures analysis of variance was used to analyse the effect of time on L-[^{35}S]methionine incorporation ($p>0.05$).

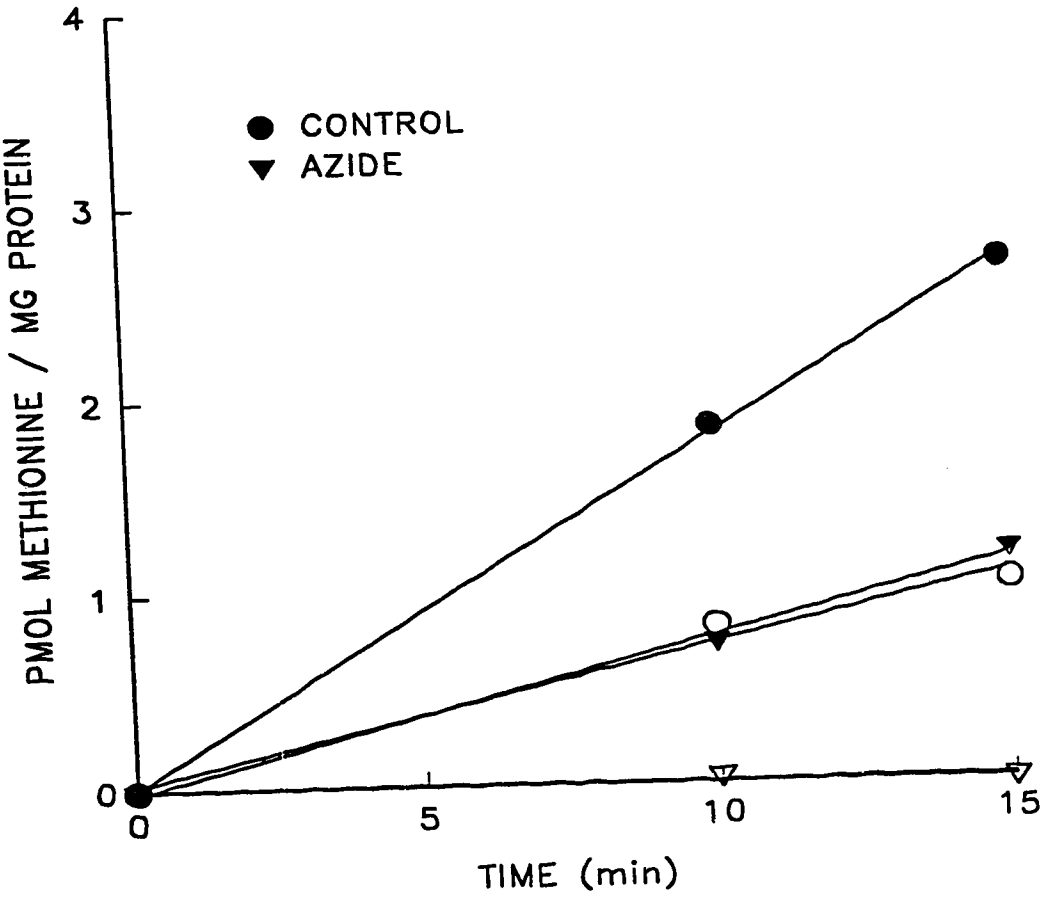

Figure 3-3. *In vitro* time course of L-[^{35}S]methionine incorporation in SS (n=1) and IMF (n=1) mitochondrial fractions in the presence and absence of sodium azide at a final concentration of 15mM. Closed symbols represent IMF and open symbols represent SS mitochondria.

CHAPTER 4

GENERAL DISCUSSION AND CONCLUSIONS

A. GENERAL CONCLUSIONS

The study of regional variation in mitochondrial biosynthesis is in its infancy. Despite clear evidence of distinct morphological and biochemical characteristics, in addition to physiological and pathological adaptations, little research has been conducted in attempt to explain these differences. The primary aim of this investigation was to examine protein synthesis, encompassing two genetic systems, in two distinct subpopulations of skeletal muscle mitochondria. Additionally, the intent of the dissertation was to provide insight into the underlying basis for the differential adaptability of SS and IMF mitochondria to various physiological and pathological stresses imposed on the organelles. The major conclusions of this research are summarized below:

1. Skeletal muscle mitochondria isolated into SS and IMF fractions display distinct biochemical characteristics.
2. Mitochondrial protein synthesis (*in vitro*) was greater in the IMF than in the SS mitochondrial fractions, which in part concurs with the differences in respiratory capacity between these two subpopulations of mitochondria.

3. Cytoplasmic protein synthesis (*in vivo*) was greater in the SS than in the IMF mitochondrial fractions, suggesting different functional roles.

4. Acute ethanol administration to isolated skeletal muscle mitochondria resulted in a distinct inhibitory effect on protein synthesis in SS and IMF mitochondrial fractions, suggesting that the use of large concentrations of chloramphenicol be avoided in the study of mitochondrial protein synthesis.

Mitochondrial biosynthesis is exceedingly complex in that it requires not only the cooperative effort of two genetic systems but also the integration of multiple processes in the biosynthesis pathway. These include mitochondrial and nuclear gene expression, mitochondrial and cytoplasmic protein synthesis, phospholipid synthesis, protein import into the organelle, and protein assembly within mitochondria. The findings presented in this dissertation portray only one aspect of this process - cytoplasmic and mitochondrial protein synthesis - to explain the disparity within skeletal muscle mitochondria. The following discussion examines processes in mitochondrial biosynthesis which may be implicated in the uniqueness and in the differential adaptability of the two subpopulations of skeletal muscle mitochondria.

B. GENE EXPRESSION

Question of distinct mitochondrial populations

Based on the data of this thesis, differential rates of protein synthesis in SS and IMF mitochondria account for another distinction between the two subpopulations of skeletal muscle mitochondria. The term subpopulation was adopted in the thesis to define a single population of mitochondria comprised of two functionally morphologically, and biochemically distinct sub-groups. It seems improbable that this regional disparity is a function of genetically different groups of mitochondria. To be certain, however, the question of two genetically distinct populations could be approached using different DNA probes. A recent study on human HeLa cells lends support to the existence of a genetically single population of mitochondria (Hayashi et al., 1994). The results provided direct evidence for frequent mitochondrial fusion of host HeLa cells entirely lacking mitochondrial DNA (mtDNA) with transferred HeLa mitochondria with mtDNA, which led to the rapid diffusion of mtDNA into the host cell. This resulted in a homogeneous distribution of mtDNA throughout the mitochondria in a cell.

Based on these results, it was suggested that mitochondria function as a single dynamic unit in a cell (Hayashi et al., 1994). Extensive research on mitochondrial organization in skeletal muscle using electron microscopy also supports the existence of a mitochondrial reticulum of

varying complexity, as discussed in chapter one. It can be concluded that mitochondria exist as ubiquitous and pleomorphic organelles which are capable of branching, dividing and fusing, in addition to changing their position inside a cell (Bereiter-Hahn, 1990). Thus, it would seem reasonable to suggest that mitochondria are in a continual state of flux between the SS and IMF regions of the cell depending on the metabolic state of the cell.

Coordination of nuclear and mitochondrial gene expression:

The proliferation of functional mitochondria requires concerted regulation of two cellular genetic compartments. It is hypothesized that genetic signalling mechanisms in response to chronic muscle stimulation are initially sensed within the nucleus and mitochondrial gene expression takes place as a secondary event, regulated by nuclear gene products associated in replication and/or transcription of mtDNA (Annex et al., 1991; Williams et al., 1987; Williams and Harlan, 1987). The regulatory mechanisms that coordinate gene expression in mitochondria could operate at numerous stages of the biosynthesis pathway.

One issue of recent debate is whether the oxidative capacity of a tissue is regulated by mitochondrial gene dosage or by transcriptional and/or post-transcriptional regulatory mechanisms (Nagley, 1992; Wiesner, 1992). Support for regulation at the level of mitochondrial gene

dosage (Hood et al., 1994; Nagley, 1991; Williams, 1986) is based on the positive correlation demonstrated between mtDNA and mitochondrial mRNA in response to chronic muscle stimulation (Williams, 1986; Williams et al., 1986). The nuclear respiratory factor 1 (NRF-1), a trans-activator of nuclear genes encoding structural components of the mammalian electron transport chain and the mtDNA replication machinery, is suggested as a possible mechanism for the coordinated regulation at the level of gene dosage (Evans and Scarpulla, 1990). It is argued, however, that this coupling between replication and transcription of mtDNA is not one of cause-and-effect, but may reflect a general increase in mitochondria under conditions of increased muscle use (Wiesner et al., 1992). A growing body of experimental evidence does not support the concept that gene dosage is rate limiting and thus regulates mitochondrial gene expression (Van den Bogert et al., 1993; Wiesner et al., 1992; Wiesner et al., 1994). Recently, it was postulated that mitochondrial transcription factor A (a nuclear product) may be the rate limiting determinant in the regulation of mitochondrial gene expression at the level of transcription (Garstka et al., 1994).

How may these potential regulatory mechanisms of mitochondrial gene expression relate to the experimental data? Regardless of the various proposed mechanisms for coordinated regulation of the two genetic systems, control

is initiated by the nucleus via nuclear-encoded components. Thus, it may be reasonable to suggest that the availability of these nuclear-encoded proteins is greater for the SS mitochondria due to its location in the muscle cell, in close proximity to the nucleus, or possibly due to differences between SS and IMF mitochondria in the protein import machinery responsible for the translocation of these proteins into the organelle. Although this interpretation substantiates the greater *in vivo* fractional rate of protein synthesis in the SS mitochondria, it does not explain the larger *in vitro* rate of protein synthesis found in the IMF mitochondria. It is difficult to assess the significance of this finding due to the removal of humoral factors and more importantly nuclear control with *in vitro* experimentation. This issue could be addressed with an *in vivo* study to estimate rates of mitochondrial protein synthesis attributed to the mitochondrial genome with the incorporation of an inhibitor of translation from cytoplasmic ribosomes such as cycloheximide.

C. PROTEIN IMPORT INTO MITOCHONDRIA

Nuclearly encoded polypeptides are primarily translated on free cytoplasmic ribosomes after which they are targeted to mitochondria in a post-translational manner (Glover and Lindsay, 1992). The first step in the translocation of mitochondrial proteins into the organelle requires the

recognition of precursor proteins by receptors on the mitochondrial surface (Stuart et al., 1994). This could be a potential mechanism for the distinction between SS and IMF *in vivo* fractional rates of protein synthesis. If this is the case, SS mitochondria would require either an increased number or activity of receptors compared to the IMF mitochondria. A disparity in the sensitivity of protein import receptors between SS and IMF mitochondria may also result in distinct levels of inhibition of *in vivo* fractional rates of protein synthesis following acute administration of ethanol. This remains to be tested.

D. PHYSIOLOGICAL IMPLICATIONS

The intracellular distribution of skeletal muscle mitochondria is characterised by a radial gradient with a greater concentration of mitochondria located in the SS region (Bell et al., 1992; Martin and Edgerton, 1992; Socha et al., 1993). The physiological significance of this distribution is related to the large demand for oxygen and substrates by the mitochondria for the production of ATP for muscle contraction. The greater concentration of SS mitochondria reduces the diffusion distance for the transfer of oxygen from the capillaries to the mitochondria, allowing for the subsequent rapid delivery to the IMF mitochondria where it is required in high concentrations during muscle contraction. In response to endurance training, the SS

mitochondria which exhibit a greater steady state *in vivo* fractional rate of protein synthesis (interpreted as a greater rate of protein turnover) can adapt more readily in order to meet the immediate increase in metabolic demand. Research on heart muscle suggests that the adaptation to acute exercise is one of increased protein synthesis (Hamberger et al., 1969; Mayer et al., 1981). No such studies have yet examined the effect of acute exercise on mitochondrial protein synthesis in skeletal muscle. It would be interesting to estimate rates of protein synthesis in both subpopulations of mitochondria following acute exercise to determine if the adaptation is a result of increased protein synthesis and to see if the relative difference between SS and IMF mitochondria remains.

E. CONCLUSIONS

In conclusion, the experimental findings in this thesis demonstrate considerable regional variation in rates of protein synthesis in skeletal muscle mitochondria. These simple models are just initial attempts at understanding the mechanisms potentially responsible for the disproportionate adaptive response between SS and IMF mitochondria cited in the literature. Although much has been recently explained concerning the details of mitochondrial gene expression, experiments are needed to identify the precise level(s) of regulation that bring about

the accompanying distinct changes in gene expression. Also, future research directions could focus on determining the effect of acute exercise on mitochondrial protein synthesis, both *in vivo* and *in vitro*. It is with hope that this and future research will eventually lead to the understanding of how mitochondria and the nucleus collaborate in order to bring about distinct changes in mitochondrial content in different areas of the muscle cell.

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APPENDIX A

MITOCHONDRIAL ISOLATION PROCEDURE AND DETERMINATION OF MITOCHONDRIAL PURITY AND INTEGRITY

ISOLATION OF TWO SUBPOPULATIONS OF MITOCHONDRIA

The objective of the isolation procedure was to isolate two distinct subpopulations of skeletal muscle mitochondria: subsarcolemmal (SS) and intermyofibrillar (IMF). The two subpopulations of mitochondria were isolated according to the method of Kreiger et al. (1980) as illustrated in Figure A-1.

In order to minimize bacterial contamination for the in vitro study, all glassware was acid bathed and dried for several hours at 100°C. In addition, all buffers must be filtered (0.2 micron) or autoclaved, and gloves should be worn at all times. An aseptic environment is highly recommended.

Solutions

Table A-1. Composition of buffers used in the mitochondrial isolation procedure.

Buffer I	Buffer II	Buffer III
100 mM KCl	100 mM KCl	100 mM KCl
10 mM MOPS	10 mM MOPS	10 mM MOPS
5 mM EDTA	5 mM EGTA	5 mM EDTA
5 mM NaPPi	5 mM NaPPi	0.2% BSA
	5 mM $MgCl_2$	

A. Buffer I (pH 7.4)

To make 1 L (≈ 250 mL per isolation):

7.46 g KCl (FW 74.55)
2.09 g MOPS (FW 209.3)
1.46 g EDTA (FW 292.25)
1.33 g Na_4PPi (FW 265.9)

- dissolve reagents in 990 mL of distilled water (dH_2O)
- adjust pH to 7.4 with KOH
- bring volume to 1 L with dH_2O
- refrigerate at 4°C and pH once again at 4°C
- solution may be used for 1 week

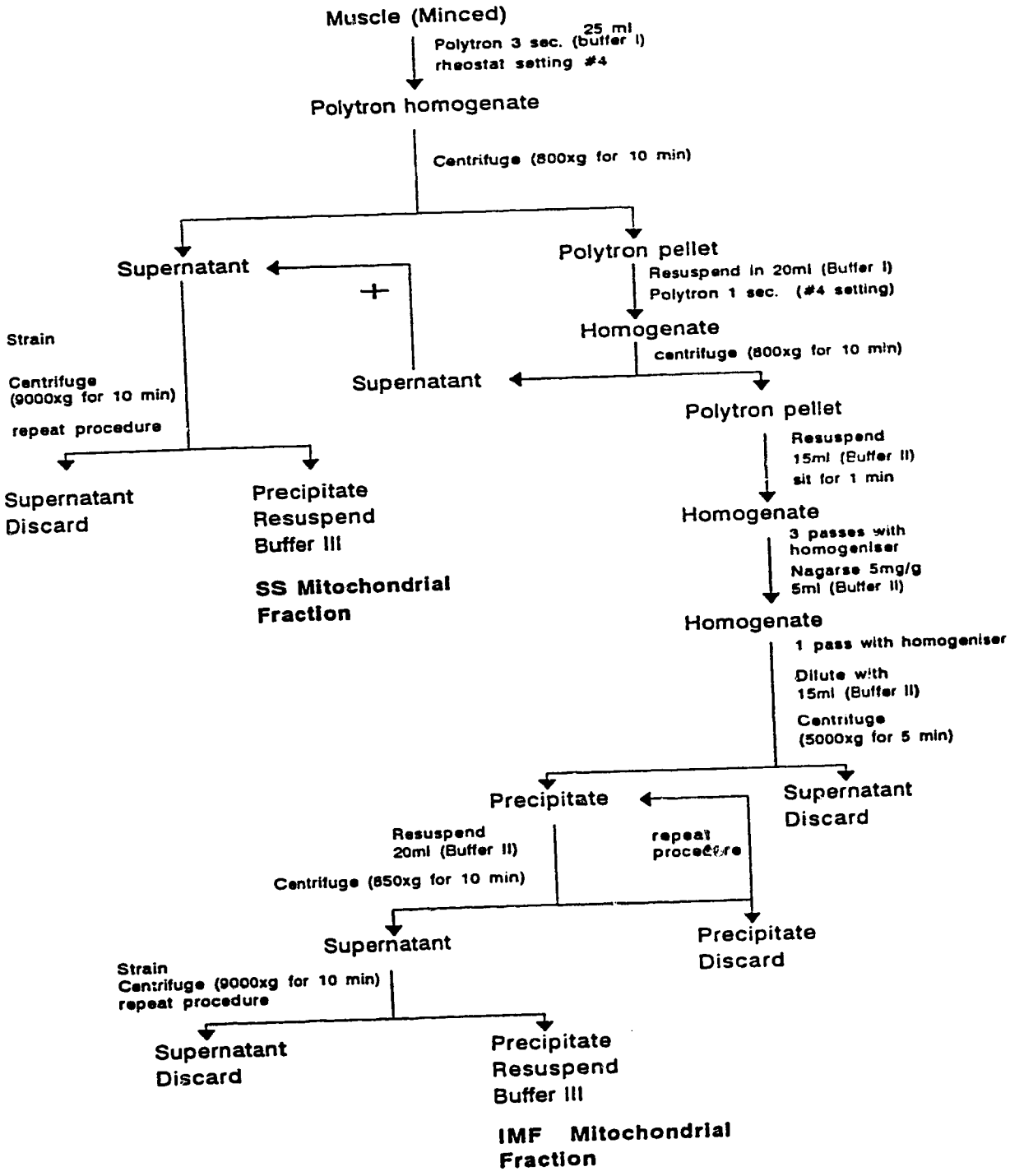

Figure A-1. Flow diagram for separation of mitochondrial fractions

B. Buffer II (pH 7.4)

To make 400 mL (≈ 200 mL per isolation):

2.984 g KCl (FW 74.55)
0.837 g MOPS (FW 209.3)
0.761 g EGTA (FW 380.4)
0.532 g Na_4PPi (FW 265.9)
0.190 g $MgCl_2$ (FW 95.21)

- dissolve EGTA in 350 mL of dH_2O by adding 6 N KOH one drop at a time
- once most of the EGTA is in solution, add NaPPi followed by the remaining reagents
- adjust pH to 7.4 with KOH
- bring volume to 400 mL with dH_2O
- refrigerate at 4°C and pH once again at 4°C
- solution may be used for 3-4 days (check that the buffer has not come out of solution prior to use)

C. Buffer III (pH 7.4)

To make 25 mL (< 10 mL per isolation):

127 mg KCl (FW 74.55)
52 mg MOPS (FW 209.3)
95 mg EGTA (FW 380.4)
50 mg BSA

- dissolve EGTA followed by KCl and MOPS in 20 mL of dH_2O
- adjust pH to 7.4 with KOH
- sprinkle BSA over buffer and let sit until dissolved (do not stir)
- bring volume to 25 mL with dH_2O
- refrigerate at 4°C and adjust pH once again at 4°C
- solution may be used for 3-5 days

Procedure

On the day of the experiment, animals were weighed, placed in a CO_2 chamber for 30 s and killed by cervical dislocation. All procedures were carried out on ice. The right hindlimb was rapidly excised and placed in 50 mL of ice-cold Buffer I. The muscles were removed from the hindlimb, trimmed of connective tissue, blotted dry, weighed, and divided equally by weight into two beakers each containing 25 mL of Buffer I.

The muscles were finely minced with scissors and the resulting mince was homogenized with a PT-20 Polytron tissue processor for 3 s at a rheostat setting of 4. The homogenate was centrifuged at 800 g for 10 min, and the resulting supernatant was filtered through a double layer of cheesecloth. The precipitate was resuspended in 20 ml of Buffer I and the first step repeated. The precipitate was subsequently used for the preparation of the IMF mitochondria. The supernatant was centrifuged at 9,000 g for 10 min and the resulting pellet containing the SS mitochondria was resuspended in 10 ml of Buffer I in a Potter-Elvehjem homogenizer using a teflon pestle. Three 10 μL aliquots were removed to determine mitochondrial fraction protein concentration, and the homogenate was then centrifuged at 9,000 g for 10 min. The final mitochondrial pellet was resuspended in Buffer III at an approximate concentration of 13 $mg \cdot mL^{-1}$ and kept on ice.

The IMF pellet from the 800 g centrifugation was resuspended in 15 mL of Buffer II and allowed to sit for 1 min to enhance the effect of the 'relaxing' buffer. The homogenate was then homogenized in a 55 mL THOMAS apparatus with three passes using a teflon pestle. Nagarse (Sigma P4789) (5 $mg \cdot g$ wet muscle $weight^{-1}$) was suspended in 5 mL of Buffer II and at once added to the homogenate, after which it was homogenized with 1 pass of the teflon pestle and immediately diluted with 15 mL of Buffer II. Without delay, the homogenate was centrifuged at 5,000 g for 5 min, and the resulting supernatant quickly discarded. The pellet was resuspended in 20 mL of Buffer II with a 55 mL Potter-Elvehjem homogenizer and centrifuged at 850 g for 10 min. This procedure was repeated a second time, after which the pellet was discarded. The supernatant containing the IMF mitochondria was carefully filtered through a double layer of cheesecloth into a clean cold centrifuge tube and pelleted at 9,000 g for 10 min. Care was taken not to contaminate the supernatant with the loose pellet. The resulting pellet was resuspended in 10 mL of Buffer I, and three 10 μl aliquots were removed for mitochondrial fraction protein determination. Following a second centrifugation at 9,000 g for 10 min., the pellet was resuspended in Buffer III at an approximate concentration of 13 $mg \cdot mL^{-1}$ and kept on ice.

RESPIRATORY CONTROL MEASUREMENTS

The objectives of the respiratory measurements were to assess the degree of intactness and coupling between electron transport and oxidative phosphorylation. In addition, state 3 respiration was measured in SS and IMF

mitochondrial fractions to verify the presence of distinct mitochondrial populations.

Solutions

Table A-2. Composition of the 1.5 mL reaction medium for measuring mitochondrial respiration

Reagent	Concentration
Trizma base	25 mM
Sucrose	45 mM
Glucose	20 mM
KH_2PO_4	30 mM
KCl	15 mM
Mannitol	12 mM
$MgCl_2$	5 mM
EDTA	7 mM
NAD^+	0.4 mM
BSA	0.2 %
Mitochondria	0.2 mg
Pyruvate	10 mM
Malate	2.5 mM
ADP	200 nmol

A. O_2 electrode buffer (pH 7.4)

To make 25 mL (1.2 mL aliquots):

94.6 mg Trizma Base (FW 121.1)
481.4 mg Sucrose (FW 342.3)
112.6 mg Glucose (FW 180.2)
127.6 mg KH_2PO_4 (FW 136.1)
34.9 mg KCl (FW 74.55)
68.3 mg Mannitol (FW 182.17)
14.9 mg $MgCl_2$ (FW 95.21)
63.9 mg EDTA (FW 292.25)
8.3 mg NAD^+ (FW 663.4)
62.5 mg BSA

- dissolve reagents into 20 mL of dH_2O in the order written with the exception of the BSA
- adjust pH to 7.4 with HCl
- sprinkle BSA over buffer and let sit until dissolved (do not stir)
- bring volume to 25 mL with dH_2O
- adjust pH once again at room temperature if necessary
- solution may be used for 3-5 days

B. Pyruvate/Malate solution (1.5 mL final volume)

To make 20 mL (0.2 mL aliquots):

165.0 mg pyruvic acid (Sigma P-2256) (FW 110.0)
50.3 mg malate (FW 134.1)

- dissolve reagents in 20 mL of dH_2O
- store required aliquots at -20°C

C. Adenosine 5'-diphosphate (ADP)
$C_{10}H_{15}N_5O_{10}P_2$ (Sigma A-8271) (FW 427.2)

To make 10 mL (50 µL aliquots):

- dissolve 17.09 mg ADP in 10 mL of dH_2O
- store required aliquots at -20°C

Procedure

Mitochondrial respiration was assayed polarographically at 30°C for each mitochondrial preparation as described by Davies et al. (1981) with a Clark type oxygen electrode by means of a Yellow Springs model 53 oxygen monitor (Yellow Springs Instruments, Yellow Springs, OH). Briefly, approximately 0.2 mg (50 µL) of mitochondrial protein was added to 1.2 mL of O_2 electrode buffer following 4 min of temperature equilibration. Then 200 µL of substrate was added and following depletion of endogenous mitochondrial substrates, oxidation was initiated by the addition of 50 µL of ADP. The final assay volume was 1.5 mL. All assays were performed in duplicate.

CALCULATION OF STATE 3 RESPIRATION AND RESPIRATORY CONTROL RATIOS (RCR)

Respiratory measurements and respiratory control ratios (RCR) were quantified from polarographic tracings by standard procedures according to Chance and Williams (1955a,b), and Estabrook (1967).

An estimate of oxygen content of the reaction medium was calculated from the model 53 oxygen monitor's user manual.

$$V = \frac{a\ v\ p}{P_{Bar}}$$

where: V = volume of O_2 in electrode solution
a = solubility coefficient of O_2 in Ringers solution at 30°C
v = volume of electrode solution
p = partial pressure of O_2 ($0.2093 \cdot P_{Bar}$)
P_{Bar} = 760 mm Hg

Therefore, $$V = \frac{(0.026)(1\ mL)(0.2093 \cdot 700\ mmHg)}{760\ mmHg}$$

$$= 5.01\ \mu L\ O_2 \cdot mL\ buffer^{-1}$$

Since 1 mL O_2 = 1.42857×10^{-3} g O_2

$5.01\ \mu L\ O_2 \cdot mL\ buffer^{-1} = 7.16 \times 10^{-3}\ mg\ O_2 \cdot mL\ buffer^{-1}$

$7.16 \times 10^{-3}\ mg\ O_2 \cdot mL\ buffer^{-1} \div 16\ atoms\ O \cdot O_2^{-1}$

$= 447.3\ ng\ atoms\ O \cdot mL\ buffer^{-1}$

This final value was utilised for the calculation of mitochondrial state III or IV respiratory rates in the following manner:

447.3 ng atoms O·mL buffer^{-1} x volume of reaction medium x rate of change in O_2 saturation ÷ mg mitochondrial protein

Respiratory control ratio, an index of intactness and coupling between electron transport and oxidative phosphorylation, was calculated as the state III rate [respiration rate in the presence of added substrate and phosphate acceptor (ADP)] divided by the state IV rate (rate after consumption of ADP). Mitochondrial preparations with RCRs greater than five were utilised for in vitro protein synthesis measurements.

$$\mathbf{RCR} = \frac{\text{State III (ng atoms O} \cdot \text{min}^{-1} \cdot \text{mg mitochondrial protein}^{-1})}{\text{State IV (ng atoms O} \cdot \text{min}^{-1} \cdot \text{mg mitochondrial protein}^{-1})}$$

OLIGOMYCIN-SENSITIVE MITOCHONDRIAL F_1-ATPase ACTIVITY

The objective of the assay was to determine mitochondrial purity. Oligomycin directly inhibits the activity of the mitochondrial F_1-adenosinetriphosphatase (ATPase) enzyme of complex V. To determine the amount of

non-mitochondrial ATPase present in the isolated fractions, the ATPase activity was measured in the presence and absence of 1 μg oligomycin. The rate observed in the presence of oligomycin represents non-mitochondrial ATPases. These may be derived from the sarcolemma, sarcoplasmic reticulum, and/or myofibrils.

The mitochondrial ATPase activity was measured by the method of Kreiger et al. (1980). The Mg^{2+}-dependent hydrolysis of ATP by the mitochondrial preparations was monitored by a continuous recording spectrophotometer (Pye Unicam PU8800) through a linked-enzyme system following the oxidation of NADH at 340 nm (Figure A-2). The assay measures the reduction rate of NADH to NAD which is limited by the amount of ATPase present in the mitochondrial fraction.

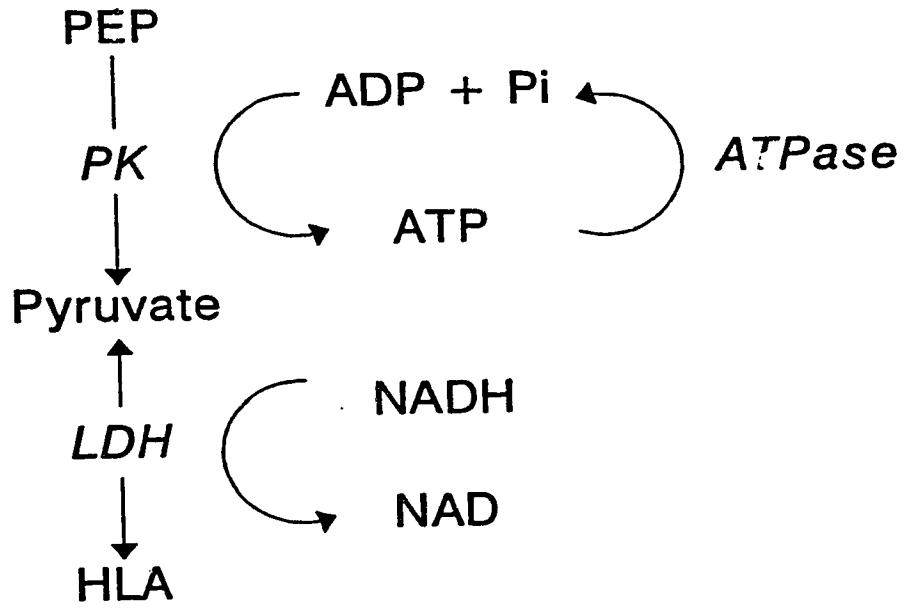

Figure A-2. Linked-enzyme system used in the ATPase assay

Solutions

Table A-3. Composition of the 1 mL reaction medium for measuring F_1-ATPase activity

Reagent	Concentration
Tris maleate	40 mM
KCl	100 mM
$MgCl_2$	5 mM
EGTA	2 mM
Phosphoenolpyruvate	2 mM
Pyruvate kinase	20 $U \cdot mL^{-1}$
Lactate dehydrogenase	20 $U \cdot mL^{-1}$
NADH	0.4 mM
Mitochondria	12 μg
Na_2ATP	2 mM

A. Tris-maleate buffer (pH 7.4)

To make 15 mL:

1.423 g Tris-maleate (FW 237.2)
1.141 g KCl (FW 74.55)
0.153 g $MgCl_2$ (FW 95.21)
0.118 g EGTA (FW 380.4)

- dissolve reagents in 10 mL of dH_2O
- adjust pH to 7.4 with KOH
- bring volume to 15 mL
- re-adjust pH
- store buffer in the refrigerator

B. Phosphoenolpyruvate (PEP)
$C_3H_4O_6PK$ (Sigma P-7127) (FW 206.1)

5.453 $mg \cdot mL^{-1}$

C. Adenosine 5'-triphosphate (ATP)
$C_{10}H_{14}N_5O_{13}P_3Na_2$ (Sigma A-5394) (FW 551.1)

121 $mg \cdot mL^{-1}$

D. Oligomycin (Sigma O-4876)

0.2 $mg \cdot mL^{-1}$ in ETOH (100%)

E. Reaction mixture

1 mL Buffer solution
1 mL PEP
3 mL dH_2O

- Prepare fresh reaction mixture daily

F. ß-Nicotinamide adenine dinucleotide, reduced (NADH)
$C_{21}H_{27}N_7O_{14}P_2Na_2 \cdot 4H_2O$ (Sigma 340-13) (FW 781)

0.3 $mg \cdot vial^{-1}$

G. Pyruvate kinase (PK) (Sigma P-1506)

H. $_L$-Lactic dehydrogenase (LDH) (Sigma L-2500)

Procedure

1. Prepare cuvettes (2 per sample: 1 with oligomycin and 1 without)
2. Set up spectrophotometer: 340 nm
   water bath : 30°C
   read 2 s intervals for 5 minutes
3. Add 1.07 mL reaction mix and 1.03 mL dH_2O to NADH vial and vortex
4. Remove 0.975 mL aliquots from the NADH vial and place into each cuvette
5. Solubilize 20 µL (0.1 mg protein) of the mitochondrial sample in 180 µL of 0.2 % deoxycholate.
   Vortex sample lightly to mix and place on ice while adding the remainder to the cuvette
6. Add 10 µL of PK and 7.3 µL of LDH enzymes to cuvette pair
7. Add 25 µL of ETOH (100%) to the first cuvette (no oligomycin)
8. Add 25 µL of oligomycin to second cuvette
9. Add 25 µL of mitochondrial sample (diluted in deoxycholate) to each cuvette and stir
10. Incubate in a shaking water bath for 5 min
11. Place cuvette in the spectrophotometer and begin the reaction by adding 10 µL of ATP mixture to cuvette and stir.

Calculations

Calculate the percent not inhibited by oligomycin as the measure of non-mitochondrial ATPase activity.

$$\% \text{ contamination} = \frac{\text{rate (absorbance} \cdot \text{min}^{-1}\text{) with oligomycin}}{\text{rate (absorbance} \cdot \text{min}^{-1}\text{) without oligomycin}} \times 100$$

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APPENDIX B

MEASUREMENT OF IN VITRO PROTEIN SYNTHESIS

In vitro rates of protein synthesis were estimated in skeletal muscle mitochondria by the filter disc technique of Mans and Novelli (1961) as modified by McKee et al. (1990a,b).

MITOCHONDRIAL PROTEIN SYNTHESIS MEDIUM

Solutions

Solutions must be filtered through 0.2 μm filters before use in order to minimize bacterial contanimation.

Table B-1. Composition and final concentration of incubation medium

Component	Concentration
Mitochondrial protein	4.0 $mg \cdot ml^{-1}$
[^{35}S]methionine	0.2 $mCi \cdot ml^{-1}$
KCl	90.0 mM
$MgSO_4 \cdot 7H_2O$	4.0 mM
KH_2PO_4	2.5 mM
MOPS	25.0 mM
Methionine	20.0 μM
Amino Acids	0.1 mM
Pyruvate	20.0 mM
Malate	0.5 mM
ADP	2.0 mM
BSA	1.0 $mg \cdot ml^{-1}$
Cycloheximide	0.1 $mg \cdot ml^{-1}$
pH	7.4
Temperature	30.0 °C

A. Protein synthesis medium (pH 7.4)

To make 50 mL (600 μL aliquots):

559.13 mg KCl (FW 74.55)
82.16 mg $MgSO_4 \cdot 7H_2O$ (FW 246.48)
28.35 mg KH_2PO_4 (FW 136.10)
436.04 mg MOPS (FW 209.30)
186.33 mg Pyruvic acid (FW 110.0)

5.59 mg	Malic acid	(FW 134.10)
71.20 mg	ADP	(FW 427.2)
83.33 mg	BSA	
8.33 mg	Cycloheximide	

- dissolve reagents in 40 mL of dH_2O with the exception of BSA and cycloheximide
- adjust pH to 7.4 with KOH
- sprinkle BSA over solution and let sit until dissolved (do not stir)
- add cycloheximde (the cycloheximide is added last as it is sensitive to pH)
- adjust volume to 50 mL with dH_2O
- adjust pH once again
- store required aliquots at -20°C

B. Amino acid solution

To make 100 mL (80 µL aliquots):

16.64 mg	Asp	(MW 133.1)	
14.89 mg	Thr	(MW 119.1)	
13.14 mg	Ser	(MW 105.1)	
16.51 mg	Asn	(MW 132.1)	
18.26 mg	Gln	(MW 146.1)	
18.39 mg	Glu	(MW 147.1)	
9.39 mg	Gly	(MW 75.1)	
11.14 mg	Ala	(MW 89.1)	
15.15 mg	Cys	(MW 121.2)	
14.63 mg	Val	(MW 117.1)	
3.69 mg	Met	(MW 149.2)	(LABEL)
16.40 mg	Ile	(MW 131.2)	
16.40 mg	Leu	(MW 131.2)	
22.65 mg	Tyr	(MW 181.2)	
20.65 mg	Phe	(MW 165.2)	
26.20 mg	His	(MW 209.6)	
22.83 mg	Lys	(MW 182.6)	
25.53 mg	Trp	(MW 204.2)	
26.34 mg	Arg·HCl	(MW 210.7)	
14.39 mg	Pro	(MW 115.1)	

- dissolve amino acids in dH_2O and bring volume to 100 mL
- store required aliquots at -20°C

Procedure

The rate of mitochondrial protein synthesis in skeletal muscle was estimated by a modification of the technique described by McKee et al. (1990a,b). The mitochondrial fractions were incubated in open 25 ml polycarbonate test

tubes at 30 °C in a shaking water bath at a protein concentration of approximately 4 $mg \cdot ml^{-1}$ with approximately 0.22 $mCi \cdot ml^{-1}$ L-[^{35}S]methionine (1000 $Ci \cdot mmol^{-1}$, ICN Biomedicals). Protease inhibitors were not added to the incubation medium, as they have been previously demonstrated not to affect mitochondrial protein synthetic activity in vitro during a 60 minute incubation period (Marzuki et al., 1988). Amino acid incorporation was supported by intramitochondrial synthesis of ATP through the addition of pyruvate-malate and ADP. At various times, 10 µL aliquots were removed in triplicate, spotted onto filter paper discs (Whatman 3MM, 2.3 cm in diameter) and briefly exposed to a stream of air in order to facilitate complete absorption of the sample which significantly reduces sample losses (Mans and Novelli, 1961). The diameter of the discs closely approximates the inside diameter of the glass counting vials, which allows the discs to lie flat on the bottom of the vial. To terminate the enzymic reaction and precipitate the protein into the matrix of the cellulose fibres, the filter discs were subsequently immersed in a 5 $ml \cdot disc^{-1}$ volume of ice-cold solution containing 5% trichloroacetic acid (TCA) and 5 mM methionine (4°C) (Bollum, 1959). The addition of non-labelled methionine helps dilute the specific activity of the added radioactive methionine which results in reduced background incorporation (zero-time control). Five minutes following the last time point sampled, the TCA was decanted and a fresh 5% TCA - 5 mM methionine solution was added and heated to 90°C. The solution was subsequently decanted following 5 min of incubation. The discs were washed twice by swirling (2 to 5 min per wash) in ice-cold 5% TCA - 5 mM methionine and twice in ice-cold ethanol:ether (3:1) to remove residual TCA and water. The discs were removed to a hood and air-dried. McKee et al. (1984) demonstrated through the use of proteins of known specific radioactivity that less than 2% of the protein originally present is lost during the steps of the aforementioned protocol.

Background incorporation

Samples removed from the reaction medium prior to incubation served as zero-time control. The background L-[^{35}S]methionine incorporation was measured in triplicate and subtracted from the rest of the results. Methionine incorporated was calculated by dividing the incorporated activity by the specific radioactivity of the medium. Specific radioactivity was measured by removing two 10 µL aliquots at time 0, diluting each aliquot with 100 µL of deionized water and subsequently placing three 10 µL samples in scintillation vials. A 0.5 ml of Soluene-350 and 15 ml of Hionic-fluor was added to each vial prior to counting. This procedure was performed in order to maintain

counts below an accurate counting limit of approximately 1 000 000 dpm.

Scintillation counting

Air-dried filter paper discs containing [^{35}S]-methionine labelled protein were placed in 20 ml glass-counting vials, dampened with 100 μL of deionized water, and the protein solubilised for 45 min in 0.5 ml of Soluene-350 (Packard) prior to counting. The purpose of this treatment is to elute the protein from the disc and to enhance the counting efficiency (93%). Fifteen ml of Hionic-fluor (Packard) scintillation fluid was added, and the vials capped and shaken vigorously. Samples were counted for 20 min or until 40,000 counts were accumulated using a Beckman liquid scintillation counter with a [^{14}C] window setting.

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APPENDIX C

MEASUREMENT OF IN VIVO FRACTIONAL RATE OF PROTEIN SYNTHESIS

The detailed methodology is a slight modification of that of Samuels (1993).

INJECTION OF FLOODING-DOSE OF [^{3}H] PHENYLALANINE (Phe)

Solutions

A. Phosphate buffered saline (PBS) (pH 7.4)

To make 100 mL:

845.0 mg	(145.0 mM)	NaCl	(MW 58.44)
227.2 mg	(16.0 mM)	Na_2HPO_4	(MW 142.0)
14.0 mg	(1.2 mM)	NaH_2PO_4	(MW 120.0)

- dissolve reagents in 75 mL Milli Q (MQ) water
- adjust to pH 7.4 with HCl
- bring volume to 100 mL with MQ water
- Autoclave 20 min or filter through a 0.2 μm Millipore filter

B. [^{3}H] Phe solution

The final solution contained 150 μCi $\cdot$ 100 g body weight^{-1} and 150 μmol Phe $\cdot$ 100g body weight^{-1} in 1.25 mL PBS $\cdot$ 100 g body weight^{-1}. Four percent additional solution was prepared in order to account for dead space in the syringe. This solution was made no more than one to two days in advance.

To make 25 mL:

563.2 mg Phe ($C_9H_{11}NO_2$) (MW 165.2)
PBS
[^{3}H] Phe (1 mCi $\cdot$ mL^{-1})

- dissolve Phe in 25 mL PBS
- this solution will take approximately one hour to dissolve
- draw out from isotope stock bottle 156 μL [^{3}H] Phe per 100 g body weight
- filter 1.144 mL of Phe solution per 100 g body weight through a 0.2 μ Millipore filter, and mix with the isotope

- draw up solution into a sterile syringe. The above volumes take into account the added four percent for dead space in the syringe.

Procedure

On the day of the experiment, animals were weighed and the syringe of radioactive solution prepared. Following administration of the intraperitoneal injection, the rat was placed in a CO_2 chamber at 19.5 minutes, after which it was euthanized by cervical dislocation at precisely 20 minutes. The rat was immersed in ice water for one minute, during which time the skin of both hindlimbs was quickly removed. This procedure was performed with the aim of rapidly halting isotope incorporation.

Once the carcass was withdrawn from the ice bath, one person removed the gastrocnemius muscle and placed it in liquid nitrogen for rapid freezing. This muscle was stored at -70°C and was later used to determine the intracellular specific radioactivity. The second individual rapidly extracted the whole contralateral hindlimb and placed it in ice-cold Buffer I. This procedure was performed to minimize variations in incorporation period in the different muscles. The muscles removed from the hindlimb were blotted dry, weighed, and used to isolate two subpopulations of mitochondria. Mitochondrial pellets were stored at -70°C and were later utilised to determine specific radioactivities of the protein-bound amino acids. See Appendix A for mitochondrial isolation procedures.

INTRACELLULAR FRACTION

Tissue homogenization

The objective of homogenizing the gastrocnemius muscle was to separate free from protein bound amino acids prior to determining the intracellular specific radioactivity.

Solution

2% Perchloric Acid ($HClO_4$)

To make 1 L:

- 28.6 mL 70% $HClO_4$
- bring volume to 1 L with dH_2O

Procedure

A 150 mg section of the gastrocnemius was homogenized using a motorised ground glass tissue homogenizer. The muscle was further cut into 2-3 pieces with a razor blade

while frozen, and then homogenized, on ice, at high speed with 3 mL of ice-cold 2% $HClO_4$. The muscle was homogenized until smooth. The homogenate was poured into a 15 mL screw capped Pyrex test tube. The homogenizer was rinsed twice with 0.5 mL 2% $HClO_4$ and the washings were combined with the homogenate. The homogenates were stored on ice until centrifuged.

The homogenate was centrifuged at 1000 x g for 15 min. The supernatant was decanted into a 10 mL test tube and saved for analysis of intracellular Phe. The pellet was discarded.

Intracellular fraction

The objective of this step was to precipitate perchlorate ions and neutralise the solution for subsequent enzymic conversion.

Solution

Saturated potassium citrate ($C_6H_5O_7K_3$)

To make 50 mL:

70 g potassium citrate

- bring volume to 50 mL with dH_2O
- keep adding 5 g of potassium citrate until saturation is achieved
- gently heat until dissolved
- cool

Procedure

Half the intracellular fraction volume of saturated potassium citrate was added to the intracellular fraction. A whitish crystalline precipitate formed. Samples were centrifuged at 2000 x g. The supernatant (intracellular fraction) was decanted into a 10 mL test tube and stored frozen. The pH was adjusted to between 6 and 7 when required. The precipitate was discarded.

MITOCHONDRIAL PROTEIN-BOUND FRACTION

The purpose of this step was to remove any free amino acids, then hydrolyse the protein-bound amino acids, remove the acid, and neutralize the solution for subsequent enzymic conversion.

Solutions

A. 2% $HClO_4$ (see above)

B. 6 N HCl

To make 500 mL:

250 mL concentrated HCl
250 mL dH_2O

- pour acid into water
- cool

C. 0.5 M Tri-sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) (pH 6.3)

To make 100 mL:

14.71 g tri-sodium citrate

- bring volume up to 100 mL with dH_2O
- adjust pH to 6.3 with HCl

Procedure

The protein pellet was washed with 4 mL ice-cold 2% $HClO_4$ for one hour, transferred to a 15 mL screw capped Pyrex test tube, and subsequently centrifuged for 15 min at 3000 x g. The supernatant was discarded. The washing procedure was performed twice after which 5 mL of 6 N HCl was added to the protein pellet. Test tubes were purged with nitrogen, capped with teflon lined screw caps, and heated to 105°C for 24 h. In order to prevent evaporation, test tubes were checked for chips on lips of test tubes. Following 0.5 h, caps were tightened and checked for evaporation, and tubes were vortexed. When evaporation occurred, lost volumes were replaced with 6 N HCl.

Following the 24 hr incubation, the acid was removed by vacuum at 65°C. The amino acids were resuspended in 0.5 mL of sodium citrate (pH 6.3). The pH was adjusted to 6-7 using NaOH, and stored frozen. The solution is ready for enzymic conversion.

ENZYME CONVERSION

The objective of this step was to convert Phe to ß-phenylethylamine (PEA) so that it may be separated from other amino acids such as tyrosine and tyramine that may have become labelled during the period of incorporation.

Solutions

A. 0.5 M sodium citrate (pH 6.3) (see above)

B. Enzyme suspension

The enzyme suspension contains 2 Units$\cdot mL^{-1}$ L-tyrosine decarboxylase (Sigma T4379), 1 mg$\cdot mL^{-1}$ pyridoxal phosphate (Sigma P9255) in 0.5 mL of 0.5 M sodium citrate (pH 6.3). The use of tyrosine decarboxylase can replace Phe decarboxylase because it is contaminated with the former, in addition to being less costly than Phe decarboxylase.

To make 25 mL of enzyme suspension:

50 units tyrosine decarboxylase
25 mg pyridoxal phosphate
0.5 M sodium citrate buffer (pH 6.3)

- combine ingredients
- bring volume to 25 mL with sodium citrate buffer
- the enzyme does not dissolve
- the suspension may be stored at - 20°C
- mix well prior to use

Procedure

One half a mL of enzyme suspension was added to 1.0 mL of supernatant fraction or 0.5 mL of neutralized hydrolysate. The tubes were stoppered and incubated for 17 h in a shaking water bath at 50°C. It is important to note that sufficient water must be added to the water bath to account for evaporation over the 17 h incubation period.

SOLVENT EXTRACTION

The purpose of this step was to separate the PEA from other amino acids. Beta-phenylethylamine was extracted by the method of Suzuki and Yagi (1976) as modified by McAllister (1987).

Solutions

A. 3 M NaOH (MW 40.01)

To make 100 mL:

12 g NaOH

- dissolve in dH_2O and bring volume to 100 mL

B. 0.1 N H_2SO_4

To make 500 mL

1.4 mL concentrated sulphuric acid

- add to 500 mL of dH_2O

C. Chloroform:n-heptane (1:3 v/v)

Use distilled reagent grade chloroform and heptane. Do not use HPLC grade chloroform.

Procedure

One half a mL of 3 M NaOH was added to the entire incubated enzyme solution. Amino acids become basic and polar; PEA becomes neutral and non-polar. Five mL of chloroform-n-heptane was subsequently added. Tubes were screw capped, shaken, and centrifuged at 500 g for 5 min. Amino acids remain in the aqueous phase because they are polar; PEA is extracted into the organic phase because it is non-polar. The upper organic layer was carefully removed by pipette. Care was taken not to contaminate the organic layer with any of the bottom aqueous phase. Chloroform is more dense than water; the organic layer is on top because n-heptane, which is miscible in chloroform, makes the organic solution less dense. The aqueous phase was discarded.

Five mL of chloroform and 2 mL of H_2SO_4 were added to the organic phase. Tubes were screw capped, shaken, and subsequently centrifuged at 500 x g for 5 min. Under acidic conditions PEA is polar and is extracted into the aqueous phase. The top aqueous phase was removed by pipette. The organic layer settles to the bottom because the added chloroform makes the organic solution more dense. The organic layer was discarded. The 0.1 N H_2SO_4 containing PEA was left uncovered at room temperature to ensure all traces of chloroform were removed. Residual chloroform results in poor pipetting. Once pipetted, solutions were tightly covered and stored frozen.

High Performance Liquid Chromatography (HPLC)

The purpose of this step was to measure the concentration of PEA in the final aqueous extract.

Solutions

All solutions were made up in glassware that was rinsed with HPLC grade water. Chemicals were always weighed on the same balance, and pipettes were calibrated prior to use.

A. 25 nmol·mL^{-1} Ethanolamine (EA)
(C_2H_7NO·HCl) (MW 97.54)
Internal Standard

To make 100 mL:

0.04877 g EA

- dissolve EA in HPLC grade water and bring volume to 100 mL
- this stock solution (5 µmol·mL^{-1}) is stored at 0-5°C and may be used for 3 d

To make working solution:

- combine exactly 0.500 mL of stock solution with 100 mL of HPLC grade water
- the working solution is made daily

B. 25 nmol·mL^{-1} ß-Phenylethylamine (PEA)
($C_8H_{11}N$·HCl) (MW 157.6)
Internal / External Standards

To make 100 mL:

0.0788 g PEA

- dissolve PEA in HPLC grade water and bring volume to 100 mL
- this stock solution (5 µmol·mL^{-1}) is stored at 0-5°C and may be used for 3 d

To make working solution:

- combine exactly 0.500 mL of stock solution with 100 mL of HPLC grade water
- the working solution is made daily

C. Buffer A

To make 2 L:
11.5 mL glacial acetic acid
8.0 g NaOH
180 mL HPLC grade methanol
10 mL tetrahydrofuran

- dissolve acid and base in 1600 mL HPLC grade water
- adjust pH to 7.2 with NaOH
- add methanol and tetrahydrofuran
- bring volume to 2 L with HPLC grade water

* alternately, 27.2 g sodium acetate (NaAc) can replace the glacial acetic acid and the NaOH. The pH is adjusted with glacial acetic acid rather than NaOH.

D. O-phthaldehyde (OPA) solution

To make 250 mL:

1 g OPA (Sigma P1378)
25 mL HPLC grade methanol
5 mL Brij 35
1 mL 2-mercaptoethanol
219 mL 0.4 M sodium borate buffer (see below)

- dissolve OPA in methanol in a dark bottle
- add Brij 35, 2-mercaptoethanol, and 0.4 M sodium borate buffer
- refrigerate
- solution may be used for 1 or 2 weeks
- non refrigerated OPA may be used for 1 d

E. 0.04 M Sodium borate buffer ($Na_2B_4O_7 \cdot (10H_2O)$) pH 9.5

To make 1 L:

15.234 g sodium borate

- dissolve sodium borate in 900 mL HPLC grade water
- adjust pH to 9.5 with NaOH
- bring volume to 1 L with HPLC grade water

F. Saturated potassium borate

To make 1 L:

30 g potassium borate ($K_2B_4O_7 \cdot (4H_2O)$)

- add potassium borate to 1 L HPLC grade water
- mix
- add solute until no more will dissolve

G. Standard (for free PEA)

EA (25 $nmol \cdot mL^{-1}$)
PEA (25 $nmol \cdot mL^{-1}$) in saturated potassium borate buffer
0.1 N H_2SO_4

- combine in ashed or new HPLC vials in proportions of 1:2:2

- cover with septa and cap
- shake and vortex

These proportions may vary slightly depending upon the concentration of PEA in the samples.

H. Standard (for mitochondrial protein-bound PEA)

To make amino acid standard solution:

50 μL EA
(10X dilution of stock solution: 500 nmol$\cdot$mL^{-1})
50 μL PEA
(10X dilution of stock solution: 500 nmol$\cdot$mL^{-1})
25 μL 0.1 N H_2SO_4
1 mL HPLC grade water

- combine in a test tube
- vortex

To make standard:

0.25 mL amino acid standard solution
0.20 mL saturated potassium borate buffer
0.80 mL HPLC grade water

- combine in ashed or new HPLC vials
- cover with septa and cap
- shake and vortex

I. Free PEA sample

sample
EA (25 nmol$\cdot$mL^{-1}) in saturated potassium borate buffer
HPLC grade water

- combine in ashed or new HPLC vials in proportions of 2:2:1
- cover with septa and cap
- shake and vortex

These proportions may vary slightly depending upon the concentration of PEA in the samples.

J. Mitochondrial protein-bound PEA sample

To make sample amino acid solution:

25 μL sample
50 μL EA
(10X dilution of stock solution: 500 $nmol \cdot mL^{-1}$)
1.05 mL HPLC grade water

To make sample for injection:

0.25 mL sample amino acid solution
0.20 mL saturated potassium borate buffer
0.80 mL HPLC grade water

- combine in ashed or new HPLC vials
- cover with septa and cap
- shake and vortex

These proportions may vary slightly depending upon the concentration of PEA in the samples.

Procedure

The HPLC consisted of a Varian 5000 high performance liquid chromatograph coupled to a Varian Fluorochrome detector. The fluorometer was set at an excitation range of 340-380 nm and an emission cutoff at 390 nm. Beta-phenylethylamine and EA were derivitized with OPA immediately prior to sample injection onto the column (Jones and Gilligan, 1983). A C-18 reverse phase column (Supelco) (75 x 4.6 mm I.D.; 40-60 μm particle size) was utilized for all samples. Chromatographic peaks were recorded and integrated using a Shimadzu Ezchrom Chromatography Data System. Two solvents, Buffer A and methanol (B), were used to form the following gradient: 40% B at 0 min to 90% at 4.5 min, 90% B from 4 min to 4.5 min, and 40% B from 4.5 min to 10.3 min. The flow rate was 1.5 $mL \cdot min^{-1}$.

Each sample was measured in duplicate. When the percent difference between duplicates was greater than 5%, the analysis was repeated. One standard was utilized for every 10 samples. There must only be two main peaks; other peaks signify poor solvent extraction of PEA. High performance liquid chromatography was performed prior to liquid scintillation counting in case re-extraction was necessary.

LIQUID SCINTILLATION COUNTING

Pipettes were calibrated carefully. A volume of 150 to 500 μL of the final extract containing PEA in 0.1 N H_2SO_4 was pipetted into a 7 mL plastic scintillation vial. Samples were counted in duplicate. Five mL of scintillation fluid (Eco-Lite) was added, and vials were capped and shaken vigorously. Vortexing does not work. Samples were kept away from any form of light source for approximately 1 h, and subsequently counted for 20 min or until 40,000 counts were accumulated using a Beckman liquid scintillation counter. Samples were monitored for two phases, chemiluminescence, and photoluminescence. In practice this was not necessary. The coefficients of variation between duplicates were less than 5%.

SPECIFIC RADIOACTIVITY OF PHE

The specific radioactivity (SRA) of Phe is equal to that of PEA, assuming the tritium atom on (^{3}H)Phe has no effect on its conversion to PEA. The SRA of PEA was calculated by dividing the radioactivity of the final extract by the concentration of PEA in the final extract:

$$\text{SRA of PEA} = (\text{DPM} \cdot \text{mL}^{-1}) \div (\text{nmol} \cdot \text{mL}^{-1})$$
$$= \text{DPM} \cdot \text{nmol}^{-1}$$

CALCULATION OF FRACTIONAL RATE OF PROTEIN SYNTHESIS

The fractional rate of protein synthesis (k_{syn}) ($\% \cdot \text{day}^{-1}$) for each tissue was calculated from the formula of McNurlan et al. (1979) and Garlick et al. (1980):

$$k_{syn} = S_b \div (S_i \cdot t) \cdot 100$$

where S_b is the specific radioactivity of mitochondrial protein-bound Phe, S_i is the specific radioactivity of tissue free Phe, and t is time of incorporation expressed in days.

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