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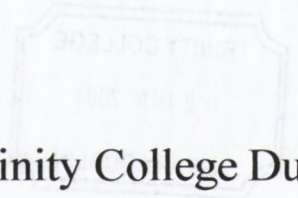
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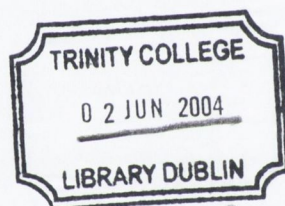
Oliver James Patrick Joyce B.A. (Mod)

A thesis submitted for the degree of Doctor of Philosophy
at the University of Dublin



Trinity College Dublin

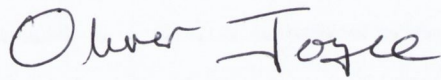
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Declaration

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A handwritten signature in black ink, reading "Oliver Joyce". The signature is written in a cursive style with a large 'O' and a long horizontal stroke for the 'y'.

Oliver J.P. Joyce

Dedicated to Lorna

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Summary

Experiments were designed to investigate for the first time *in situ* synaptosomal mitochondrial bioenergetic function using synaptosomes prepared from the whole brain of rats and to compare bioenergetic functions in synaptosomes isolated from young (3 month) and old (24 month) whole rat brain. The investigation describes for the first time the pattern of control of mitochondrial energy metabolism based on the kinetic responses of the substrate oxidation, proton leak and phosphorylation systems to changes in the mitochondrial membrane potential ($\Delta\Psi_m$) in synaptosomes isolated from the whole brain of rat. In addition, a comparison was made of the aforementioned kinetic responses, in synaptosomes isolated from old and young animals.

There was a significantly lower rate of oxygen consumption in synaptosomes isolated from the old rats in the presence of uncoupler, when compared with synaptosomes isolated from young rats. The essential difference between synaptosomes isolated from old rats, when compared with synaptosomes isolated from young rats, is an impaired flux through the synaptosomal substrate oxidation system. There were no significant age differences in the kinetic responses of the substrate oxidation system, the mitochondrial proton leak and the phosphorylation system to changes in the proton electrochemical gradient. However, as synaptosomes from whole rat brain were used in these studies, it is possible that greater levels of impairment may occur in specific brain regions.

This was the first time metabolic control analysis was applied to synaptosomal *in situ* mitochondria. Metabolic control analysis was applied to synaptosomal *in situ* mitochondria isolated from young and old rats to determine if the flux control coefficients change with age. The pattern of control was similar in synaptosomes isolated from young and old whole rat brain. The flux control coefficients show that the control of flux through the substrate oxidation system, the proton leak and control of the mitochondrial membrane potential was mainly by the substrate oxidation system at maximal ATP-producing capacity. Proton leak, as expected, had little control at maximal ATP-producing capacity. Control of the flux through the phosphorylation system was mainly by the substrate oxidation system in young rat brain synaptosomes, but mainly by

the phosphorylating system itself in the old rat brain synaptosomes, however, the substrate oxidation system has a similar degree of control of flux through the phosphorylating system. The control over the mitochondrial membrane potential lies with the substrate oxidation system in synaptosomal *in situ* mitochondria isolated from young and old rat brains. The P/O ratio was controlled mainly by the phosphorylating system in young and old rat brain synaptosomes.

Chapter 5 showed that the use of nagarse in the isolation of muscle mitochondria, namely beef heart, altered the polypeptide pattern of mitochondrial membrane proteins and in particular complexes I, II, III, IV and V when resolved using blue native/tricine-SDS-PAGE two-dimensional electrophoresis. Any work on muscle tissue mitochondria isolated using nagarse will have to consider that mitochondrial proteins are digested so that the characteristic protein pattern of mitochondrial proteins resolved in two dimensions is absent when compared with mitochondria isolated without using nagarse.

The results in Chapter 6 are novel in that this is the first time blue native/tricine-SDS-PAGE two-dimensional electrophoresis was used in an aging comparison or for a comparison of mitochondria isolated from euthyroid and hyperthyroid animals. In the age comparison, significant differences were found in a comparison of mitochondria isolated from young rat liver with mitochondria isolated from old rat liver. The levels of complexes I, III, IV and V, when normalised per mg protein and when normalised to citrate synthase activity, were shown to be significantly less in liver mitochondria isolated from old rats compared with liver mitochondria isolated from young rats. However, only complex IV showed a significant decrease when normalised to the in gel complex V in liver mitochondria isolated from old rats compared with liver mitochondria isolated from young rats. The results found in the rat liver mitochondria comparison were surprising given that liver is a mitotic tissue and aging effects are hypothesized to be manifest greater in post mitotic tissues. The levels of complexes I, III, IV and V were shown to have increased in synaptic mitochondria isolated from old rats compared with synaptic mitochondria isolated from young rats when the absorbances were normalised to citrate synthase activity. When the levels of complexes I, III and V were normalised to

the in gel complex IV, there were no significant differences in mitochondria isolated from young and old rat brain synaptosomes. The results obtained for synaptic mitochondria were novel in that this was the first time proteins from synaptic mitochondria isolated from whole brain were resolved using blue native/tricine-SDS-PAGE two-dimensional electrophoresis. Previously, Schägger and Ohm (1995) used blue native/tricine-SDS-PAGE two-dimensional electrophoresis to compare hippocampal mitochondrial proteins of control and Alzheimer's brain, that is to say, there was no distinction between synaptic and non-synaptic mitochondria.

The results shown in Chapter 7 are novel because this is the first time mitochondria isolated from rat heart, rat kidney, rat brown adipose tissue, rat skeletal muscle mitochondria and rat brain synaptic mitochondria were resolved using blue native/tricine-SDS-PAGE two-dimensional electrophoresis. Rat brown adipose tissue mitochondria were different when compared to mitochondria from the other rat tissues examined and mitochondria from beef heart. In rat brown adipose tissue mitochondria, complex I and complex V were greatly reduced relative to the level of complex IV. The relative level of complex I in rat brown adipose tissue was compared to the relative levels of complex I in the other rat tissues and in beef heart mitochondria. It was found that relative to complex IV, the level of complex I in rat brown adipose tissue was significantly less than the level of complex I found in rat heart mitochondria, rat kidney mitochondria, rat liver mitochondria and beef heart mitochondria. The finding that rat brown adipose tissue mitochondria have a reduced content of complex I, as shown by blue native/tricine-SDS-PAGE two-dimensional electrophoresis is novel and unexpected as there have been no reports of reduced amounts of complex I in BAT mitochondria.

Our results showing a decreased level of complex V in rat brown adipose tissue mitochondria confirm previous reports that showed the content of complex V was exceptionally low in brown adipose tissue (Cannon and Vogel, 1977, Houstek et al., 1978). Here is the first time that the low content of complex V in BAT was demonstrated using blue native/tricine-SDS-PAGE two dimensional electrophoresis. The relative level of complex V in rat brown adipose tissue was compared to the relative levels of complex

IV in the other rat tissues and in beef heart mitochondria. It was found that relative to complex IV, the level of complex V in rat brown adipose tissue was significantly less than the level of complex V found in rat heart mitochondria, rat kidney mitochondria, rat liver mitochondria, rat skeletal muscle mitochondria, rat synaptic mitochondria, and beef heart mitochondria.

The finding that different amounts of maltoside were required to solublize mitochondrial proteins from different mitochondria was novel. Based on a comparison of the work of Rolfe *et al.* (1994) who determined the lipid: protein ratio (mg/mg) of mitochondria isolated from rat liver, kidney, brain and skeletal muscle, it would seem that the higher the lipid: protein ratio of mitochondria, the more maltoside required to solubilize mitochondrial membrane proteins. In general the polypeptide patterns of the mitochondrial membrane proteins were different in all of the mitochondria examined, however the significance of these differences isn't known because the identity of many of the proteins isn't known. Specifically, the polypeptide pattern of complexes I, III, IV and V was identical when resolved using blue native/tricine-SDS-PAGE two-dimensional electrophoresis in all of the mitochondria examined.

Abbreviations and Glossary

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
A6	A6 subunit of complex V
B_{max}	Maximum binding
BSA	Bovine serum albumin
COIII	Subunit III of complex IV
DNA	Deoxyribonucleic acid
DTNB	5,5-dithio-bis-nitrobenzoic acid
DTT	Dithiothreitol
Δp	Proton electrochemical gradient
ΔΨ	Mitochondrial membrane potential
EDTA	Ethylenedinitrilo tetraacetic acid
EGTA	Ethyleneglycol-bis(aminoethylether)-N,N-tetraacetate
FADH	Flavin adenine dinucleotide (reduced)
FCCP	Carbonyl cyanide <i>p</i> -trifluoromethoxyphenylhydrazone
Hepes	<i>N</i> -2-Hydroxyethylpiperazine- <i>N'</i> -2-ethane sulfonic acid
J	Flux through a pathway; measured as rate of oxygen consumption
kDa	Kilodaltons
mtDNA	mitochondrial DNA
mV	millivolts
β-NADH	β-nicotinamide adenine dinucleotide (reduced form)
OSCP	Oligomycin sensitivity conferring protein
PBS	phosphate-buffered saline
P/O	Adenosine diphosphate/oxygen ratio
RNA	Ribonucleic acid
RCR	Respiratory control ratio (state 3/state 4)
ROS	Reactive Oxygen Species
SD	Standard deviation

SEM	Standard error of the mean
TPB⁻	Tetraphenyl boron ion
TPMP⁺	Triphenylmethyl phosphonium
UV	Ultraviolet
v/v	Volume per unit volume
V_c	Cytoplasmic volume of synaptosomes
V_m	The volume of synaptosome that is mitochondrial
V_{max}	Maximum velocity
w/v	Weight per unit volume
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

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Chapter 1

Introduction

Chapter 1 is an introduction explaining the theory behind the research in this thesis and an explanation of the methodology used, and the aim of the project. Chapter 2 is the materials and methods chapter, listing the reagents and methods used. Each results chapter starts with a brief introduction, followed by results and ends with a discussion of those results. Chapter 3 is the first of five results chapters and deals with the investigation of *in situ* synaptosomal mitochondrial bioenergetic function using synaptosomes prepared from the whole brain of rats and compares bioenergetic functions in synaptosomes isolated from young (3 month) and old (24 month) rat brains. In Chapter 4, top-down metabolic control analysis was used to acquire a general overview of the control structure of the energy pathways in synaptosomes isolated from young (3 month) and old (24 month) rat brains using some of the results in Chapter 3. Chapter 5 determined the effect that nagarse had when used in the isolation of beef heart mitochondria on the mitochondrial polypeptide pattern when the mitochondrial proteins were resolved using blue native/tricine-SDS-PAGE two-dimensional electrophoresis. In Chapter 6 mitochondria were isolated from young (3 month) and old (24 month) rats and compared using blue native/tricine-SDS-PAGE two dimensional electrophoresis to determine if there were any differences in the polypeptide pattern of the mitochondrial proteins when resolved in the second dimension and to compare the levels of complexes I, III, IV and V in mitochondria isolated from young (3 month) and old (24 month) rats. Chapter 7 deals with the comparison of mitochondria isolated from various rat tissues in an approach similar to that taken in Chapter 6. Mitochondria were isolated from rat tissues and compared using blue native/tricine-SDS-PAGE two-dimensional electrophoresis to determine if there were any differences in the polypeptide pattern of the mitochondrial proteins when resolved in the second dimension and to compare the levels of complexes I, III, IV and V in mitochondria isolated from rat tissues. Chapter 8 highlights the conclusions drawn from the research and outlines the main results, what is novel about the results and puts the results into perspective.

1.1 Energy Metabolism and Oxidative Phosphorylation

Oxidative phosphorylation is the process by which aerobic organisms make ATP and it occurs in the mitochondria of cells (Fig. 1.1.1). ATP is the main energy intermediate in the cell and is thus used to distribute free energy to the energy requiring reactions in the cell (Rolfe and Brown, 1997). Reducing equivalents such as NADH and FADH₂, derived from the oxidation of reduced compounds (the carbohydrate or fatty acid molecules derived directly from the diet or released from fuel stores in the body), feed electrons into the electron transport chain, which is located within the mitochondrial inner membrane. The ultimate acceptor of these electrons is O₂, which goes on to form water. The electron transport chain is made up of complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), coenzyme Q, complex III (cytochrome *c* reductase), cytochrome *c* and complex IV (cytochrome *c* oxidase). As electrons pass down the electron transport chain, protons are translocated across the mitochondrial inner membrane from the matrix to the intermembrane space, setting up a delocalised proton electrochemical gradient termed Δp . Δp can be used to do useful work such as (1) transport metabolites across the mitochondrial inner membrane (*e.g.* ADP/ATP exchange and phosphate/proton symport) and (2) synthesis of ATP from matrix ADP and phosphate by driving protons through the ATP synthase (complex V), which is located in the mitochondrial inner membrane and extends into the matrix. Hence, ATP synthesis is coupled to oxygen consumption via the proton electrochemical gradient.

Oxidative phosphorylation is the principle source of ATP at rest. However, studies of oxidative phosphorylation have shown that ATP synthesis is not perfectly coupled to oxygen consumption in intact mitochondria (Porter *et al.*, 1996, Porter and Brand, 1993). The reason is that the mitochondrial inner membrane is not absolutely impermeable to protons, *i.e.* there is an inefficiency, namely a leakage of protons across the mitochondrial inner membrane (Brand *et al.*, 1994).

It seems that the *in situ* mitochondria in cells and tissues are in between state 3 and state 4. State 3 is the maximum phosphorylating condition and is defined as the oxygen

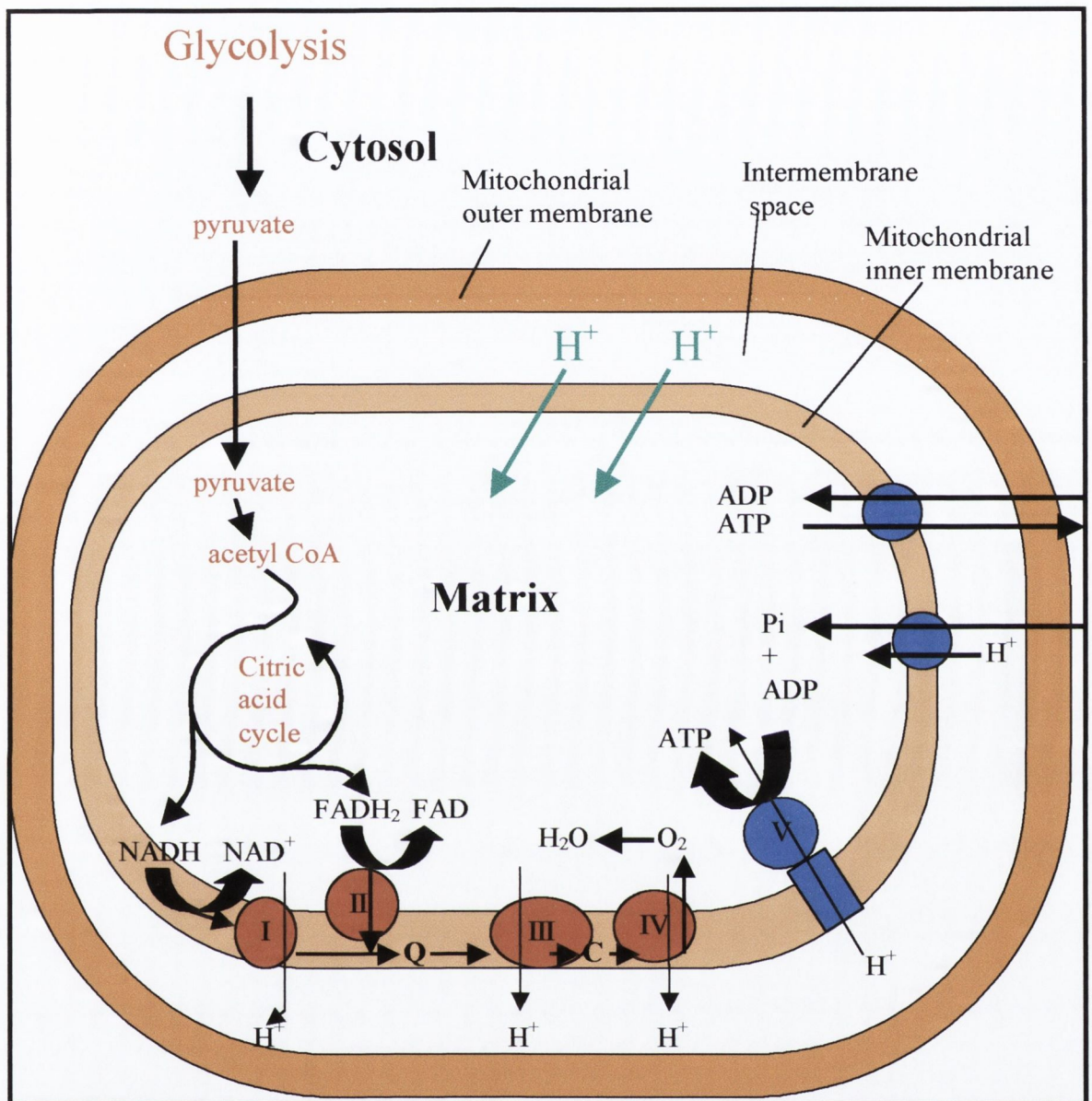


Figure 1.1.1 Schematic representation of oxidative phosphorylation. See text for details. Diagram labels are as follows: I: complex I (NADH dehydrogenase); II: complex II (succinate dehydrogenase); III: complex III (cytochrome *c* reductase); IV: complex IV (cytochrome *c* oxidase); V: complex V (ATP synthase); Q: coenzyme Q; C: cytochrome *c*. The substrate oxidation system is in red, the proton leak in green and the phosphorylation system is in blue.

consumption rate in the presence of substrate, ADP and inorganic phosphate. State 4 is the non-phosphorylating condition. The state 4 oxygen consumption rate is due to proton leak across the mitochondrial inner membrane. Hence, it has been shown that the oxygen consumed by *in situ* mitochondria in hepatocytes, thymocytes and lymphocytes is not due solely to ATP demand, but also a significant proportion of it is consumed by proton leak (Brand *et al.*, 1994).

1.2 Aging

Throughout adult life, many physiological functions gradually decline with age. There is a diminished capacity for cellular protein synthesis, a decline in immune function, an increase in fat mass, a loss of muscle mass and strength, and a decrease in bone mineral density (Lamberts *et al.*, 1997). Aging appears to be a highly complex biological process and is associated with a progressive decline in the performance of most organs. The possible role of mitochondria as pacemakers of aging has been increasingly investigated as they are the organelles responsible for energy production in humans and throughout animal phyla. Across species, the mitochondria of old organisms have been reported to show a decrease in number, an increase in size and the occurrence of structural abnormalities (Bittles, 1989).

1.2.1 Changes in mitochondria associated with aging

The free-radical theory of aging says that free radicals cause the progressive and irreversible damage and deterioration of cellular function (Harman, 1956). Free radicals are compounds with an unpaired electron in their outer orbital that renders them highly reactive, and they tend to attack neighbouring compounds autocatalytically. Free radicals can result from the exposure of cells and organelles to ionizing radiation but the free-radical theory of aging emphasizes the role of oxygen-radical-initiated tissue damage. A number of reactive species are derived from molecular oxygen, including the superoxide and hydroperoxyl radicals, hydrogen peroxide, hydroxyl radical and singlet oxygen. Polyunsaturated fatty acid side chains of cell and organelle membranes serve as highly susceptible targets for the action of oxygen radicals. Lipids undergo a chain reaction, known as lipid peroxidation, that may result in severe damage and the eventual death of

the cell (Gutteridge *et al.*, 1986). Due to the close association of lipids and proteins in membranes, oxidative damage to mitochondrial proteins as a direct result of oxidative stress or as a result of lipid peroxidation can result in protein cross-linking, degradation of proteins and loss of function (Van Remmen and Richardson, 2001).

Enzymic reactions involved in the respiratory chain serve as primary sources of free radicals. Lesser levels of free radicals are formed during phagocytosis, in prostaglandin synthesis and in the cytochrome P-450 system. The inner mitochondrial membrane is thought to be the site of maximal, potential free radical damage as it is the location for oxidative phosphorylation. Even though there are defences to deal with free radicals, they are not perfect and it is estimated that 1-4% of electrons escape the electron transport chain and go on to form free radicals (Gadaleta *et al.*, 1999). However, it has been suggested that only irreversibly differentiated (post-mitotic) cells, such as those of the heart, blood vessels and brain, are severely affected, since, in fast replicating (mitotic) cells, frequent turn-over of macromolecules can take place during mitochondrial division at mitosis (Miquel and Fleming, 1986). ROS (Reactive Oxygen Species) have been shown to be produced at a higher rate in mitochondria of organisms with short maximum life spans (Herrero and Barja, 1998), suggesting that ROS production is related to aging. Nohl *et al.* (1978) showed that coupled heart mitochondria from old rats produce oxygen radicals more actively than those from young controls.

Most human cells have hundreds of mitochondria, and each mitochondrion contains multiple mitochondrial DNAs which code for 13 essential polypeptides of the oxidative phosphorylation pathway plus the 22 transfer RNAs and 2 ribosomal RNAs for mitochondrial protein synthesis (Wallace *et al.*, 1995) (Fig. 1.2.1.1). Thus, the proteins of oxidative phosphorylation are assembled from gene products encoded by both the nuclear DNA and mitochondrial DNA. The 13 polypeptides encoded by the mitochondrial DNA include 7 subunits of complex I, 1 subunit of complex III, 3 subunits of complex IV and 2 subunits of complex V (Anderson *et al.*, 1981; Wallace *et al.*, 1995).

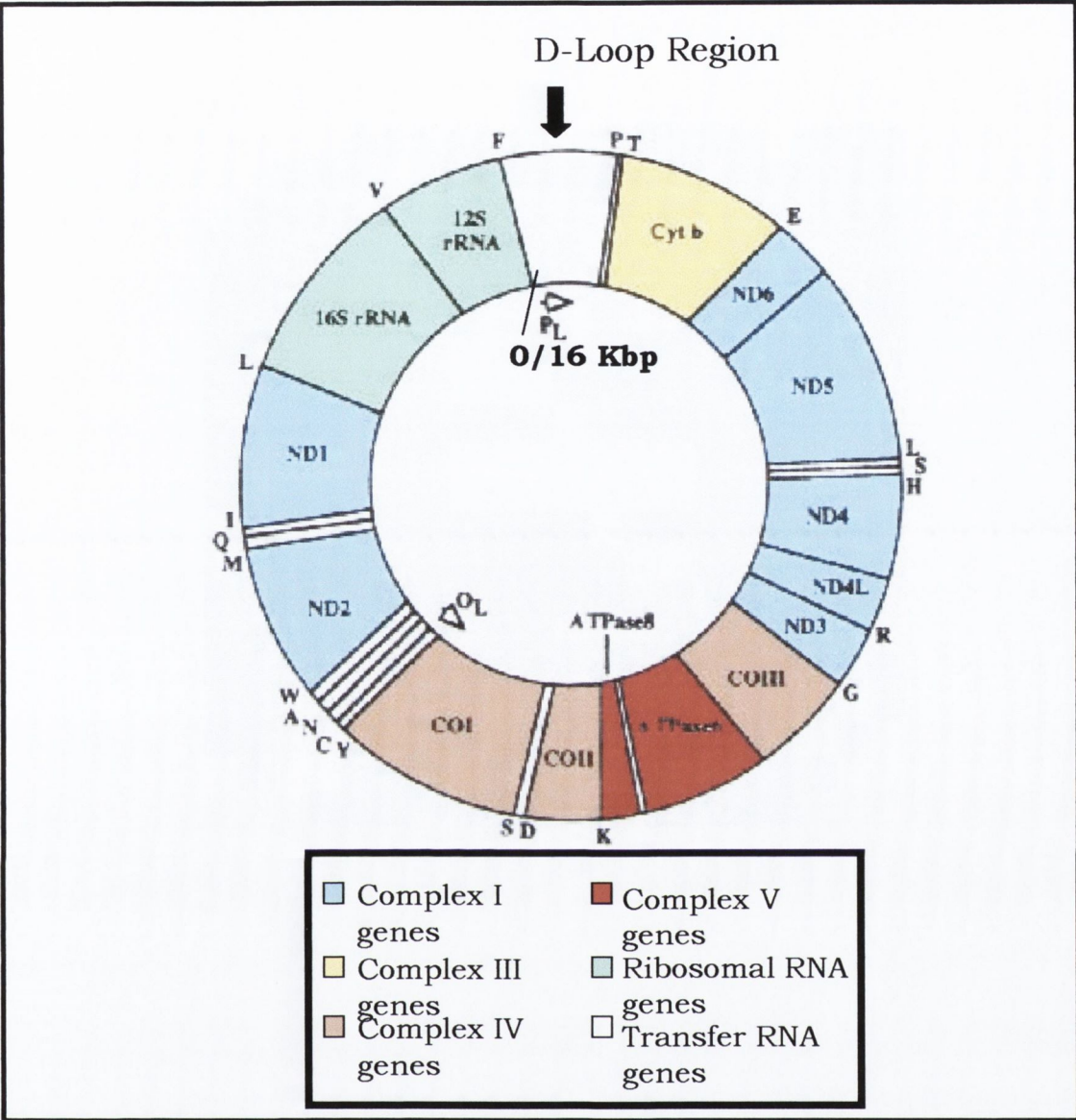


Figure 1.2.1.1 Schematic representation of the human mtDNA. The mtDNA codes for 37 genes: 13 oxidative phosphorylation polypeptides, 12S and 16S rRNAs and 22 tRNAs that punctuate the polypeptide and rRNA genes. The positions of the genes coding for components of the oxidative phosphorylation pathway are highlighted by various types of shading. The 13 polypeptides encoded by the mitochondrial DNA include 7 subunits of complex I, 1 subunit of complex III, 3 subunits of complex IV and 2 subunits of complex V. The single letters on the outside of the schematic represent the relevant transfer RNA.

Mitochondrial DNA is located in the mitochondrial matrix near the mitochondrial inner membrane where reactive oxygen species, which cause oxidative damage of DNA, are generated by the respiratory chain. The DNA in mitochondria lacks protective proteins like histones (Richter, 1992) and mitochondria lack the capacity to repair UV-induced pyrimidine dimers (Clayton *et al.*, 1974). Mitochondria can, however, repair oxidative damage to mitochondrial DNA, e.g. damage to bases and single-strand breaks (Croteau and Bohr, 1997). It has been estimated that mitochondrial DNA has a 10-times higher rate of mutation than the nuclear genome (Wallace *et al.*, 1995). The mitochondrial DNA undergoes a continuous turnover both in mitotic and post-mitotic cells and this increases the chance of mutations (Richter, 1992). As a result, mutated DNA molecules can accumulate with age in post-mitotic tissues. There is evidence showing that maternally inherited and somatic mutant mitochondrial DNA molecules are preferentially amplified in post-mitotic tissues (Wallace *et al.*, 1995).

The accumulation of mitochondrial DNA mutations in post-mitotic tissues may contribute to a number of age-associated diseases such as Alzheimer's (Davis *et al.*, 1997). While this explains why mutated mitochondrial DNA molecules accumulate with age and can determine oxidative phosphorylation decline, the molecular mechanism by which their preferential amplification takes place is not known.

A wide variety of mitochondrial DNA mutations have been identified in degenerative diseases of the brain, heart, skeletal muscle, kidney and endocrine system (Wallace *et al.*, 1995). Generally individuals who inherit these mutations are relatively normal in early life, develop symptoms in childhood, midlife or old age, depending on the severity of the maternally inherited mitochondrial DNA mutation and then undergo a progressive decline. The features of these mitochondrial DNA diseases are proposed to be a product of the high dependence of the target organs on mitochondrial bioenergetics, the cumulative oxidative phosphorylation defect caused by the inherited mitochondrial DNA mutation, together with the age-related accumulation of mitochondrial DNA mutations in post-mitotic tissues.

Hudson *et al.* (1998) reported a decrease in the rate of mitochondrial protein synthesis in old rat heart mitochondria when compared to those from young animals. A decrease in the rate of mitochondrial protein synthesis may contribute to a decline in those complexes that have mitochondrially encoded subunits but especially complex IV activity since the mitochondrial encoded subunits of complex IV make up the catalytic core of the enzyme (Kim *et al.*, 1995). An age-associated increase in oxidative damage to mitochondrial protein has been observed (Sohal and Dubey, 1994). The accumulation of oxidized dysfunctional protein with reactive carbonyl groups could lead to inter- and intramolecular cross-links with protein amino groups, which would then cause loss of function in mitochondria. Thus, the age-related accumulation of protein oxidation products in mitochondria may also lead to a loss of energy production and increased production of oxidants.

Aging has been correlated with decreased inner membrane cardiolipin and linoleic acid levels (Paradies *et al.*, 1997). Cardiolipin is unique to bioenergetic membranes. It is localised within the mitochondrial inner membrane where it is synthesized (Hostetler and Van den Bosh, 1972) and is essential for proper enzyme and transporter conformation and activity in the inner membrane. A decrease in the levels of cardiolipin with age would seem to imply that the enzymes and transporters with which cardiolipin is associated would show a decline or a dysfunction with age. Linoleic acid is necessary for the optimal interactions of cardiolipins with enzymes and transporters of the mitochondria. It has been hypothesized that decreases in inner membrane lipids containing linoleic acid and altered cardiolipin-protein interactions may lead to decreased State 3 and State 4 respiration rates and contribute to the loss of efficiency in mitochondrial function (Shigenaga *et al.*, 1994). Aging may also affect cardiolipin acylation, the effects of which could result in apoptosis through cardiolipin's interaction with cytochrome *c* (McMillin and Dowhan, 2002). In addition, it has been reported that there is an increase in the mitochondrial inner membrane long-chain fatty acids with age. An increase in long chain fatty acids has been positively correlated with an increase in mitochondrial proton leak (Paradies *et al.*, 1997; Porter, 1996). An age-related increase in mitochondrial inner-membrane fatty acids would suggest that there could be an

increase in the proton leak rate across the mitochondrial inner membrane with age. Brand (2000) put forward the idea, seeing that proton leak occurs in ectotherms, that thermogenesis is probably not the primary function of proton leak. Instead, the function of proton leak could be to decrease the production of superoxide and other ROS. A decrease in the production of ROS could be important in minimising oxidative damage to mitochondrial DNA and slowing aging. Mitochondria are the major source of cellular ROS. Increased mitochondrial proton leak leads to the oxidation of ubiquinone and decreased ROS production in isolated mitochondria. A study of mouse hepatocyte *in situ* mitochondria showed an increase in mitochondrial proton leak in hepatocytes isolated from old (30 months) compared with young (3 month) mice (Harper *et al.*, 1998).

A study on energy thresholds in brain mitochondria used non-synaptic brain mitochondria to examine the relationship between individual respiratory-chain complexes and oxidative phosphorylation (Davey *et al.*, 1998). Complexes I, III and IV were titrated with specific inhibitors and the extent to which the activity of a complex could be inhibited before causing impairment of mitochondrial energy metabolism was determined. Complex I, III and IV activities were decreased by approximately 25, 80 and 70% respectively before changes in the rates of oxygen consumption and ATP synthesis were observed. The decrease of 25% in complex I activity suggests that complex I activity has a major control of oxidative phosphorylation, such that, when a threshold of 25% inhibition is exceeded, energy metabolism is impaired resulting in reduced synthesis of ATP. The study implies that complex I is very important in oxidative phosphorylation as a decrease in complex I activity of just 25% will impair oxidative phosphorylation. In fact, Ventura *et al.* (2002) found that the flux control coefficients of complex I increased with age in old (30 month) rat liver mitochondria compared with young (4 month) rat liver mitochondria, meaning that complex I becomes more rate-limiting with age. Ventura *et al.* (2002) also found that state 3 and state 4 respiration decreased significantly with age. The threshold study by Davey *et al.* (1998) also used the catecholaminergic PC12 cell line, which is depleted of GSH, to observe the consequences of GSH depletion on mitochondrial function. Depletion of GSH in PC12 cells reduces complex I activity and abolishes the threshold effect. The study also highlights the importance of

antioxidants in mitochondrial function, findings that have implications with regard to aging.

Analysis of the enzymes of oxidative phosphorylation in post-mitotic tissue has revealed that the specific activities of the respiratory complexes decline with age (Trounce *et al.*; 1989, Yen *et al.*, 1989). The decline of the specific activities of the respiratory complexes with age has been correlated with the progressive accumulation of somatic mitochondrial DNA mutations in post-mitotic tissues. Nohl *et al.* (1978) reported a decrease in ATP hydrolysis catalyzed by heart mitochondria when 23-month-old rats were compared with 3-month-old controls. It has also been shown that there is an age-related decrease in the ATPase activity of the F_0F_1 ATP synthase in sonicated submitochondrial particles isolated from 24-month-old rat brain and rat heart compared to those isolated from 3- and 6-month-old rats (Guerrieri *et al.*, 1992). The B_{max} for cytochrome *c* binding to cytochrome oxidase decreases in synaptosomal and non-synaptic mitochondria isolated from cerebral cortex of 30-month-old rats compared with 4-month-old rats (Curti *et al.*, 1990). In mice synaptic mitochondria, a significant decrease was found in the activity of complexes IV and V in old compared with young, and non-synaptic mitochondria also showed a decrease in activity in complexes I, II, III and IV, suggesting a mitochondrial dysfunction in brain aging (Ferrandiz *et al.*, 1994).

Based on the above reports of aging-associated changes in mitochondria, an investigation was undertaken to establish the relationship between proton leak, the substrate oxidation system and the phosphorylation system and changes in the mitochondrial membrane potential in synaptosomal *in situ* mitochondria isolated from the whole brain of rats. The relationship was characterized and compared in synaptosomal *in situ* mitochondria isolated from whole brains of young (3 month) and old (24 month) rats. Synaptosomes represent neurons that are a post-mitotic tissue. One would expect that accumulation of free radical damage with age might have a more pronounced effect in post-mitotic tissue compared with a highly regenerative tissue.

1.3 Synaptosomes as a model system of neuronal function

When regions of brain are homogenised under conditions of moderate shear, a high number of nerve endings reseal and can be isolated as synaptosomes (Gordon-Weeks, 1987). Synaptosomes have been used as *in vitro* systems for the study of neurotransmitter uptake, metabolism and release. Synaptosomes function as small anucleate cells, they retain the enzymes of glycolysis, usually have one or more mitochondria and possess extremely active ion transport systems across their membranes (Scott and Nicholls, 1980). They also serve as the parent fraction for preparations of synaptic vesicles and other synaptic components (Fig 1.3.1). Synaptosomes can be prepared in high yield from brain tissue and also in decreasing yield from spinal cord, retina, sympathetic ganglia, myenteric plexus and electric organs (Whittaker, 1993). Synaptosome preparations are widely used to study drugs and toxins that owe their actions to their ability to block uptake of transmitter precursors in a selective manner. Synaptosomes are sealed structures, which, under metabolizing conditions, respire, take up oxygen and glucose, extrude Na^+ , accumulate K^+ , maintain a normal membrane potential and, on depolarisation, release neurotransmitter in a Ca^{2+} -dependent manner (Fig 1.3.2) (Whittaker, 1993). Thus, they provide an excellent preparation with which to investigate synaptic function without the complications encountered with synapses *in situ* and have been shown to be good model for studying neuronal mitochondria in a cellular environment (Scott & Nicholls, 1980; Deutsch *et al.*, 1981; McMahon & Nicholls, 1991; Erecińska *et al.*, 1996). Many amino acid transmitters such as noradrenaline, dopamine, serotonin and precursors such as choline (for acetylcholine synthesis) and tyrosine (for catecholamine synthesis) are also taken up by carrier-mediated mechanisms located in the plasma membrane.

Freshly prepared synaptosomes have a high internal Na^+ and low K^+ concentration relative to living neurons (Whittaker, 1993). Incubation under metabolizing conditions causes an uptake of K^+ and an exclusion of Na^+ and the establishment of a resting membrane potential equal to that of living neurons. The Na^+/K^+ -ATPase necessary for these ionic translocations is localised in the synaptosomal plasma membrane (Fig 1.3.2).

The presence of a normal resting membrane potential in respiring and metabolizing synaptosomes has been inferred from the distribution of $^{86}\text{Rb}^+$ across the plasma membrane of synaptosomes (Scott and Nicholls, 1980).

Transmitter release from synaptosomes requires the exocytosis of the transmitter-storing synaptic vesicles; this is induced by a rise in the free Ca^{2+} concentration by the terminal cytoplasm, which, in turn, may be brought about by Ca^{2+} ionophores such as ionomycin or by activation of voltage-sensitive Ca^{2+} channels present in the synaptic plasma membrane. Depolarisation needed to activate these channels may be achieved by electrical stimulation, by increasing the external K^+ concentration, by increasing the permeability of the membrane to Na^+ with veratridine or by the K^+ -channel blockers 4-aminopyridine or dendrotoxin.

There are many advantages to using synaptosomes as a model system to study neuronal *in situ* mitochondrial function with age. (1) Synaptosomes represent a post-mitotic tissue. In a post-mitotic tissue, no new cells are made, as opposed to a highly regenerative tissue *e.g.* liver. As a result, one would expect that accumulation of free-radical damage might have a more pronounced effect in post-mitotic tissue compared with a highly regenerative tissue. (2) Synaptosomes also have the advantage in an *in situ* mitochondrial aging study in that the mitochondria are in a physiological environment. Mitochondrial ATP demand can be varied by altering the rate of ion cycling at the plasma membrane; in this way, the consequences of a more 'physiological' transition away from state 4 can be readily investigated, allowing the kinetic response of the phosphorylation system to changes in the mitochondrial membrane potential *in situ* to be investigated. The synaptosomal system used in this study has been used by Scott and Nicholls (1980) to measure the synaptosomal plasma membrane potential and *in situ* mitochondrial membrane potential and respiration, however, this is the first time the system has been used in an old/young comparison using the titrations we have used.

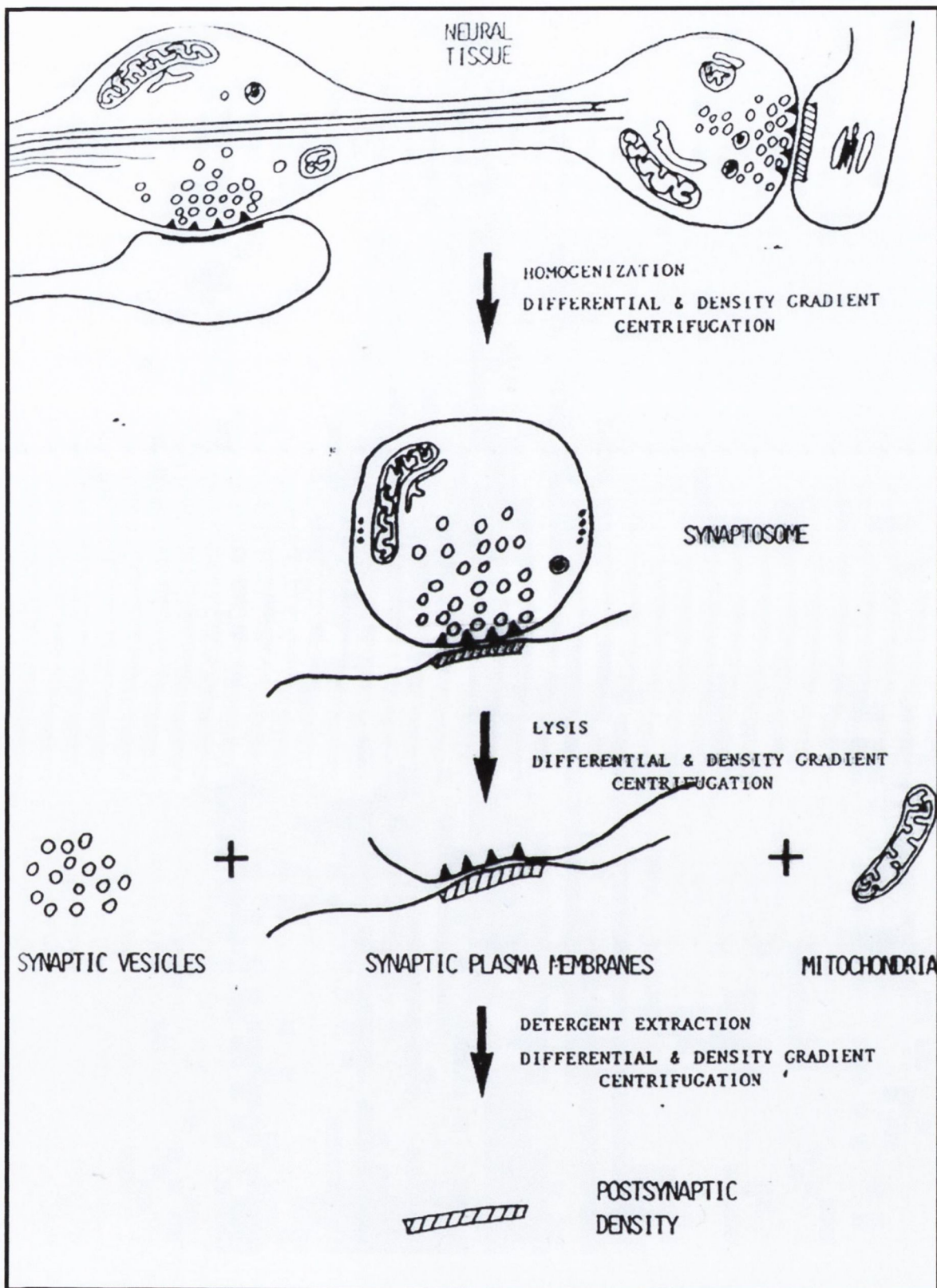


Figure 1.3.1 Diagram illustrating the derivation of synaptosomes and their subcellular fractions from a homogenate of neural tissue. The diagram is taken from Gordon-Weeks (1987).

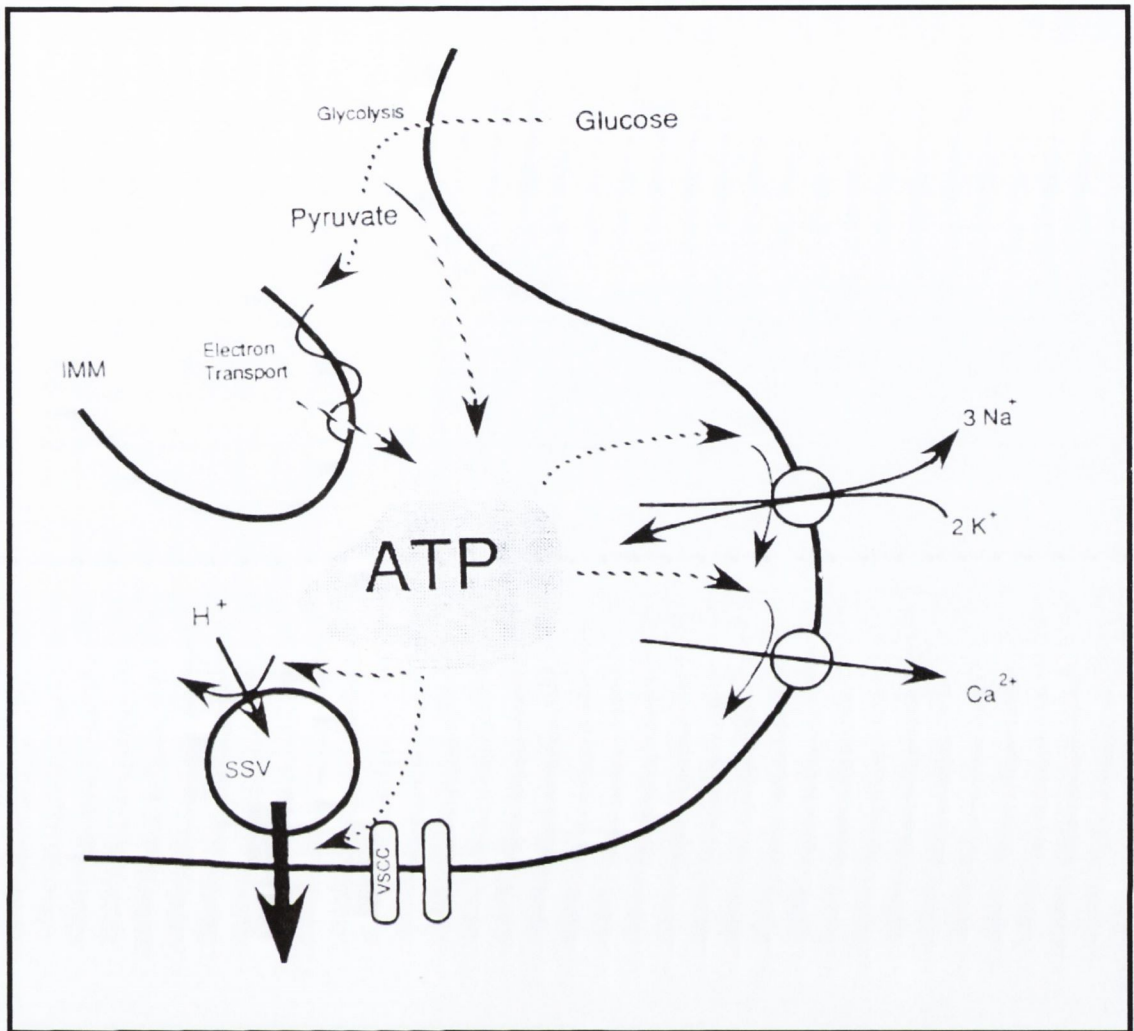
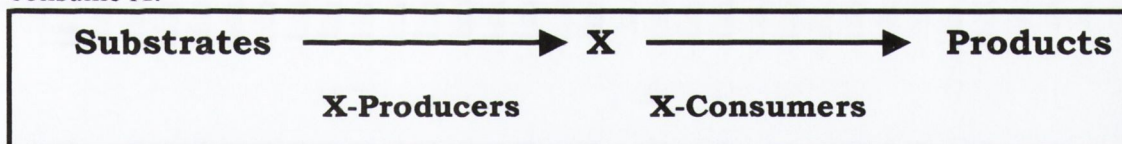


Figure 1.3.2. Some of the major pathways for the synthesis and utilization of ATP in synaptosomes. The major ATP-utilising process is the Na⁺/K⁺-ATPase, especially if Na⁺ conductance of the membrane is increased, when dissipative Na⁺ cycling can utilize most of the mitochondrial respiratory capacity. ATP is also required for the plasma membrane Ca²⁺-ATPase and for the exocytotic process. SSV, small synaptic vesicle; VSCC, voltage-sensitive Ca²⁺ channel. The diagram is taken from McMahon and Nicholls (1991).

1.4 Top-down elasticity control analysis

Control analysis has previously been applied to rat hepatocytes (Harper and Brand, 1993). Control analysis quantifies the control of particular steps in a pathway on the flux through the pathway (flux-control coefficients). The flux control coefficient is the percent change in the steady state divided by the percent change in the concentration of the enzyme. The top-down approach divides the system into two or three simple blocks and then solves this simplified system. The 'bottom up' approach measures the control exerted by a single step. Repeated application of either approach yields the same information, but one may be more appropriate than the other in the initial stages of the experimental analysis of a real system. Previously it had been assumed that all metabolic pathways would have a single rate-limiting step, but extensive investigations of control within isolated mitochondria have shown that control is shared by a number of reactions and that the distribution of control changes with conditions.

To use the top-down approach, a suitable pathway intermediate, X, is chosen and the system is conceptually divided into those reactions that produce X and those that consume X:



By varying the concentration of X and measuring the rate of the pathway, the overall kinetic responses (elasticities) of the X producers and the X consumers to X can be calculated. The intermediate that is measured must be representative of the intermediate that the system uses, *i.e.* it must not be channelled, compartmentalised or diffusion-limited. Also, it must be the only significant connecting link between X-producers and X-consumers, otherwise, changes in the X-producers might change the rate of the X-consumers by routes other than through the intermediate X. The intermediate need not refer to defined molecular species, much of the top-down analysis of control of the oxidative phosphorylation has been done using the membrane potential as the intermediate (Hafner *et al.*, 1990).

The overall control coefficients of the two ends of a pathway are always equal and opposite. In other words, the processes that produce a substance always have exactly the same overall control over the level of that substance as the processes that consume that substance. The sum of all the flux control coefficients is equal to one. If an enzyme has little control over the flux through a pathway then it will have a control coefficient close to zero. An enzyme that has a high degree of control over a pathway flux will have a control coefficient closer to one. The ability to measure two of the fluxes and partially (but specifically) inhibit one of the branches is sufficient to determine all the control coefficients.

For the purposes of comparing synaptosomal *in situ* mitochondria isolated from young and old rats, oxidative phosphorylation was divided into producers and consumers of a common metabolite, Δp (mitochondrial proton motive force) across the mitochondrial inner membrane (fig. 1.4.1). Δp is produced by the substrate oxidation system. The substrate oxidation system is composed of all those reactions involved in converting an initial fuel substrate into Δp , namely glycolysis, the citric acid cycle and the electron transport chain (red in colour in fig. 1.1.1). The reactions that consume Δp can be divided into two groups, the proton leak system (green in colour in fig. 1.1.1) and the phosphorylation system. The phosphorylation system consists of the ATP synthase, the inorganic phosphate carrier and the adenine nucleotide carrier (blue in colour in fig. 1.1.1). The overall flux through the substrate oxidation system is the sum of the fluxes through the proton leak system and the phosphorylation system:

$$J_S = J_L + J_P$$

Where S represents the substrate oxidation system, L is the proton leak and P is the phosphorylation system. The flux, J , is measured as the rate of oxygen consumption in nmol O_2 /min/mg synaptosomal protein.

In this study, the flux through each of these pathways in relation to changes in Δp , the major component of which is the membrane potential ($\Delta\Psi_m$, measured in mV), was investigated.

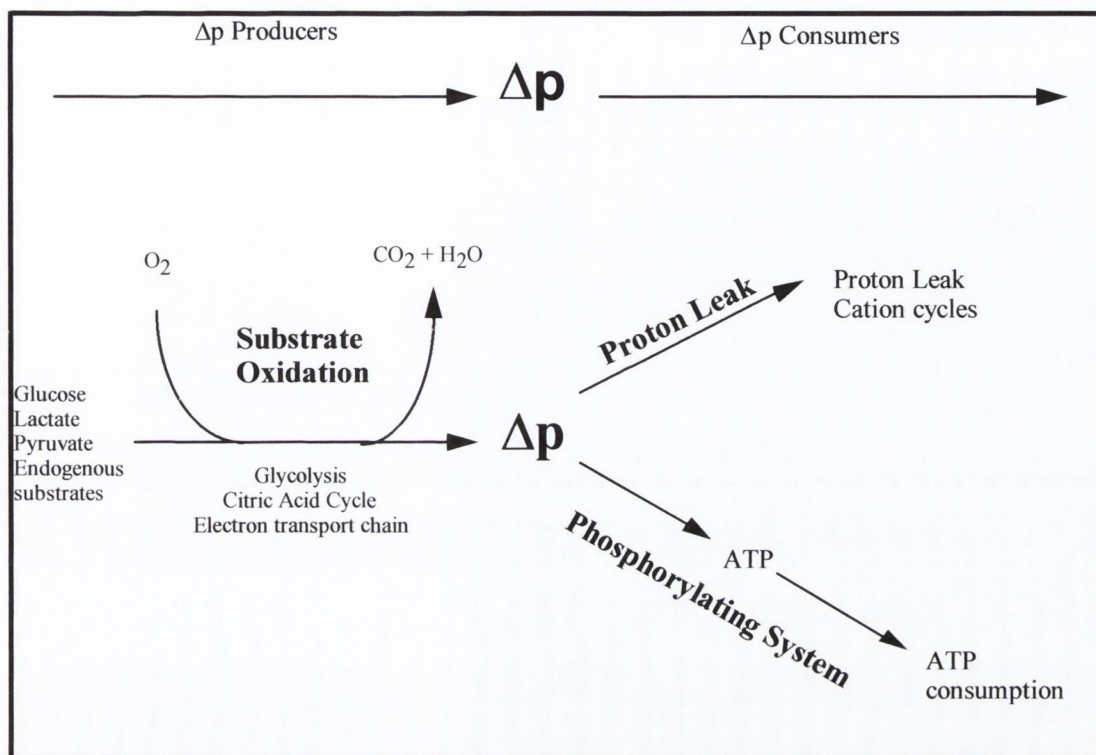


Figure 1.4.1 Schematic representation of oxidative phosphorylation in synaptosomes. The system is branched. Mitochondrial membrane potential *in situ* ($\Delta\Psi_m$), which is the major component of the mitochondrial proton motive force (Δp), is produced by substrate oxidation and is consumed by the phosphorylation system and proton leak.

By measuring oxygen consumption and $\Delta\Psi_m$ (mitochondrial membrane potential) *in situ* under conditions where $\Delta\Psi_m$ changes, we can measure the kinetic response of each of the three subsystems to changes in $\Delta\Psi_m$, and this indicates where the control of flux through each subsystem lies.

The equations needed to calculate the elasticity and control coefficients that describe the control of oxygen flux by synaptosomes were those given in Hafner *et al.* (1990) (see Table 1.4.1).

Elasticity coefficients were defined by the following equation;

$$E_{\Delta\Psi_m}^x = dJ_x/d\Delta\Psi_m \cdot \Delta\Psi_m/J_x$$

where x = the substrate oxidation system (S), the phosphorylation system (P) or the proton leak system (L). The elasticity coefficients were then used in the list of equations given in Table 1.4.1 to generate the flux control coefficients of the three reaction subsystems over the rate of each subsystem and to generate their concentration control coefficients over $\Delta\Psi_m$, which describe the control in the system.

Top-down elasticity analysis of *in situ* synaptosomal mitochondria in state 3 was determined for young (3 month) and old (24 month) rats using the strategy outlined below (fig. 1.4.2). The kinetic response of proton leak to changes in Δp can be investigated as follows. To begin with, oligomycin is used to place the *in situ* mitochondria into state 4. Oligomycin is an antibiotic that inhibits proton flow through the F_o part of the ATP synthase and hence, ATP synthesis. After addition of oligomycin, the mitochondrial oxygen consumption is due solely to proton leak. To see how the flux through proton leak changes with changes in Δp , the substrate oxidation system can be inhibited by titrating with electron transport inhibitors such as myxothiazol (which inhibits the cytochrome bc_1 complex of complex III). As the rate of substrate oxidation decreases, the rate of proton pumping decreases and so the value of Δp falls. Analysis of the relationship between proton leak rate and Δp shows that the proton leak rate has a non-linear dependence on Δp : at high membrane potential, there is a dramatic change in proton leak rate for a small change in membrane potential.

$$\varepsilon_{\Delta\Psi_m}^S = dJ_S / d\Delta\Psi_m \cdot \Delta\Psi_m / J_S$$

$$\varepsilon_{\Delta\Psi_m}^L = dJ_L / d\Delta\Psi_m \cdot \Delta\Psi_m / J_L$$

$$\varepsilon_{\Delta\Psi_m}^P = d(J_S - J_L) / d\Delta\Psi_m \cdot \Delta\Psi_m / J_S - J_L$$

$$C_S^{J_S} = (J_P \cdot \varepsilon_{\Delta\Psi_m}^P + J_L \cdot \varepsilon_{\Delta\Psi_m}^L) / (J_P \cdot \varepsilon_{\Delta\Psi_m}^P + J_L \cdot \varepsilon_{\Delta\Psi_m}^L - J_S \cdot \varepsilon_{\Delta\Psi_m}^S)$$

$$C_P^{J_S} = J_P \cdot (1 - C_S^{J_S} / J_S)$$

$$C_L^{J_S} = J_L \cdot (1 - C_S^{J_S} / J_S)$$

$$C_S^{J_P} = J_S \cdot \varepsilon_{\Delta\Psi_m}^P / (J_P \cdot \varepsilon_{\Delta\Psi_m}^P + J_L \cdot \varepsilon_{\Delta\Psi_m}^L - J_S \cdot \varepsilon_{\Delta\Psi_m}^S)$$

$$C_P^{J_P} = 1 - (J_P \cdot C_S^{J_P} / J_S)$$

$$C_L^{J_P} = -J_L \cdot (C_S^{J_P} / J_S)$$

$$C_S^{J_L} = J_S \cdot \varepsilon_{\Delta\Psi_m}^P / (J_P \cdot \varepsilon_{\Delta\Psi_m}^P + J_L \cdot \varepsilon_{\Delta\Psi_m}^L - J_S \cdot \varepsilon_{\Delta\Psi_m}^S)$$

$$C_P^{J_L} = -J_P \cdot (C_S^{J_L} / J_S)$$

$$C_L^{J_L} = 1 - (J_L \cdot C_S^{J_L} / J_S)$$

$$C_S^{\Delta\Psi_m} = J_S / (J_P \cdot \varepsilon_{\Delta\Psi_m}^P + J_L \cdot \varepsilon_{\Delta\Psi_m}^L - J_S \cdot \varepsilon_{\Delta\Psi_m}^S)$$

$$C_P^{\Delta\Psi_m} = -J_P \cdot (C_S^{\Delta\Psi_m} / J_S)$$

$$C_L^{\Delta\Psi_m} = -J_L \cdot (C_S^{\Delta\Psi_m} / J_S)$$

Table 1.4.1 The equations needed to calculate the elasticity and control coefficients that describe the control of oxygen flux by synaptosomes as given in Hafner *et al.* (1990). $C_S^{J_S}$ refers to the control the substrate oxidation system exerts over the flux through the substrate oxidation system. ε^S refers to the elasticity of the substrate oxidation system. J_S refers to the flux through the substrate oxidation system and $\Delta\Psi_m$ refers to the mitochondrial membrane potential.

The kinetic response of the substrate oxidation system to changes in Δp was investigated using oligomycin and veratridine (Fig. 1.4.2). Oligomycin is an antibiotic that inhibits proton translocation through the F_o part of the ATP synthase and thus prevents ATP synthesis. Oligomycin was used to titrate *in situ* synaptosomal mitochondria from a resting (R) oxygen consumption rate and mitochondrial membrane potential to a non-phosphorylating state. The result was a decrease in the rate of oxygen consumption rate and an increase in $\Delta\Psi_m$. Under non-phosphorylating conditions, oxygen consumption by mitochondria is due solely to proton leak. Veratridine is an alkaloid ionophore that can be used to uncouple the plasma membrane. It works by stabilizing the high conductance conformation of the potential-dependent ionophore in the plasma membrane and so induces a long-lasting depolarisation of the plasma membrane. The *in situ* mitochondria have to generate more ATP due to the demand of the Na^+/K^+ ATPase, which works harder to re-establish the sodium gradient across the plasma membrane. The *in situ* mitochondria are pushed closer and closer to state 3 due to an increased cytosolic demand for ATP. As a result the mitochondrial membrane potential ($\Delta\Psi_m$) is used to make ATP i.e. the value of $\Delta\Psi_m$ decreases. Thus, the changes in substrate oxidation rate in response to changes in $\Delta\Psi_m$ are investigated by using increasing amounts of oligomycin and veratridine.

The kinetic response of the phosphorylation system to changes in Δp were determined by titration with myxothiazol in the presence of that amount of veratridine required to place the synaptosomal *in situ* mitochondria in state 3. The phosphorylating system was studied as a function of decreasing membrane potential.

Not all oxygen consumption is mitochondrial and therefore coupled to oxidative phosphorylation and a significant portion of mitochondrial oxygen consumption is not coupled to ATP synthesis (Rolfe and Brown, 1997). A large number of oxidases function in metabolism (e.g. in peroxisomal fatty acid oxidation). Estimates of oxygen consumption not due to oxidative phosphorylation can be made by adding specific

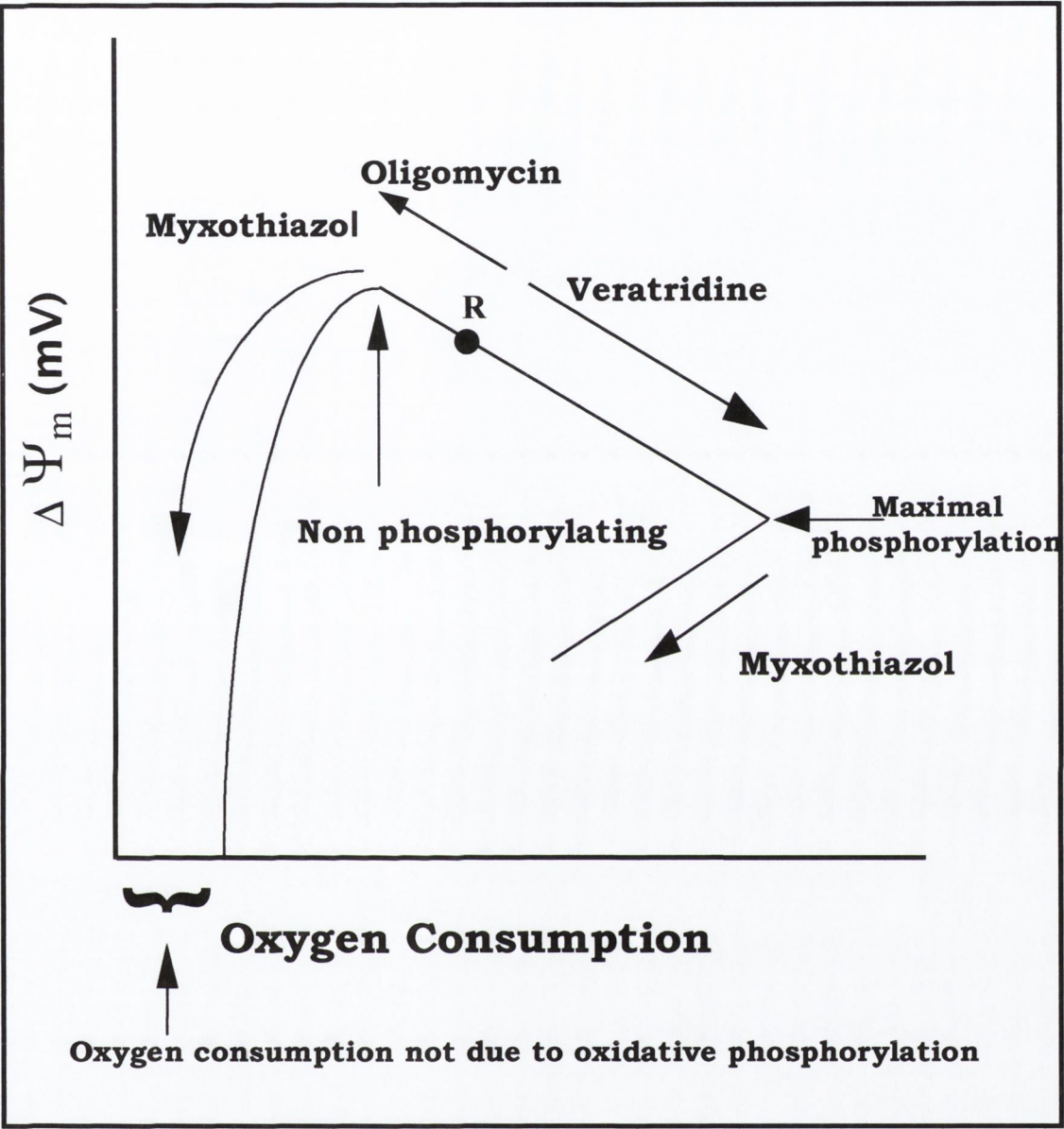


Figure 1.4.2.

Figure 1.4.2. Schematic representation of the results for the titration of proton leak, substrate oxidation and the phosphorylation system in synaptosomes from whole brain of young and old rats. Oligomycin was used to titrate *in situ* synaptosomal mitochondria from a resting (R) oxygen consumption rate and mitochondrial membrane potential ($\Delta\Psi_m$) to a non-phosphorylating state. The result was decrease in the rate of oxygen consumption rate and an increase in $\Delta\Psi_m$. Under non-phosphorylating conditions, oxygen consumption by mitochondria is due solely to proton leak. Oligomycin inhibits proton flow through the F_o component of the ATP synthase and thus prevents ATP synthesis. The kinetic response of the (oxygen consumption rate due to) proton leak to a decrease in $\Delta\Psi_m$ is non-linear and is achieved by titrating the electron transport chain with increasing amounts of the complex III inhibitor, myxothiazol. Once the oxygen consumption due to mitochondria has been inhibited, the synaptosomal oxygen consumption not due to oxidative phosphorylation can be estimated. Addition of veratridine to resting (R) synaptosomes increased ATP turnover in the synaptosomes with the result that mitochondria increased ATP production. Veratridine is a sodium ionophore that is used to uncouple the plasma membrane. The consequence was an increase in oxygen consumption and a decrease in $\Delta\Psi_m$ as the mitochondria headed towards a maximal phosphorylating state (state 3). The oligomycin and veratridine titrations combined gave a measure of the kinetic response of the substrate oxidation system. At maximal phosphorylating state, titration with myxothiazol gave a measure of the kinetic response of the phosphorylation system (with proton leak) to a decrease in $\Delta\Psi$. Once oxygen consumption due to proton leak was subtracted at corresponding $\Delta\Psi_m$ values, the true kinetic response of the phosphorylating system to $\Delta\Psi_m$ was revealed.

inhibitors of the mitochondrial respiratory chain and ionophores that affect the mitochondrial inner membrane and then measuring the residual oxygen consumption of the tissue or cell. To determine the oxygen consumption by synaptosomes that is not due to oxidative phosphorylation, it was necessary to use an excess of a mitochondrial electron chain inhibitor. The inhibitor used was myxothiazol, which is an inhibitor of mitochondrial electron transport and acts on the bc_1 complex of complex III. Oligomycin is an antibiotic that inhibits proton translocation through the F_o part of the ATP synthase. Valinomycin is a mobile K^+ ionophore and FCCP is a proton ionophore.

1.5 Blue native/tricine-SDS-PAGE two dimensional electrophoresis.

Blue native PAGE can be used to resolve membrane protein complexes in the 1000kDa to 100kDa region. Therefore, mitochondrial protein complexes can be solubilized using dodecyl maltoside and aminocaproic acid and can then be resolved using blue native PAGE. Blue native PAGE is a charge shift method. The electrophoretic mobility of proteins is affected by the binding of negatively charged Serva blue G, which is included in the cathode buffer and added to the sample just before electrophoresis. The fact that the catalytic activity of the oxidative phosphorylation complexes is maintained after blue native PAGE has been exploited using histochemical staining methods. The combination of blue native PAGE and catalytic staining was used in the detection of complex deficiencies in heart, skeletal muscle, liver and cultured skin fibroblasts (Van Coster *et al.*, 2001). Catalytic staining was also used after blue native PAGE to investigate complex activities in mitochondria isolated from mouse spinal cord, cerebellum and forebrain. (Jung *et al.*, 2000).

Once the mitochondrial complexes have been resolved in the first dimension, the complexes can be further resolved into their constituent proteins using a second dimension tricine-SDS-gel. To this end, the blue native/tricine-SDS-PAGE two-dimensional technique has been used in the comparison of mitochondria from fibroblasts isolated from patients with a complex IV deficiency (Klement *et al.*, 1995) with mitochondria from fibroblasts isolated from a control patient. It has also been used to characterise the preprotein translocase of the outer mitochondrial membrane (Dekker *et*

al., 1996) and the intramitochondrial dimerisation of citrate synthase (Reif *et al.*, 2000). The blue native/tricine-SDS-PAGE two-dimensional technique has also been used to identify novel mitochondrial proteins in *Arabidopsis* (Kruft *et al.*, 2001). In this thesis, blue native/tricine-SDS-PAGE two-dimensional electrophoresis was used in the comparisons outlined below.

1.5.1 Comparison of mitochondria isolated from young and old rats

Based on the reports of changes associated with aging in mitochondria, (section 1.2.1), blue native/tricine-SDS-PAGE two-dimensional electrophoresis was used to resolve mitochondria isolated from young (3 month) and old (24 month) rat tissues to see if there were any differences in (a) the polypeptide pattern in the second dimension and (b) the levels of complexes I, III, IV and V. Rat heart mitochondria, rat liver mitochondria and rat synaptic mitochondria isolated from young and old mitochondria were compared using blue native/tricine-SDS-PAGE two dimensional electrophoresis.

1.5.2 Comparison of liver mitochondria isolated from rats of different thyroid status.

There are a number of reports of the effects of different thyroid status on mitochondria. It has been shown that hyperthyroid rat liver mitochondria have greater state 3 rates than rat liver mitochondria isolated from euthyroid rats. Cote and Boulet (1985) found that there was an increase in the rate of mitochondrial protein synthesis in heart mitochondria isolated from hyperthyroid rats when compared to the rate of mitochondrial protein synthesis in heart mitochondria isolated from euthyroid rats. Guerrieri *et al.* (1998) found that the decrease observed in catalytic activity of the ATP synthase in rat liver mitochondria isolated from hypothyroid rats was accompanied by a decrease in the amount of subunits F1 β and OSCP of the ATP synthase. Based on the above reports, one might expect to see an increase in the level of complexes I, III, IV and V in rat liver mitochondria isolated from hyperthyroid rats compared with rat liver mitochondria isolated from euthyroid rats. Blue native/tricine-SDS-PAGE two dimensional electrophoresis was used to compare liver mitochondria isolated from euthyroid rats with liver mitochondria isolated from hyperthyroid rats.

1.5.3 Comparison of mitochondria isolated from different rat tissues

Rolfe *et al.* (1994) examined the control of oxidative phosphorylation in mitochondria in the major oxygen consuming tissues of the rat, namely, liver, kidney, brain and skeletal muscle. The substrate oxidation system was found to be more active in kidney, brain and skeletal muscle than in liver (per mg of mitochondrial protein). Liver and kidney phosphorylating systems were found to be less active than brain and skeletal muscle (per mg mitochondrial protein). Other reports have examined the content of mitochondrial ATP synthase, which has been shown to be exceptionally low in brown adipose tissue (Cannon and Vogel, 1977; Houstek *et al.*, 1978). Based on these reports, it might be expected that there would be a difference in the polypeptide pattern of the various tissue mitochondria when resolved on a second dimension gel or in the levels of complexes. With this in mind, blue native/tricine-SDS-PAGE two-dimensional electrophoresis was used to resolve mitochondria isolated from various rat tissues to determine if (a) there were any differences in the polypeptide pattern in the second dimension and (b) the levels of complexes I, III, IV and V. The mitochondria were isolated from rat heart, kidney, liver, brown adipose tissue, skeletal muscle, whole brain synaptosomes and beef heart mitochondria.

1.6 Aims

1. To characterise and compare *in situ* mitochondrial function from old rat brain synaptosomes (neurons) compared with those from young rat brain synaptosomes.
2. To quantify and compare the control of the flux through the system exerted by the proton leak, the substrate oxidation system and the phosphorylation system with respect to changes in the mitochondrial membrane potential in *in situ* mitochondria from old (24 month) rat brain synaptosomes (neurons) compared with *in situ* mitochondria from young (3 month) rat brain synaptosomes using top down elasticity analysis.
3. To resolve mitochondria isolated from young (3 month) and old (24 month) rat tissues and from euthyroid and hyperthyroid rat liver using blue native/tricine-SDS-PAGE two-dimensional electrophoresis to determine if there were any differences in

the polypeptide pattern in the second dimension and the levels of complexes I, III, IV and V in old compared with young mitochondria.

4. To resolve mitochondria isolated from rat tissues and beef heart to determine if there were any differences in the polypeptide pattern in the second dimension and the levels of complexes I, III, IV and IV using blue native/tricine-SDS-PAGE two-dimensional electrophoresis.

Chapter 2

Materials And Methods.

2.1 Materials

Acetic acid, acetyl CoA, acrylamide, aminocaproic acid, ATP, bisacrylamide, Bistris, bromophenol blue, bovine serum albumin, calcium chloride, Coomassie blue R, copper sulfate 5-hydrate, DTNB, DTT, EDTA, EGTA, FCCP, formaldehyde, glucose, glycerol, glycine, Hepes, lead acetate, mercaptoethanol, methanol, myxothiazol, nagarse, β -NADH, nitro blue tetrazolium, oligomycin, oxaloacetate, percoll, potassium dihydrogen phosphate, potassium chloride, potassium hydroxide, silver nitrate, sodium carbonate, sodium dodecyl sulphate, sodium hydroxide, sodium potassium tartrate, sodium thiosulfate, sucrose, tetraphenol boron, tricine, Tris-succinate, Triton, Trizma, Tween, veratridine, valinomycin and the goat anti-rabbit secondary antibody were all obtained from **Sigma, Poole, Dorset, U.K.**

Dow Corning 550 fluid, hydrochloric acid, magnesium chloride 6-hydrate, sodium dihydrogen orthophosphate, sodium carbonate and perchloric acid were obtained from **BDH Laboratory Supplies, Poole, Dorset, U.K.**

Sodium chloride was obtained from **Merck KGaA, 64271 Darmstadt, Germany.**

$[^3\text{H}]\text{TPMP}^+$, $^{86}\text{Rb}^+$, $[^{14}\text{C}]$ -labelled Methoxy inulin, $^3\text{H}_2\text{O}$ were obtained from **New England Nuclear, 449 Albany St., Boston, MA 02118, U.S.A.**

Ecoscint scintillation fluid was obtained from **National Diagnostics, 305 Patton Drive, Atlanta, Georgia 30336, U.S.A.**

Immobilon (PVDF) was obtained from **Millipore, c/o Sigma Chemical Co. Ltd., Fancy Rd., Poole, Dorset, U.K.**

Blotting paper was obtained from **Whatman Ltd., Maidstone, U.K.**

Dodecylmaltoside was obtained from **Roche Analytical Ltd., Sandford Industrial Estate, Foxrock, Dublin 18, Ireland.**

Serva blue G was obtained from **AMS Biotech, 185 A/B, Milton Park, Abingdon, OX14 4SR, U.K.**

Complex IV COIII subunit antibody and the complex V A6 subunit antibody were produced by **Eurogentec s.a., Parc Scientifique du Sart Tilman, 4102 Seraing, BELGIUM.**

ECL reagent was obtained from Amersham International, **Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, U.K.**

Marvel was obtained from **Chivers Ireland Ltd., Coolock, Dublin 5, Ireland.**

2.2 Methods

2.2.1 Synaptosomal methods

2.2.1.1 Preparation of whole rat brain synaptosomes

Whole brain synaptosomes were prepared from young (3 month) or old (24 month) Wistar rats as described by Gordon-Weeks (1987). Solutions were prepared as follows:

Solution A: 0.32 M sucrose, 5 mM Hepes/NaOH buffer, pH 7.4

Krebs-Heinseleit Buffer: 145 mM NaCl, 1.3 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 5 mM KCl, 1.2 mM CaCl_2 , 10 mM Hepes, pH 7.4.

90% (v/v) Percoll: 1 volume of 3.2 M sucrose added to 9 volumes of Percoll.

For each preparation, four Wistar rats were killed by cervical dislocation and their brains were rapidly removed and placed into ice-cold Solution A. The brains were chopped into small pieces using a backed razor blade and were homogenised by hand in a Potter-Elvehjem homogeniser using 12 passes of the loose pestle followed by 12 passes of the tight pestle. The homogenate was placed in two 50-ml centrifuge tubes, adjusted to 40 ml with Solution A and centrifuged at 1000 g for 5 min at 4°C. The supernatants were decanted and stored on ice while the pellets were resuspended, homogenised and centrifuged as before. The two supernatants were pooled and centrifuged at 12,000 g for 20 min at 4°C. This step was repeated twice more. The crude synaptosomal pellet was resuspended in approximately 10 ml of Solution A and adjusted to 7.5% (v/v) Percoll with 90% (v/v) Percoll solution. The total volume of the suspension was adjusted to approximately 36 ml with Solution A.

The Percoll density gradients were made by placing 4 ml of 10% (v/v) Percoll above 4 ml of 16% (v/v) Percoll in 17 ml plastic centrifuge tubes. The 7.5% Percoll-synaptosomal suspension was then layered onto the gradient.

The Percoll gradients were centrifuged at 15,000 g for 20 min at 4°C. The synaptosomes formed a band at the 10%/16% Percoll gradient interface and were removed using a plastic Pasteur pipette. The volume of the synaptosomal suspension was adjusted to 40 ml with Krebs-Heinseleit buffer and the synaptosomes were collected by centrifugation at 15,000 g for 20 min at 4°C. The synaptosomal pellet was resuspended in Krebs-Heinseleit buffer to a final volume of approximately 3 ml. The protein concentration of the solution was measured using the method of Markwell *et al.* (1978), as described in Section 2.2.4.1.

2.2.1.2 Measurement of synaptosomal oxygen consumption rates

The rate of synaptosomal oxygen consumption was measured in a thermostated Clark-type oxygen electrode chamber made by Rank Brothers, Bottisham, Cambridge, U.K. The total volume of the electrode was 1 ml. All experiments were performed in a final volume of 0.5 ml at a temperature of 37°C. A Philips (PM 8252) dual-pen chart recorder with the maximum full scale deflection set to 1 mV was used to record oxygen consumption rates. Oxygen consumption rates were measured in duplicate by preincubating synaptosomes (1.2 mg protein) in 0.6 ml of Krebs-Heinseleit buffer in 5 ml plastic stoppered vials for 10 min in a shaking water bath at 37°C. 0.5 ml synaptosomes were then transferred to the oxygen electrode at 37°C. The suspension also contained TPMP⁺ (1.3 µM) and TPB⁻ (3 µM).

2.2.1.3 Measurement of mitochondrial membrane potential using [³H]-TPMP⁺

Synaptosomal mitochondrial membrane potentials were measured using methyltriphenylphosphonium (TPMP⁺), which equilibrates across membranes according to the magnitude of their membrane potentials. Synaptosomes (0.5 mg protein) were preincubated in 0.5 ml of Krebs-Heinseleit buffer (145 mM NaCl, 1.3 mM MgCl₂, 5 mM KCl, 1.2 mM CaCl₂, 10 mM glucose, 1.2 mM NaH₂PO₄, 20 mM Hepes, pH 7.4)

containing 1.3 μM [^3H]-TPMP $^+$ (0.2 $\mu\text{Ci}/\text{mmol}$ or 7.4 kBq/mmol), 3 μM tetraphenylboran (TPB $^-$) and $^{86}\text{Rb}^+$ (50 μM , 0.5 μCi or 18.5 kBq) in stoppered polycarbonate flasks in a shaking water bath at 37°C. After the incubation period, 0.4ml of the synaptosomal suspension was layered onto 0.4 ml of a mixture of Dow Corning 550 fluid-dinonyl phthalate (60:40, v/v) layered above 0.15 ml of Perchloric acid (1M) and centrifuged in a bench top centrifuge for 3 min at 15000 g. Aliquots of the supernatant (0.2ml) and infranatant (0.1ml) were removed and placed into separate scintillation vials containing 10 ml of Ecosint scintillation fluid. The solutions were mixed and counted for radioactivity. The mitochondrial membrane potential can be calculated according to the following equation (Scott and Nicholls, 1980):

Equation 1

$$\Delta\Psi_m = 61.5 \log \left[\frac{V_s \cdot a_m}{V_m} \left(\frac{[\text{Rb}^+]_e}{[\text{Rb}^+]_s} \frac{[\text{TPMP}^+]_s}{[\text{TPMP}^+]_e a_e} \frac{-1}{a_c} \right) \right]$$

where $\Delta\Psi_m$ is the mitochondrial membrane potential, V_s is the synaptosomal volume, V_m is the mitochondrial volume, a_e , a_c and a_m are the extracellular medium, and the cytosolic and mitochondrial matrix binding correction factors for TPMP $^+$, respectively. $[\text{Rb}^+]_e/[\text{Rb}^+]_s$ is a measure of the plasma membrane potential.

2.2.1.4 Measurement of the plasma membrane potential using $^{86}\text{Rb}^+$

Plasma membrane potential can be determined using the distribution of $^{86}\text{Rb}^+$ across the plasma membrane. Synaptosomes (0.5 mg protein) were preincubated in 0.5 ml of Krebs-Heinseleit buffer (145 mM NaCl, 1.3 mM MgCl_2 , 5 mM KCl, 1.2 mM CaCl_2 , 10 mM glucose, 1.2 mM NaH_2PO_4 , 20 mM Hepes, pH 7.4) containing 1.3 μM [^3H]-TPMP $^+$ (0.2 $\mu\text{Ci}/\text{mmol}$ or 7.4 kBq/mmol), 3 μM tetraphenylboran (TPB $^-$) and $^{86}\text{Rb}^+$ (50 μM , 0.5 μCi or 18.5 kBq) in polycarbonate flasks in a shaking water bath at 37°C. After the incubation period, 0.4 ml of the synaptosomal suspension was layered onto 0.4 ml of a mixture of Dow Corning 550 fluid-dinonyl phthalate (60:40 v/v) layered above

0.15 ml of perchloric acid (1M) and centrifuged in a bench-top centrifuge for 3 min at 15000g. Aliquots of the supernatant (0.2 ml) and infranatant (0.1 ml) were removed and placed into separate scintillation vials containing 10 ml of Ecosint scintillation fluid. The solutions were mixed and counted for radioactivity in a Tricarb 1500 liquid scintillation counter. The plasma membrane potential was calculated from the accumulation ratio using the Nernst equation as follows:

Equation 2

$$\Delta\Psi_p = 61.5 \log \frac{[Rb^+]_s}{[Rb^+]_e}$$

Where $\Delta\Psi_p$ is the plasma membrane potential, s refers to the concentration of $^{86}Rb^+$ in the synaptosome and e refers to the concentration of $^{86}Rb^+$ in the extracellular medium.

2.2.1.5 Measurement of synaptosomal volume

The synaptosomal volume was measured using [^{14}C]-methoxyinulin as an extrasynaptosomal space marker and 3H_2O as the total space marker. Synaptosomes (1 mg protein) were incubated at 37°C for 10 min with 3H_2O (0.4 μ Ci/ml or 14.8 kBq/ml) Krebs-Heinseleit buffer (145 mM NaCl, 1.3 mM $MgCl_2$, 5 mM KCl, 1.2 mM $CaCl_2$, 10 mM glucose, 1.2 mM NaH_2PO_4 , 20 mM Hepes, pH 7.4). At the end of the incubation period, [^{14}C]-methoxyinulin (0.04 μ Ci/ml or 1.48 kBq) was added. The sample was vortex-mixed and 0.4 ml of the synaptosomal suspension was layered onto 0.4 ml of a mixture of Dow Corning 550 fluid-dinonyl phthalate (60:40 v/v) layered above 0.15 ml perchloric acid (1 M) and centrifuged in a bench top centrifuge for 3 min at 15000 g. Aliquots of the supernatant (0.2 ml) and infranatant (0.1 ml) were removed and placed into separate scintillation vials containing 10 ml of Ecosint scintillation fluid. The solutions were mixed and counted for radioactivity. The total pellet volume and the extrasynaptosomal space were calculated as 3H_2O space(μ l/mg protein) - [^{14}C]-methoxyinulin (μ l/mg protein).

2.2.2 Isolation of mitochondria

2.2.2.1 Isolation of beef heart mitochondria without using nagarse

Beef heart mitochondria were isolated according to the method of Smith (1967). The beef heart was stored on ice until isolation. Isolation was started within approximately 2 h of the animal being slaughtered. The heart muscle was cut into cubes that were approximately 5 cm in length. A 300g amount of the heart muscle was passed through a domestic meat grinder that was precooled at 4°C. The minced heart muscle was placed in 400ml of a solution containing 0.25 M sucrose and 0.01 M Trizma-HCl (pH 7.8). The pH of the mixture was adjusted to 7.5 using 6 M KOH. The pH-adjusted heart muscle mince was placed in a double layer of muslin and squeezed free of the above sucrose solution. A 200 g amount of the minced heart muscle was placed in 400 ml of a solution containing 0.25 M sucrose, 0.1 M Trizma-HCl (pH 7.8), 1 mM tris-succinate and 0.2 mM EDTA. The 400 ml mixture of heart muscle mince and sucrose solution was placed in a precooled Waring blender and 1ml of 6M KOH was added. The Waring blender was turned on at high speed for 15 s, 1 ml of 6M KOH was added and the blender was turned on for a further 5 s. The pH of the homogenate was adjusted to 7.8 using KOH (1M). The homogenate was centrifuged at 1200 g for 3 min. After centrifugation, the supernatant was filtered through two layers of muslin to remove lipid granules. The filtered homogenate was readjusted to pH 7.8 using 1 M KOH and centrifuged at 26000 g for 15 min. The supernatant was discarded and the pellets were resuspended in 0.25M sucrose, 0.01M Trizma-HCl (pH 7.8), 1 mM Tris-succinate and 0.2mM EDTA, after which they were pooled and recentrifuged at 26000 g for 15 min. The final pellet was suspended in the above solution and the mitochondrial protein content was determined using the method of Markwell *et al.* (1978) as described in Section 2.2.4.1.

2.2.2.2 Isolation of beef heart mitochondria using nagarse

Beef heart mitochondria were isolated using nagarse by a modification of a method described by Bhattacharya *et al.* (1991). The beef heart was stored on ice until isolation. The isolation procedure was started within approximately 2 h of the animal being slaughtered. A 200 g amount of beef heart was chopped into thin strips using a backed

razor blade on a precooled glass plate. The 200g of chopped beef heart was placed in a beaker containing 400ml of isolation medium (100mM sucrose, 9mM EDTA, 1mM EGTA, 46 mM KCl, 100 mM Trizma-HCl pH 7.4) plus 0.08 g nagarse and 2 g BSA. The chopped beef heart was incubated with nagarse on ice with stirring for 5 min. The chopped beef heart was placed in a Waring blender and homogenised for 15 s at half the maximum speed. The homogenised beef heart was centrifuged at 4°C at 1200 g for 3 min. After centrifugation, the supernatant was filtered through two layers of muslin to remove lipid granules and centrifuged at 12000 g for 10 min at 4°C. The pellets were resuspended in ice cold isolation medium and the resuspension was centrifuged at 12000 g for 10 min at 4°C. The pellets were again resuspended in ice cold isolation medium and centrifuged at 12000 g for 10 min at 4°C. The final pellet was suspended in isolation medium and the mitochondrial protein content was determined using the method of Markwell *et al.* (1978) as described in Section 2.2.4.1.

2.2.2.3 Isolation of rat heart mitochondria

Rat heart mitochondria were isolated by a modification of a method described by Bhattacharya *et al.* (1991). The main modification to the method was that nagarse was not used in the isolation of mitochondria. For each preparation, eight Wistar rats were killed by cervical dislocation and their hearts were rapidly dissected and placed in ice-cold isolation medium (100 mM sucrose, 9 mM EDTA, 1 mM EGTA, 46 mM KCl, 100 mM Trizma-HCl pH 7.4). The rat hearts were rinsed twice in isolation medium to remove blood and were then chopped using a backed razor blade on a glass plate. The chopped rat hearts were homogenised by hand in a Potter-Elvehjem homogeniser using six passes of the loose pestle followed by 6 passes of the tight pestle. The rat heart homogenate was centrifuged at 4°C and 1200 g for 3 min. The pellets were resuspended in ice cold isolation medium and the suspension was centrifuged at 12000 g for 10 min at 4°C. The pellets were resuspended in ice cold isolation medium and centrifuged at 12000 g for 10 min at 4°C. The final pellet was suspended in isolation medium and the mitochondrial protein content was determined using the method of Markwell *et al.* (1978) as described in Section 2.2.4.1.

2.2.2.4 Isolation of rat skeletal muscle mitochondria

Rat skeletal muscle mitochondria were isolated by a modification of a method described by Bhattacharya *et al.* (1991). The main modification to the method was that nagarse was not used in the isolation of mitochondria. For each preparation, two Wistar rats were killed by cervical dislocation and the skeletal muscle was rapidly dissected from the hind legs and some from the back and placed in ice-cold isolation medium (100 mM sucrose, 9 mM EDTA, 1 mM EGTA, 46 mM KCl, 100 mM Trizma-HCl pH 7.4). The rat skeletal muscle was chopped using a scissors in a beaker containing ice-cold isolation medium. Once the rat skeletal muscle was chopped sufficiently, the volume of the isolation medium plus rat skeletal muscle was made up to 200 ml and the mixture was placed in a Waring blender which was operated at half maximum speed for 30 s. The muscle homogenate was further homogenised by hand in a Potter-Elvehjem homogeniser using six passes of the loose pestle followed by six passes of the tight pestle. The rat skeletal muscle homogenate was passed through one layer of muslin and centrifuged at 1200 g for 3 min at 4°C. The supernatant was carefully poured off and centrifuged at 12,000 g for 10 min at 4°C. The pellets were resuspended and centrifuged at 12,000 g for 10 min at 4°C. The final pellets were resuspended in isolation medium and the mitochondrial protein content was determined using the method of Markwell *et al.* (1978) as described in Section 2.2.4.1.

2.2.2.5 Isolation of rat liver mitochondria

Rat liver mitochondria were isolated by a modification of a method by Chappell and Hansford (1972). For each preparation, one Wistar rat was killed by cervical dislocation and the liver was rapidly dissected and placed in ice-cold STE (250 mM sucrose, 2 mM EGTA and 5 mM Trizma-HCl pH 7.4). The rat liver was chopped in ice-cold STE buffer using scissors. The chopped liver was washed three times in STE buffer and homogenised by hand in a Potter-Elvehjem homogeniser using six passes of the loose pestle followed by six passes of the tight pestle. The rat liver homogenate was centrifuged at 800 g for 3 min at 4°C. The supernatant was centrifuged at 14,500 g for 10 min at 4°C. The pellet was resuspended in STE medium and centrifuged at 14,500 g for 10 min at 4°C. The pellet was again resuspended and centrifuged at 14,500 g for 10 min

at 4°C. The final pellet was suspended in STE buffer and the mitochondrial protein content was determined using the method of Markwell *et al.* (1978) as described in Section 2.2.4.1.

2.2.2.6 Isolation of rat brown adipose tissue mitochondria

Rat brown adipose tissue mitochondria were isolated using nagarse by a modification of a method by Scarpace *et al.* (1990). For each preparation, two Wistar rats were killed by cervical dislocation and the brown adipose tissue (BAT) from three rats was removed from the interscapular region (behind the head, between the shoulder blades) and placed in ice-cold isolation medium (250 mM sucrose, 1 mM EGTA and 5 mM Trizma-HCl pH 7.4). The rat brown adipose tissue was chopped using a scissors in a beaker containing ice-cold isolation medium and homogenised by hand in a Potter-Elvehjem homogeniser using three passes of the loose pestle followed by six passes of the tight pestle. The homogenate was passed through four layers of muslin and centrifuged at 8600 g for 10 min at 4°C. Fat deposits on the side of the centrifuge tube were removed using clean tissue and the pellet was resuspended using an ice-cold finger. The resuspended brown adipose tissue material was centrifuged at 750 g for 10 min at 4°C. The supernatant was carefully decanted and centrifuged at 8600 g for 10 min at 4°C. The pellet was resuspended in isolation medium and the mitochondrial protein content was determined using the method of Markwell *et al.* (1978) as described in Section 2.2.4.1.

2.2.2.7 Isolation of rat kidney mitochondria

Rat kidney mitochondria were isolated by a modification of a method by Chappell and Hansford (1972). For each preparation two Wistar rats were killed by cervical dislocation and the kidneys were rapidly dissected and placed in ice cold isolation medium (250 mM sucrose, 1 mM EGTA and 5 mM Trizma-HCl pH 7.4). The kidneys were chopped using scissors and rinsed in three changes of isolation medium to remove blood. The chopped rat kidneys were homogenised by hand in a Potter-Elvehjem homogeniser using ten passes of the loose pestle. The homogenate was centrifuged at 1050 g for 3 min at 4°C. The supernatant from the 1050 g centrifugation step was stored on ice and the pellets were homogenised by hand in isolation medium with five passes of

the tight pestle. The homogenate from the second homogenisation was centrifuged at 1050 g for 3 min at 4°C. The supernatant from this 1050 g centrifugation step was pooled with the supernatant stored on ice and centrifuged at 15,000 g for 5 min at 4°C. The pellets were resuspended in isolation medium and centrifuged once more at 15,000 g for 5 min at 4°C. The pellet was resuspended in isolation medium and the mitochondrial protein content was determined using the method of Markwell *et al.* (1978) as described in Section 2.2.4.1.

2.2.2.8 Isolation of rat synaptic mitochondria

Synaptic mitochondria were isolated from synaptosomes by a modification of a method by Lai and Clark (1976). Synaptosomes were isolated from four young rat brains or three old rat brains as in Section 2.2.1. The final synaptosomal pellet was isolated by centrifugation at 15,000 g for 20 min at 4°C. A 10 ml volume of 6 mM Trizma-HCL pH 8.1, was added to the pelleted synaptosomes, which were then vortex-mixed for three min in order to lyse the synaptosomes. The volume was made up to 50 ml with 6 mM Trizma and the lysed synaptosomes were centrifuged at 11,800 g for 10 min at 4°C. The supernatant was decanted without disturbing the pellet. The lysing procedure was repeated and again the lysed synaptosomes were centrifuged at 8300 g for 10 min at 4°C. The pellet from the 8300 g centrifugation step was suspended in 2 ml of STE (320 mM sucrose, 1 mM EDTA and 10 mM Trizma-HCl pH 7.4) and centrifuged at 12,000 g for 5 min in a bench top centrifuge. The final pellet was suspended in 0.5 ml of KSH medium (100 mM KCl, 20 mM sucrose, 10 mM KH₂PO₄, 5 mM Hepes, 2 mM MgCl₂ and 1 mM EGTA, pH 7.2). The mitochondrial protein content was determined using the method of Markwell *et al.* (1978) as described in Section 2.2.4.1.

2.2.3 Electrophoretic Methods

2.2.3.1 Sample preparation for native gels

Mitochondrial membrane proteins were solubilized with dodecylmaltoside by a modification of a method by Schägger *et al.* (1994). A maltoside:protein ratio of 3:1 was found to be optimum for the solubilization of beef heart and rat skeletal muscle

mitochondria. A maltoside:protein ratio of 2:1 was found to be optimum for the solubilization of rat synaptic, brown adipose tissue and heart mitochondria. A maltoside:protein ratio of 1:1 was found to be optimum for the solubilization of rat liver and kidney mitochondria. The mitochondrial samples were prepared as follows: the mitochondrial protein was made up to 80 μ l with distilled water. An 80- μ l volume of 1.5 M aminocaproic acid, 100 mM Bistris-HCl, pH 7.0 plus 40 μ l of 10% dodecylmaltoside was added to the mitochondria. The suspension was vortex mixed for 1 min before being centrifuged at 100,000 g for 10 min at 2°C. Shortly before starting, blue native PAGE, 5 μ l of Serva blue G from a 5% stock solution in 500 mM aminocaproic acid was added. Between 0.150 mg and 0.454 mg of protein were loaded per well on the native first-dimension gel.

2.2.3.2 First dimension: Blue native gel electrophoresis

The polyacrylamide gels were prepared by a modification of a method by Schagger (1994). Non-linear gradient gels (5.5-12%) were poured using a gradient maker and peristaltic pump. A Mini-protean II from BioRad was used for first-dimension gels. The gels were overlaid with a 4% stacking gel. Electrophoresis was started at 40 V for the first 30 min until the samples had entered the stacking gel. The voltage was then increased to 80 V. Electrophoresis was continued until the dye front reached the end of the gel. Immediately after electrophoresis, the gels were either frozen for use later or fixed and stained with Serva blue G.

2.2.3.3 Second dimension: Tricine-SDS-PAGE

Tricine-SDS gels were prepared as described by Schagger (1994). Gel lanes or a series of bands that were cut out from a first dimension blue native gel were placed on a glass plate. The gel pieces were then treated with 250 μ l of a 20 mM dithiothreitol (DTT)/1% SDS solution for 10 min per side per gel lane. As much of the denaturing solution as possible was removed, and the gel lanes or pieces were placed at the usual position between two glass plates for stacking gels. An ATTO AE-6220 gel electrophoresis unit was used and two first-dimension lanes were run side by side. The resolving gel, overlay and stacking gel were then poured. Usually a 10% resolving gel was used.

Electrophoresis was started at 60 V for approximately 50 min until the samples had entered for stacking gel. The voltage was then increased to 180 V. After 3 hours the voltage was increased to 200V. The gels took approximately 4 hours to run. Immediately after electrophoresis, the gels were stained with Serva blue G, silver stained or placed in a semidry blotter for Western blot analysis.

2.2.3.4 Serva blue G staining of polyacrylamide gels

First-dimension native gels, second-dimension tricine gels and Laemmli gels were stained using Serva blue G according to the method of Schägger (1994). Gels were fixed for 30 min in a 50% methanol/10% acetic acid solution, followed by staining in 10% acetic acid/0.025% Serva blue G for 30 to 60 min. The gels were destained in a 10% acetic acid solution that contained a piece of sponge to soak up excess dye.

2.2.4 General Methods

2.2.4.1 Markwell protein assay

The protein content of synaptosomal and mitochondrial suspensions was measured by the method of Markwell *et al.* (1978). The assay required the following solutions :

Markwell A: 2% (w/v) sodium carbonate
 0.4% (w/v) sodium hydroxide
 0.16% (w/v) sodium potassium tartrate
 1% (w/v) sodium dodecyl sulfate (SDS)

Markwell B: 4% (w/v) copper sulfate

Markwell C: This solution consisted of a 1:100 (v/v)
 dilution of Markwell B with Markwell A

Folin and Ciocalteu's reagent was diluted 1:1 (v/v) with deionised water. Markwell C (3 ml) was added to 1 ml of protein sample, which had been diluted with deionised water

and left at room temperature for 15 min. Folin and Ciocalteu's reagent (0.3ml) was added and the solution was left at room temperature for 45 min before the absorbance was read at 650 nm. The amount of protein present was determined by reference to a standard curve prepared using dilutions of a 1 mg/ml BSA solution (0-100µg).

2.2.4.2 Synaptosomal titrations

The specifics of the titrations of leak system, substrate oxidation system, phosphorylation system and the determination of oxygen consumption not due to oxidative phosphorylation are given in the legends of the relevant figures of Chapter 3.

2.2.4.3 Citrate synthase assay

The citrate synthase assay was carried out according to the method of Hansford and Castro (1982). The reaction was monitored at 412nm using a Unicam Heλ105γ spectrophotometer connected to a Philips (PM 8252) dual pen chart recorder. A 50µl volume of mitochondria treated with 4mg/ml DTNB, 50ml of 2mM acetyl-CoA and 5µl of Triton (0.2% final Triton concentration) were used. The blank rate was observed for 3 min before 75µl of a 60 mM oxaloacetate solution was added. The reaction was observed for a further 3 min.

2.2.4.4 Densitometry

Images of gels were obtained using a UVP camera and UVP Darkroom. The images of the gels were analyzed using Labworks 4 software.

2.2.4.5 Source of animals

The Wistar rats used were bred by the BioResources Unit (BRU), Trinity College Dublin.

2.2.4.6 Thyroid hormone treatment of Wistar rats

Hyperthyroidism was induced in Wistar rats by 10 daily IP injections of 15 µg of triiodo-L-thyronine (T₃)/100 g body weight. Sham euthyroids were administered 10 daily IP injections of PBS, pH 7.0.

2.2.4.7 Statistical analysis of the data

Data are presented as the mean standard error of the mean (SEM). Unless otherwise stated, significance was determined using *Student's* two-tailed *t*-test with differences of $p < 0.05$ considered to be significant.

Chapter 3

Oxidative Phosphorylation by *in situ* Synaptosomal Mitochondria from Whole Brain of Young and Old Rats.

3.1 Introduction

Experiments were designed to investigate *in situ* synaptosomal mitochondrial bioenergetic function using synaptosomes prepared from the whole brain of rats and to compare bioenergetic functions in synaptosomes isolated from young (3 month) and old (24 month) rats. The analysis determined the kinetic responses of the producers of $\Delta \Psi$ (substrate oxidation) and the consumers of $\Delta \Psi$ (proton leak and the phosphorylation system) to changes in $\Delta \Psi$, the intermediate in this branched pathway (Fig. 1.4.1). Thus, flux through the substrate oxidation, the proton leak and the phosphorylation system was measured indirectly by measuring oxygen consumption at different values of $\Delta \Psi$. The *in situ* mitochondrial $\Delta \Psi$ was modulated using (i) inhibitors of the mitochondrial electron transport chain (myxothiazol), (ii) inhibitors of ATP synthase (oligomycin) and (iii) a sodium ionophore of the plasma membrane (veratridine). The titration curves expected for the measurement of oxygen consumption by *in situ* synaptosomal mitochondria as a function of the magnitude of $\Delta \Psi$ during the course of these titrations are depicted in the schematic diagram (shown in Fig. 1.4.2).

The investigation describes for the first time the pattern of control of mitochondrial energy metabolism based on the kinetic responses of the substrate oxidation, proton leak and phosphorylation systems to changes in the mitochondrial proton electrochemical gradient (Δp) in synaptosomes isolated from the whole brain of rat. In addition, a comparison was made of the aforementioned kinetic responses, in synaptosomes from old and young animals.

3.2 Results

Figure 3.2.1 shows the synaptosomal *in situ* mitochondrial oxygen consumption rate as a function of increasing veratridine concentration in synaptosomes isolated from young (3 month) and old (24 month) rat brains. The concentration range of veratridine used in this

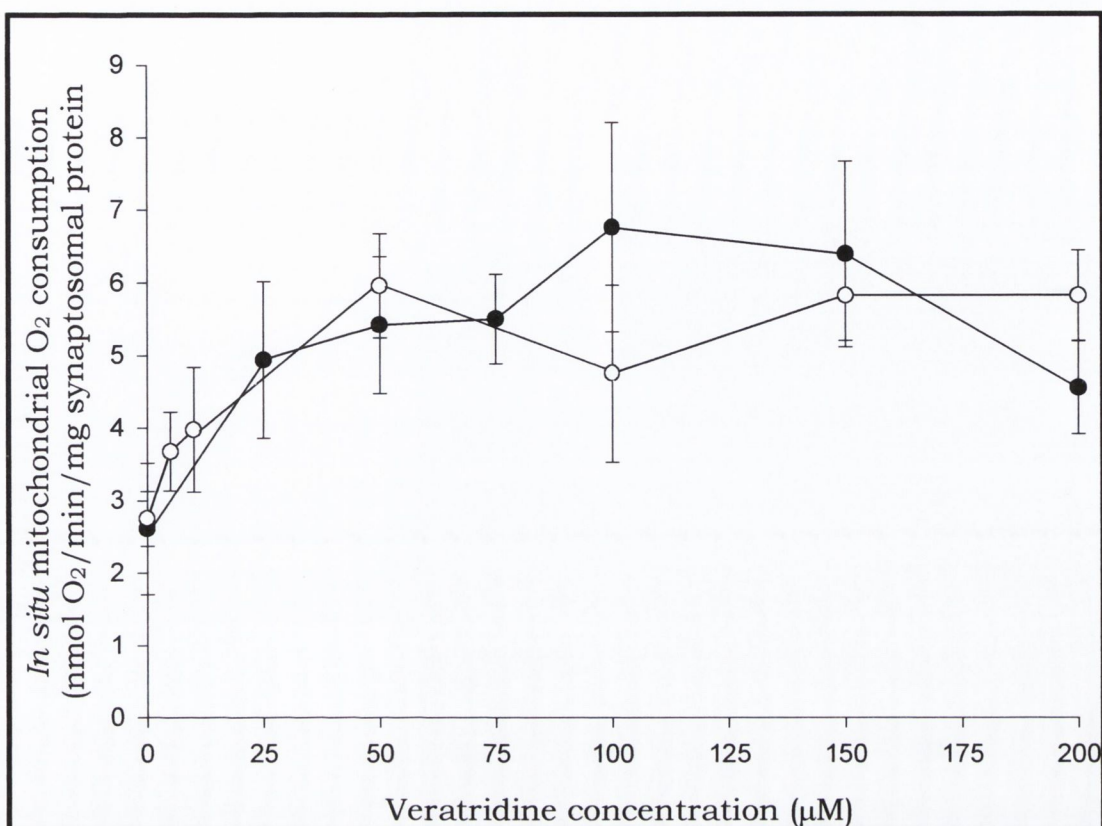


Figure 3.2.1 Effect of increasing veratridine concentration on synaptosomal *in situ* mitochondrial oxygen consumption in synaptosomes isolated from young (3 month) and old (24 month) rat brains. Synaptosomes (1.2 mg protein) isolated from young (●) and old (○) rat brains were preincubated in 0.6ml Krebs-Heinseleit buffer containing $1.3\mu\text{M TPMP}^+$ and $3\mu\text{M TPB}^-$ in stoppered polycarbonate flasks for 10 min in a shaking waterbath at 37°C . Synaptosomal *in situ* mitochondrial oxygen consumption was measured using a thermostated Clark-type oxygen electrode at 37°C , as decreased in Materials and Methods. Veratridine was dissolved in DMSO. The concentration range of veratridine added to young and old rat brain synaptosomes was 0-200 μM . Oxygen consumption not due to oxidative phosphorylation was measured as oxygen consumption in the presence of $2\mu\text{M}$ myxothiazol, oligomycin ($0.4\mu\text{g/ml}$ incubation medium), $3\mu\text{M}$ valinomycin and $20\mu\text{M}$ FCCP and was subtracted from all mitochondrial fluxes. All data represent the mean $\pm$ S.D. of at least two experiments.

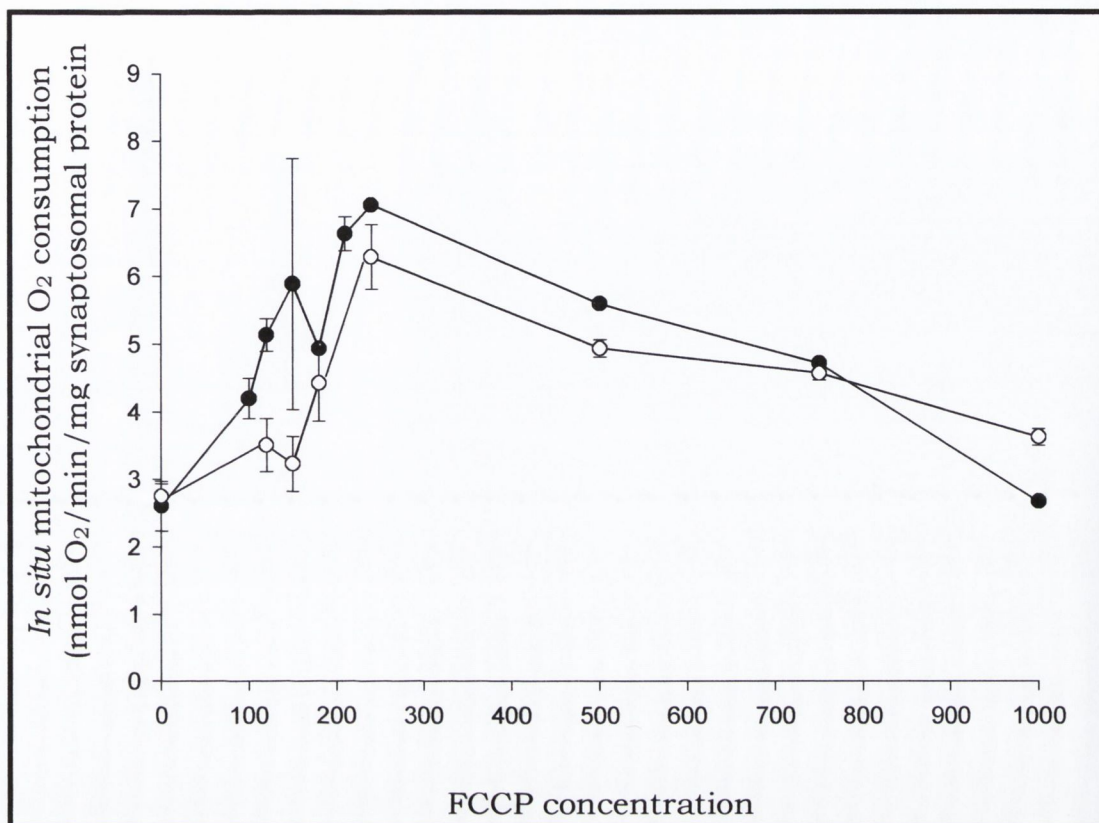


Figure 3.2.2 Effect of increasing FCCP concentration on synaptosomal *in situ* mitochondrial oxygen consumption in synaptosomes isolated from young (3 month) and old (24 month) rat brains. Synaptosomes (1.2 mg protein) isolated from young (●) and old (○) rats were preincubated in 0.6ml Krebs-Heinseleit buffer containing $1.3\mu\text{M TPMP}^+$ and $3\mu\text{M TPB}^-$ in stoppered polycarbonate flasks for 10 min in a shaking waterbath at 37°C . Synaptosomal *in situ* mitochondrial oxygen consumption was measured using a thermostated Clark-type oxygen electrode at 37°C as described in Materials and Methods. FCCP was dissolved in DMSO. The concentration range added to young and old rat brain synaptosomes was 0-1 μM . Oxygen consumption not due to oxidative phosphorylation was measured as oxygen consumption in the presence of $2\mu\text{M}$ myxothiazol, oligomycin ($0.4\mu\text{g/ml}$ incubation medium), $3\mu\text{M}$ valinomycin and $20\mu\text{M}$ FCCP and was subtracted from all mitochondrial fluxes. All data represent the mean $\pm$ S.D. of at least two experiments.

experiment ranged from 0-200 μM in the young and old rat brain synaptosomes. The optimum concentration of veratridine for placing the synaptosomal *in situ* mitochondria into state 3 was found to be 100 μM in the case of young and 50 μM in old rats (fig. 3.2.1) Thus, the state 3 oxygen consumption rate is the rate in the presence of excess veratridine when the substrate oxidation system and the phosphorylation system are together working at their maximal rate. Concentrations above these optima caused a decrease in oxygen consumption, presumably due to the secondary effects of veratridine. In addition, experiments showed that 3 min after the addition of veratridine, oxygen consumption decreased, presumably also due to the secondary effects of veratridine (results not shown).

Figure 3.2.2 shows the synaptosomal *in situ* mitochondrial oxygen consumption rate as a function of increasing FCCP concentration in synaptosomes isolated from young (3 month) and old (24 month) rat brains. The concentrations of FCCP used ranged from 0 to 1 μM in young and old rat brain synaptosomes. The oxygen consumption rate in the presence of excess FCCP is called the state 3 uncoupled (unc) rate. The optimum concentration of FCCP for placing the synaptosomal *in situ* mitochondria into state 3 uncoupled was found to be 240nM in both young and old rats. FCCP is a proton ionophore and so uncouples mitochondrial oxygen consumption from ATP synthesis by dissipating Δp (the mitochondrial membrane potential). The decrease in Δp with increasing concentrations of FCCP causes an increase in the activity of the substrate oxidation system which can be measured as an increase in the synaptosomal oxygen consumption rate. Concentrations of FCCP above this optimum caused a decrease in oxygen consumption in young rats, presumably due to the secondary effects of FCCP on the substrate oxidation system. Figure 3.2.3 shows a timecourse of the accumulation of $[^3\text{H}]\text{TPMP}^+$ by synaptosomes due to the magnitude of the plasma membrane potential and the *in situ* mitochondrial membrane potential in synaptosomes isolated from young (3 month) and old (24 month) rats. Figure 3.2.3 also shows the effect of adding veratridine and FCCP on $[^3\text{H}]\text{TPMP}^+$ accumulation in young rat brain synaptosomal *in situ* mitochondria. $[^3\text{H}]\text{TPMP}^+$ accumulation followed the expected trend. A steady state accumulation was reached within approximately 6 min. Addition of veratridine and

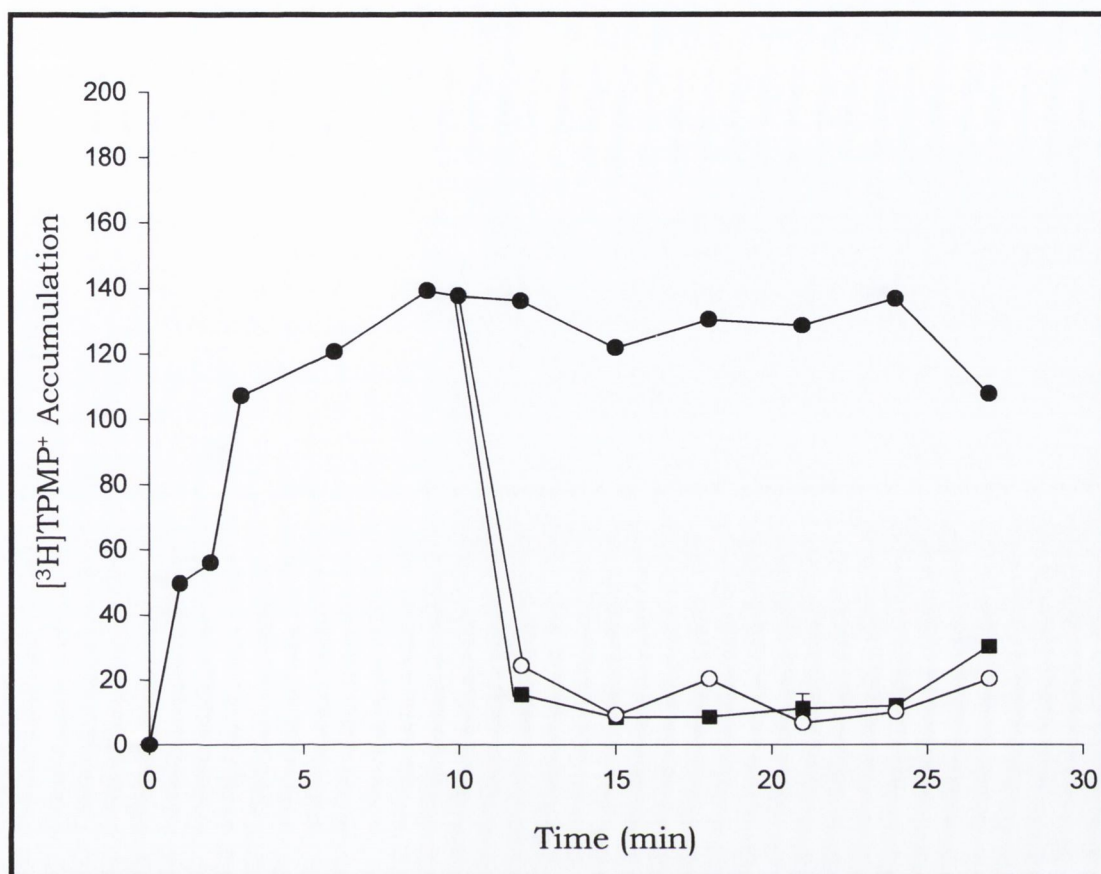


Figure 3.2.3 Time-dependent accumulation of [³H]TPMP⁺ by synaptosomes isolated from young (3 month) rat brains and the effect of veratridine and FCCP on [³H]TPMP⁺ accumulation. Synaptosomes (0.5 mg protein) from young rat brains were preincubated in 0.5ml Krebs-Heinseleit buffer containing 1.3 μM [³H]TPMP⁺ (0.2 μCi/mmol or 7.4 kBq/mmol), 3 μM TPB⁻ and ⁸⁶Rb⁺ (50 μM, 0.5 μCi or 18.5 kBq) in stoppered polycarbonate flasks in a shaking waterbath at 37°C. The synaptosomes were aerated every 10 min. After the incubation period, 0.4ml of the synaptosomal suspension was removed and the accumulation of [³H]TPMP⁺ was determined as described in Materials and Methods. The accumulation of [³H]TPMP⁺ with time is represented as ●. At 10 min, the following additions were made: (■) 100 μM veratridine; (○) 240 nM FCCP. All points represent the mean of two experiments.

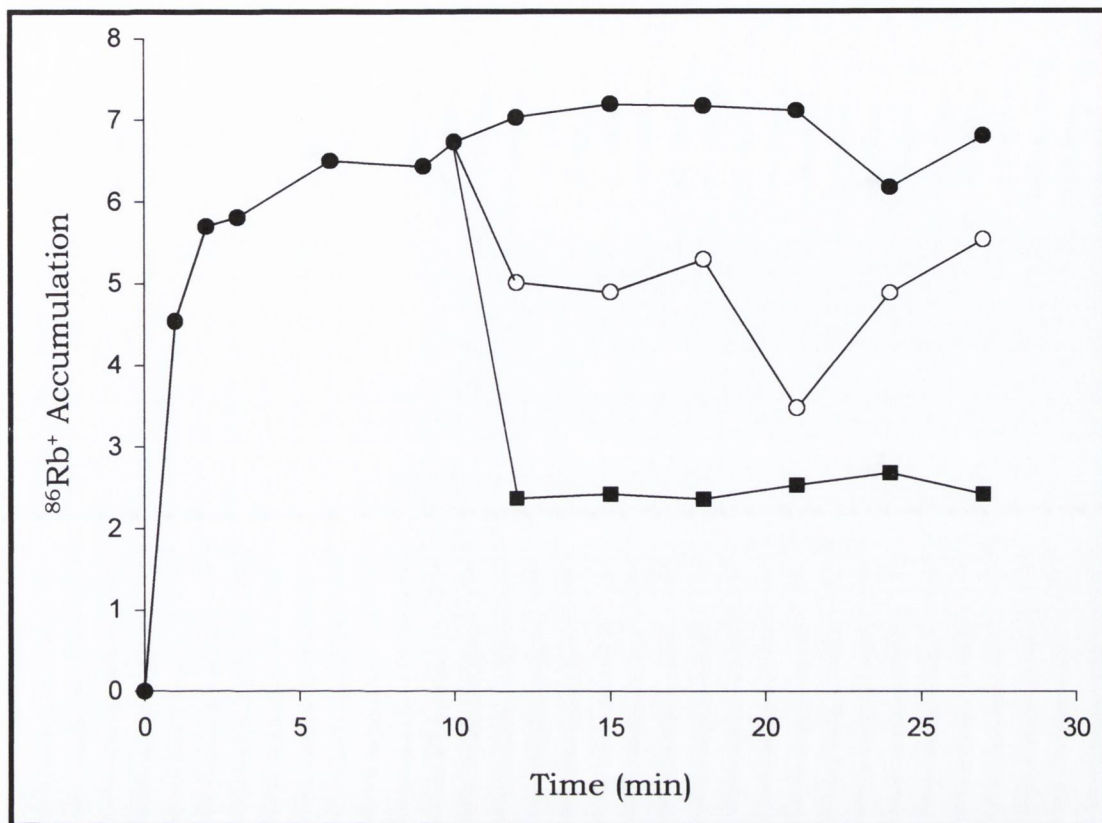


Figure 3.2.4 Time dependent accumulation of $^{86}\text{Rb}^+$ by synaptosomes isolated from young (3 month) rat brains and the effect of veratridine and FCCP on $^{86}\text{Rb}^+$ accumulation. Synaptosomes (0.5 mg protein) from young rat brains were preincubated in 0.5ml Krebs-Heinseleit buffer containing $1.3\mu\text{M}$ $[^3\text{H}]\text{TPMP}^+$ ($0.2\mu\text{Ci}/\text{mmol}$ or $7.4\text{kBq}/\text{mmol}$), $3\mu\text{M}$ TPB^- and $^{86}\text{Rb}^+$ ($50\mu\text{M}$, $0.5\mu\text{Ci}$ or 18.5kBq) in stoppered polycarbonate flasks in a shaking water bath at 37°C . The synaptosomes were aerated every 10 min. After the incubation period, 0.4ml of the synaptosomal suspension was removed and the accumulation of $^{86}\text{Rb}^+$ was determined as described in Materials and Methods. The accumulation of $^{86}\text{Rb}^+$ with time is represented as ●. At 10 min the following additions were made: (■) $100\mu\text{M}$ veratridine; (○) 240nM FCCP. All points represent the mean of two experiments.

FCCP causes a decrease in $[^3\text{H}]\text{TPMP}^+$ accumulation. Addition of veratridine or FCCP at 10 min caused a decrease in $[^3\text{H}]\text{TPMP}^+$ accumulation, as expected. In fact, $[^3\text{H}]\text{TPMP}^+$ accumulation came to a new equilibrium with the electrochemical gradient within 2 min, which is before the aforementioned secondary effects come into play. Hence, all oxygen consumption rates and $[^3\text{H}]\text{TPMP}^+$ accumulations were measured between 2 and 3 min after the addition of veratridine (fig. 3.2.3).

Figure 3.2.4 shows a timecourse of $^{86}\text{Rb}^+$ accumulation by synaptosomes. Figure 3.2.4 also shows the effect of adding veratridine and FCCP on $^{86}\text{Rb}^+$ accumulation in young rat brain synaptosomes. $^{86}\text{Rb}^+$ accumulation followed the expected trend. A steady-state accumulation was reached within approximately 6 min. The addition of veratridine and FCCP affects the plasma membrane potential. The effect of veratridine and FCCP on $^{86}\text{Rb}^+$ accumulation was showed that the changes in plasma membrane potential occurred within the '3-min window'. Veratridine and FCCP were added at 10 min, the accumulated $^{86}\text{Rb}^+$ reached equilibrium with the adjusted potentials within 2 min. Figure 3.2.3 shows that the addition of veratridine and FCCP had an effect on synaptosomal $[^3\text{H}]\text{TPMP}^+$ accumulation. Considering this in light of the information in Figure 3.2.4, some of these changes in $[^3\text{H}]\text{TPMP}^+$ accumulation are due to changes in the plasma membrane potential. Thus, when $[^3\text{H}]\text{TPMP}^+$ is used to determine the *in situ* mitochondrial membrane potential, it is necessary to correct for the $[^3\text{H}]\text{TPMP}^+$ accumulation due to the magnitude of the plasma membrane potential. The equation used to calculate the mitochondrial membrane potential (Equation 1, Section 2.2.1.3) takes into consideration the correction of the mitochondrial membrane due to the plasma membrane potential, which is calculated using $^{86}\text{Rb}^+$ accumulation across the plasma membrane.

Figure 3.2.5 shows the synaptosomal *in situ* mitochondrial oxygen consumption rate as a function of increasing oligomycin concentration in synaptosomes isolated from young (3 month) rat brains. Figure 3.2.6 shows the accumulation of $[^3\text{H}]\text{TPMP}^+$ by synaptosomes as a function of increasing oligomycin concentration in synaptosomes isolated from

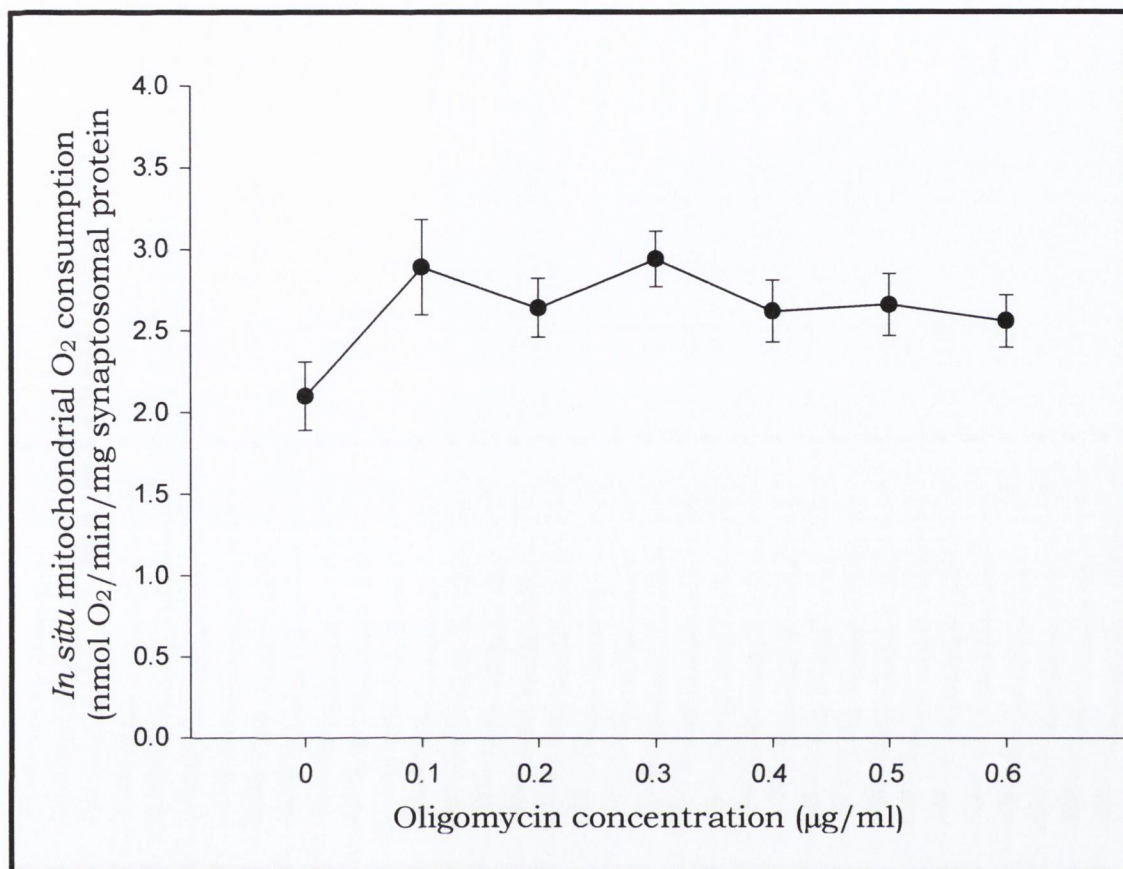


Figure 3.2.5 Effect of increasing oligomycin concentration on *in situ* mitochondrial oxygen consumption in synaptosomes isolated from young (3 month) rat brains. Synaptosomes (1.2 mg protein) isolated from young (●) rat brains were preincubated in 0.6ml Krebs-Heinseleit buffer containing $1.3\mu\text{M TPMP}^+$ and $3\mu\text{M TPB}^-$ in stoppered polycarbonate flasks for 10 min in a shaking water bath at 37°C and then for a further 10 min in the presence of oligomycin. Synaptosomal *in situ* mitochondrial oxygen consumption was measured using a thermostated Clark-type oxygen electrode at 37°C as in materials and methods. Oligomycin was dissolved in ethanol and added to young rat brain synaptosomes in the concentration range of 0.1-0.6µg/ml. Oxygen consumption not due to oxidative phosphorylation was measured as oxygen consumption in the presence of $2\mu\text{M}$ myxothiazol, oligomycin ($0.4\mu\text{g/ml}$ incubation medium), $3\mu\text{M}$ valinomycin and $20\mu\text{M}$ FCCP and was subtracted from all mitochondrial fluxes. All data represent the mean $\pm$ S.E.M. of three experiments.

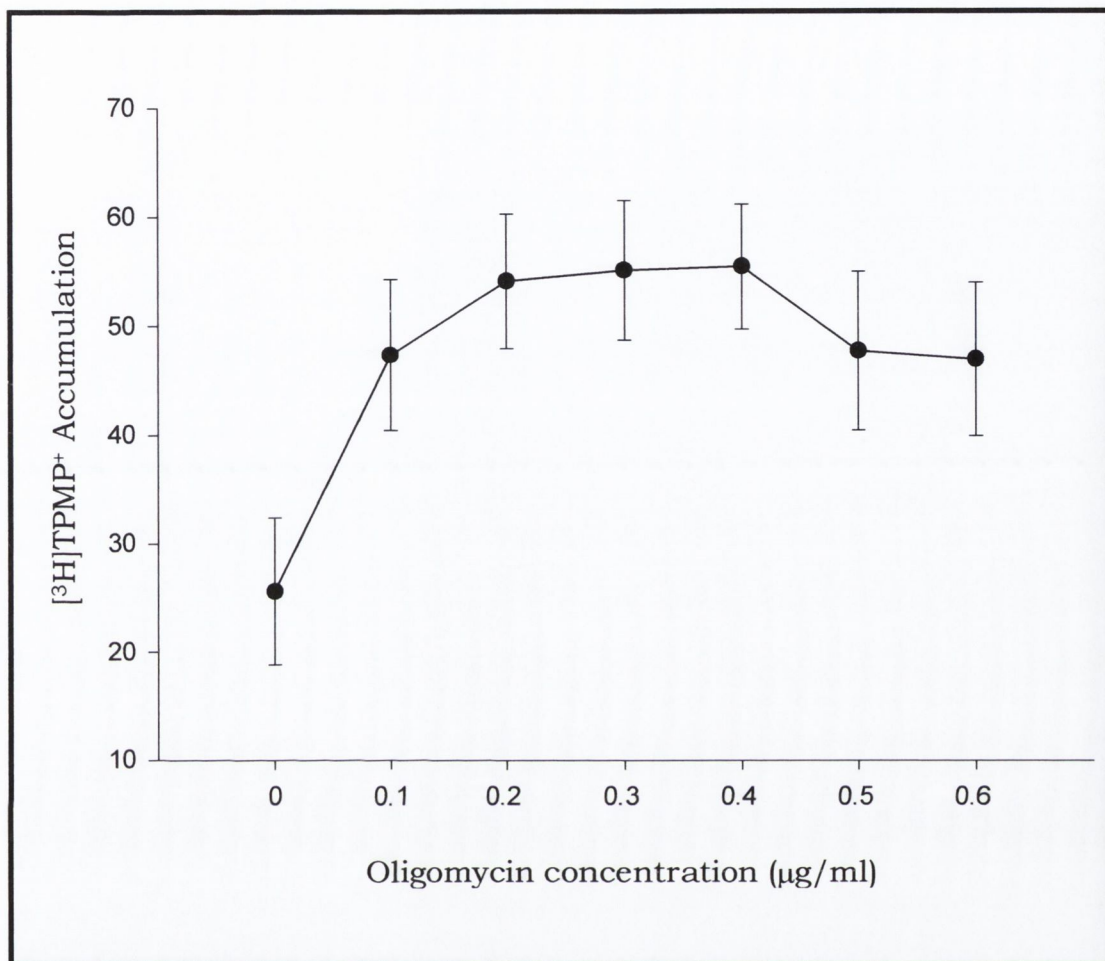


Figure 3.2.6 Effect of increasing oligomycin concentration on *in situ* mitochondrial [³H]TPMP⁺ accumulation in synaptosomes isolated from young (3 month) rat brains. Synaptosomes (0.5 mg protein) isolated from young rat brains (●) were preincubated in 0.5ml Krebs-Heinseleit buffer containing 1.3µM [³H]TPMP⁺ (0.2µCi/mmol or 7.4kBq/mmol), 3µM TPB⁻, in stoppered polycarbonate flasks in a shaking waterbath at 37°C for 10 min. After the incubation period, 0.4ml of the synaptosomal suspension was removed and the accumulation of [³H]TPMP⁺ was determined as in Materials and Methods. Oligomycin was dissolved in ethanol. The concentration range of oligomycin added to young rat brain synaptosomes was 0.1-0.6µg/ml. All points represent the mean ± S.E.M. of three experiments.

young (3 month) rat brains. In order to place *in situ* mitochondria in state 4, as a prelude to determining the proton leak kinetics, resting synaptosomes were titrated with increasing concentrations of oligomycin. Oligomycin had no significant effect on synaptosomal *in situ* mitochondrial oxygen consumption rates (fig. 3.2.5), however, oligomycin at a concentration of 0.4 μ g/ml gave a maximal increase in mitochondrial membrane potential (fig. 3.2.6). Concentrations of oligomycin above 0.4 μ g/ml decreased mitochondrial membrane potential below that of the maximal mitochondrial membrane potential achieved using 0.4 μ g/ml oligomycin. It was concluded that an oligomycin concentration of 0.4 μ g/ml was sufficient to place the synaptosomal *in situ* mitochondria into a non-phosphorylation state (state 4).

Figure 3.2.7 shows the kinetic response of the proton leak, substrate oxidation system and phosphorylation system to changes in the mitochondrial membrane potential in synaptosomal *in situ* mitochondria in synaptosomes isolated from young (3 month) rat brain mitochondria. Included is the oxygen consumption rate not due to oxidative phosphorylation. Synaptosomes isolated from young rat brains were preincubated in stoppered polycarbonate flasks for 10 min in a shaking water bath at 37°C before the titrations were carried out. In the case of the proton leak titration, the synaptosomes were incubated for a further 10 min in the presence of oligomycin. The kinetics of the proton leak in the presence oligomycin were established by titration with myxothiazol (0-0.5 μ M). The kinetics of the mitochondrial substrate oxidation system were established by changing the mitochondrial membrane potential by titration with increasing amounts of veratridine. For the phosphorylation system, the kinetics of mitochondrial membrane potential consumers were established by titrating the mitochondrial membrane potential in the presence of veratridine (100 μ M) with myxothiazol (0.05, 0.1 μ M). All data represent the mean $\pm$ S.E.M. of at least three experiments. The lines for *in situ* proton leak were drawn using an exponential fit. The lines defining the substrate oxidation system and phosphorylation system were fitted using linear regression weighted to pass through the maximal oxygen consumption rate point (*in situ* state 3 point).

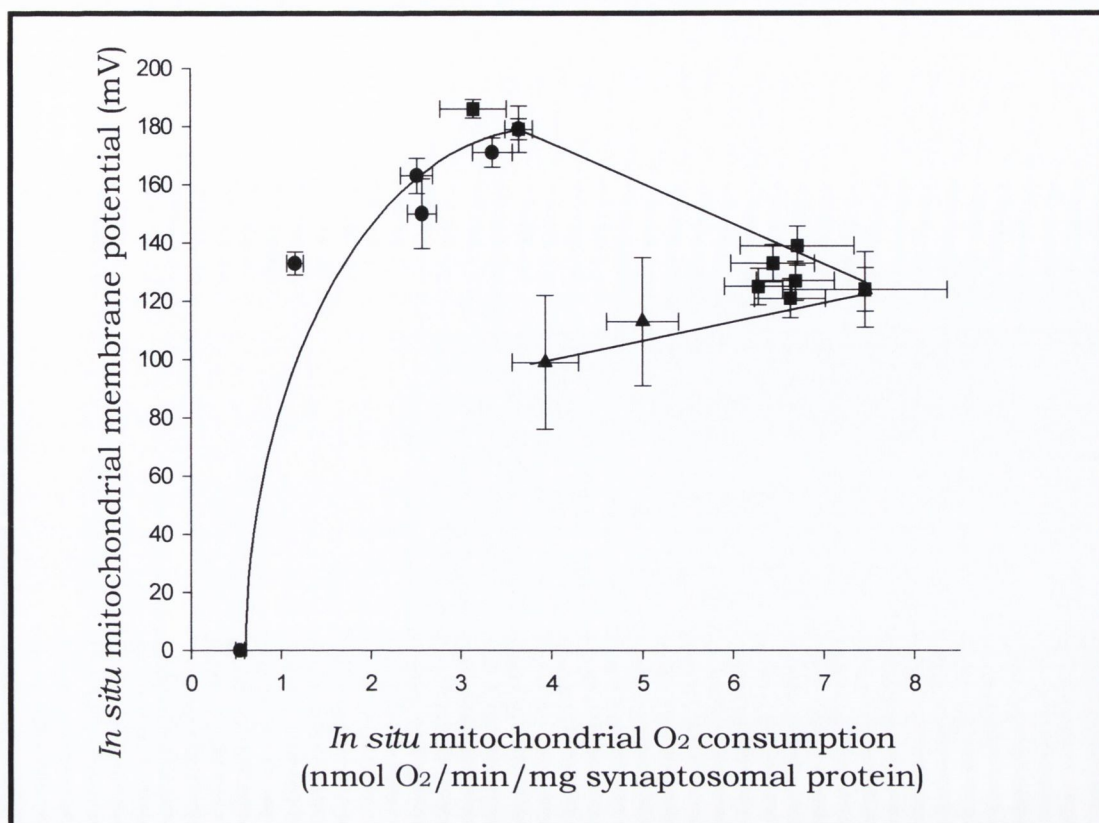


Figure 3.2.7 Comparison of the proton leak titration, substrate oxidation system titration and phosphorylation system titration in *in situ* mitochondria in synaptosomes isolated from young (3 month) rat brains. Synaptosomal *in situ* mitochondrial oxygen consumption and *in situ* mitochondrial membrane potential were determined as in Materials and Methods. Synaptosomes isolated from young rat brains were preincubated in stoppered polycarbonate flasks for 10 min in a shaking water bath at 37°C before the titrations were carried out. In the case of the proton leak titration, the synaptosomes were incubated for a further 10 min in the presence of oligomycin. Kinetics of the proton leak (●) in the presence of oligomycin were established by titration with myxothiazol (0, 0.03, 0.05, 0.075, 0.1, 0.5 μM). Kinetics of the mitochondrial substrate oxidation (■) system were established by changing the mitochondrial membrane potential by titration with increasing amounts of veratridine. For the phosphorylation system (▲), the kinetics of mitochondrial membrane potential consumers were established by titrating the mitochondrial membrane potential in the presence of veratridine (100 μM) with myxothiazol (0.05, 0.1 μM). Included is the oxygen consumption rate not due to oxidative phosphorylation. All data represent the mean ± S.E.M. of at least three experiments.

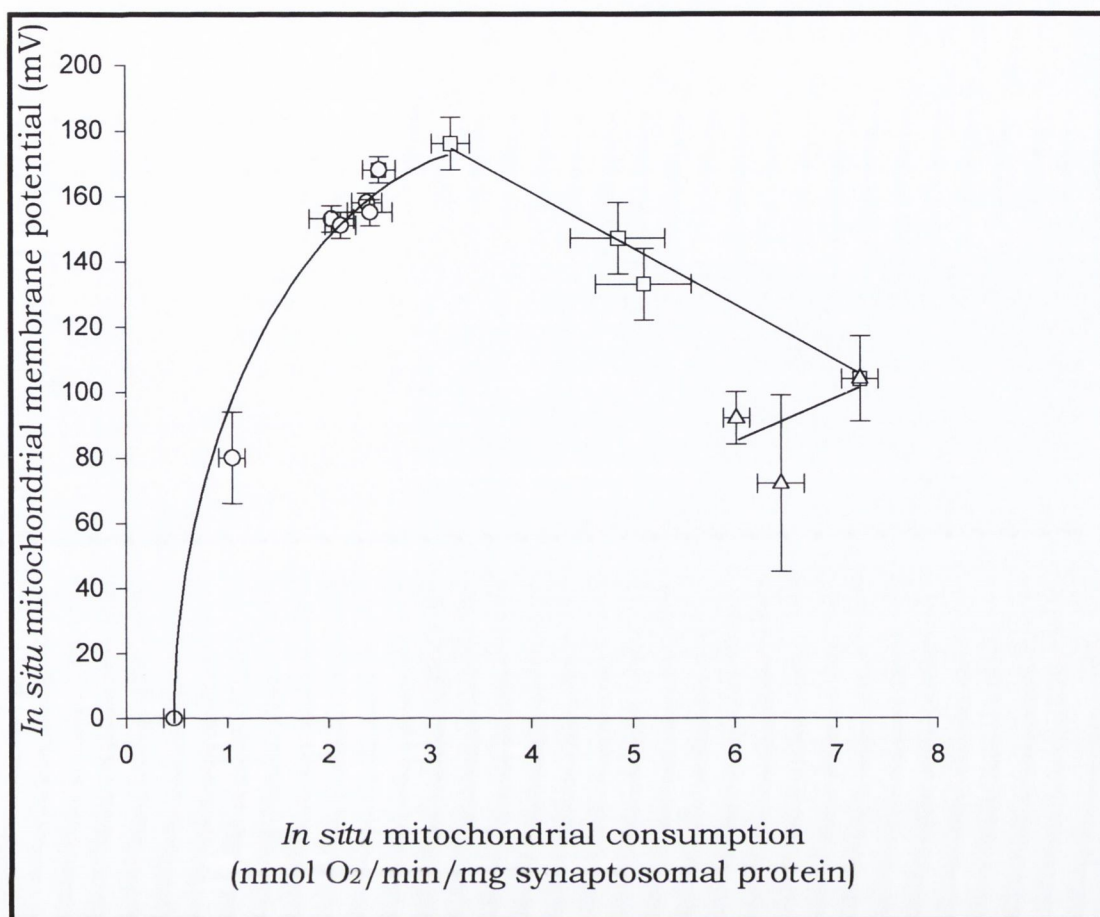


Figure 3.2.8 Comparison of the proton leak titration, substrate oxidation system titration and phosphorylation system titration in *in situ* mitochondria in synaptosomes isolated from old (24 month) rat brains. Synaptosomal *in situ* mitochondrial oxygen consumption and *in situ* mitochondrial membrane potential were determined as in Materials and Methods. Synaptosomes isolated from old rat brains were preincubated in stoppered polycarbonate flasks for 10 min in a shaking water bath at 37°C before the titrations were carried out. In the case of the proton leak titration, the synaptosomes were incubated for a further 10 min in the presence of oligomycin. Kinetics of the proton leak (○) in the presence oligomycin were established by titration with myxothiazol (0, 0.03, 0.05, 0.075, 0.1, 0.5 μM). Kinetics of the mitochondrial substrate oxidation system (□) were established by changing the mitochondrial membrane potential by titration with increasing amounts of veratridine. For the phosphorylation system (△), the kinetics of mitochondrial membrane potential consumers were established by titrating the mitochondrial membrane potential in the presence of veratridine (50 μM) with myxothiazol (0.05, 0.1 μM). Included is the oxygen consumption rate not due to oxidative phosphorylation. All data represent the mean ± S.E.M. of at least three experiments.

Figure 3.2.8 shows the kinetic response of the proton leak, substrate oxidation system and phosphorylation system to changes in the mitochondrial membrane potential in synaptosomal *in situ* mitochondria in synaptosomes isolated from old (24 month) rat brain mitochondria. Included is the oxygen consumption rate not due to oxidative phosphorylation. Synaptosomes isolated from rat brain of young rats were preincubated in stoppered polycarbonate flasks for 10 min in a shaking water bath at 37°C before the titrations were carried out. In the case of the proton leak titration, the synaptosomes were incubated for a further 10 min in the presence of oligomycin. Kinetics of the proton leak in the presence oligomycin were established by titration with myxothiazol (0-0.5 μM). Kinetics of the mitochondrial substrate oxidation system were established by changing the mitochondrial membrane potential by titration with increasing amounts of veratridine. For the phosphorylation system, kinetics of mitochondrial membrane potential consumers were established by titrating the mitochondrial membrane potential in the presence of veratridine (50 μM) with myxothiazol (0.05, 0.1 μM). All data represent the mean $\pm$ S.E.M. of at least three experiments. The lines for *in situ* proton leak were drawn using an exponential fit. The lines defining the substrate oxidation system and phosphorylation system were fitted using linear regression weighted to pass through the maximal oxygen consumption rate point (*in situ* state 3 point).

Figure 3.2.9 shows the kinetic response of the proton leak to changes in the mitochondrial membrane potential in synaptosomal *in situ* mitochondria in synaptosomes isolated from young (3 month) and old (24 month) rat brain mitochondria. Synaptosomes isolated from young and old rat brains were preincubated in stoppered polycarbonate flasks for 10 min in a shaking water bath at 37°C and then for a further 10 min in the presence of oligomycin. Kinetics of the proton leak in the presence oligomycin were established by titration with myxothiazol (0-0.5 μM). Oxygen consumption not due to oxidative phosphorylation was measured as oxygen consumption in the presence of 2 μM myxothiazol, oligomycin (0.4 $\mu\text{g/ml}$ incubation medium), 3 μM valinomycin and 20 μM FCCP and was subtracted from all mitochondrial fluxes. All data represent the mean $\pm$ S.E.M. of at least three experiments. The lines for *in situ* proton leak were drawn using

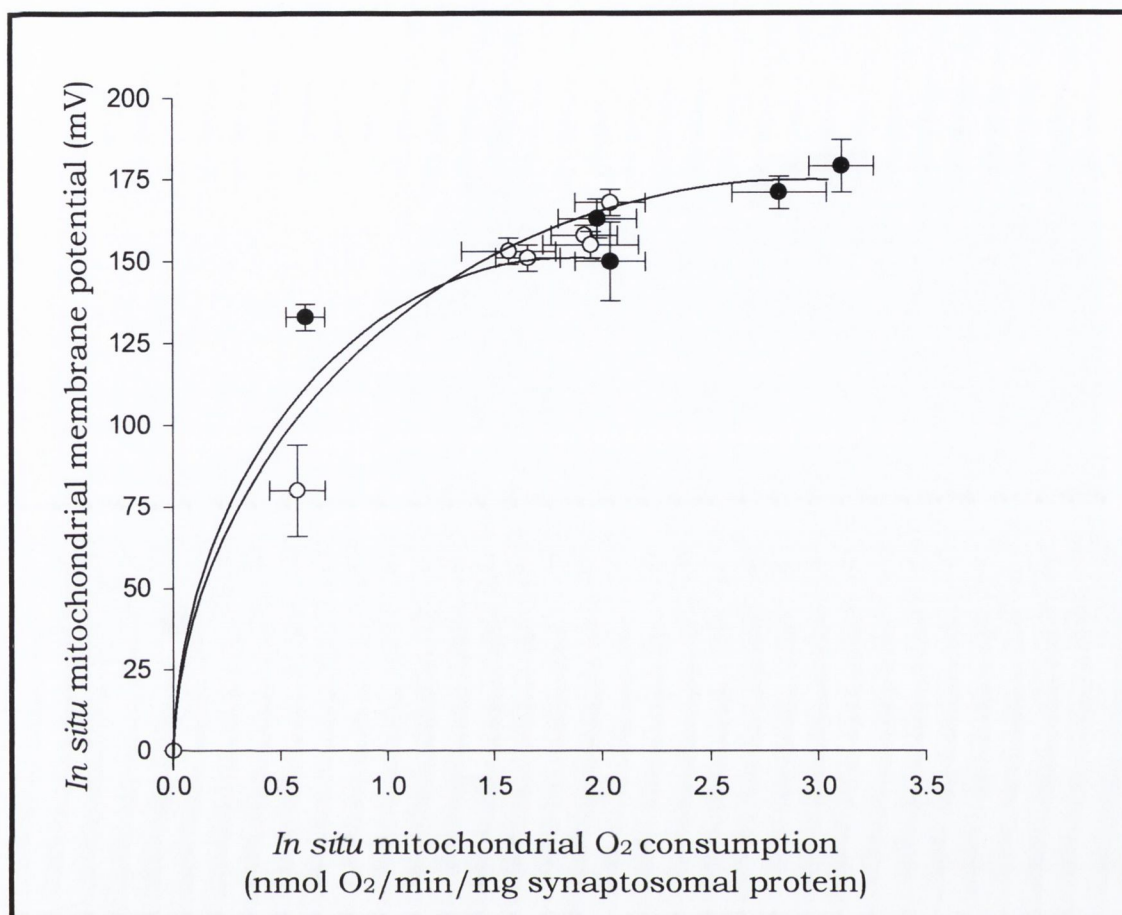


Figure 3.2.9 Comparison of the proton leak titration in *in situ* mitochondria in synaptosomes isolated from young (3 month) and old (24 month) rat brains. Synaptosomal *in situ* mitochondrial oxygen consumption and *in situ* mitochondrial membrane potential were determined as in Materials and Methods. Synaptosomes isolated from young (●) and old (○) rat brains were preincubated in stoppered polycarbonate flasks for 10 min in a shaking water bath at 37°C and then for a further 10 min in the presence of oligomycin. Kinetics of the proton leak in the presence oligomycin were established by titration with myxothiazol (0, 0.03, 0.05, 0.075, 0.1, 0.5 μM). Oxygen consumption not due to oxidative phosphorylation was measured as dioxygen consumption in the presence of 2 μM myxothiazol, oligomycin (0.4 μg/ml incubation medium), 3 μM valinomycin and 20 μM FCCP and was subtracted from all mitochondrial fluxes. All data represent the mean ± S.E.M. of at least three experiments.

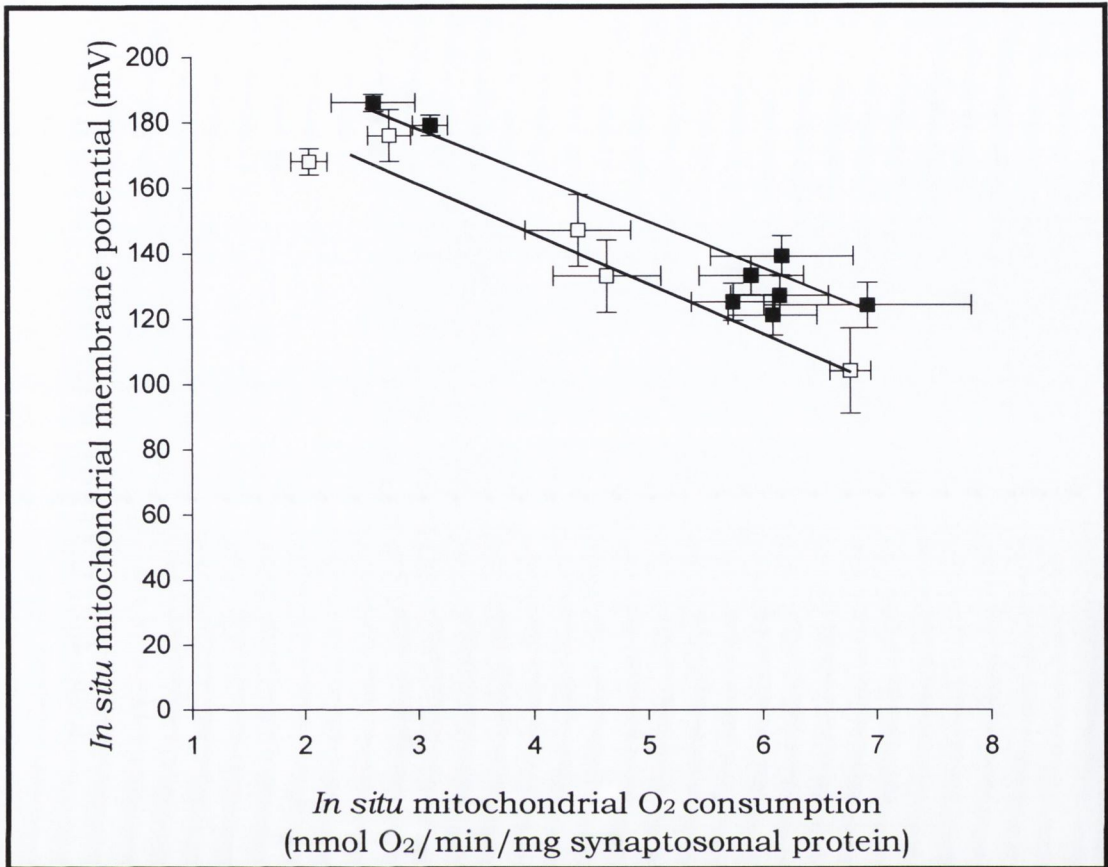


Figure 3.2.10 Comparison of the substrate oxidation titration in *in situ* mitochondria in synaptosomes isolated from young (3 month) and old (24 month) rat brains. Synaptosomal *in situ* mitochondrial oxygen consumption and *in situ* mitochondrial membrane potential were determined as in Materials and Methods. Synaptosomes isolated from young (■) and old (□) rats were preincubated in stoppered polycarbonate flasks for 10 min in a shaking water bath at 37°C before the titrations were carried out. Kinetics of the mitochondrial substrate oxidation system were established by changing the mitochondrial membrane potential by titration with increasing amounts of veratridine. Oxygen consumption not due to oxidative phosphorylation was measured as oxygen consumption in the presence of 2μM myxothiazol, oligomycin (0.4μg/ml incubation medium), 3μM valinomycin and 20μM FCCP and was subtracted from all mitochondrial fluxes. All data represent the mean ± sem of at least three experiments.

an exponential fit. The data shows the characteristic non-linear relationship between the rate of oxygen consumption due to proton leak and the driving force for that leak, Δp . Curves fitted to data for synaptosomes from old and young rats practically superimpose, suggesting that the degree of mitochondrial proton leak is similar and, as deduced by an overlap in the error bars of the points defining the curve.

Figure 3.2.10 shows the kinetic response of the substrate oxidation system to changes in the mitochondrial membrane potential in synaptosomal *in situ* mitochondria in synaptosomes isolated from young (3 month) and old (24 month) rat brain mitochondria. Synaptosomes isolated from young rat brains were preincubated in stoppered polycarbonate flasks for 10 min in a shaking water bath at 37°C. Kinetics of the mitochondrial substrate oxidation system were established by changing the mitochondrial membrane potential by titration with increasing amounts of veratridine. Oxygen consumption not due to oxidative phosphorylation was measured as oxygen consumption in the presence of 2µM myxothiazol, oligomycin (0.4µg/ml incubation medium), 3µM valinomycin and 20µM FCCP and was subtracted from all mitochondrial fluxes. All data represent the mean $\pm$ S.E.M. of at least three experiments. The lines defining the substrate oxidation system were fitted using linear regression weighted to pass through the maximal oxygen consumption rate point (*in situ* state 3 point). The data show that the kinetic response of the substrate oxidation systems for *in situ* mitochondria of synaptosomes isolated from young and old rat brains is similar, as deduced by an overlap in the error bars of the points defining the line.

Figure 3.2.11 shows the kinetic response of the phosphorylation system to changes in the mitochondrial membrane potential in synaptosomal *in situ* mitochondria in synaptosomes isolated from young (3 month) and old (24 month) rat brain mitochondria. Synaptosomes isolated from young rat brains were preincubated in stoppered polycarbonate flasks for 10 min in a shaking water bath at 37°C. For the phosphorylation system, the kinetics of mitochondrial membrane potential consumers were established by titrating the mitochondrial membrane potential in the presence of veratridine (100µM for young

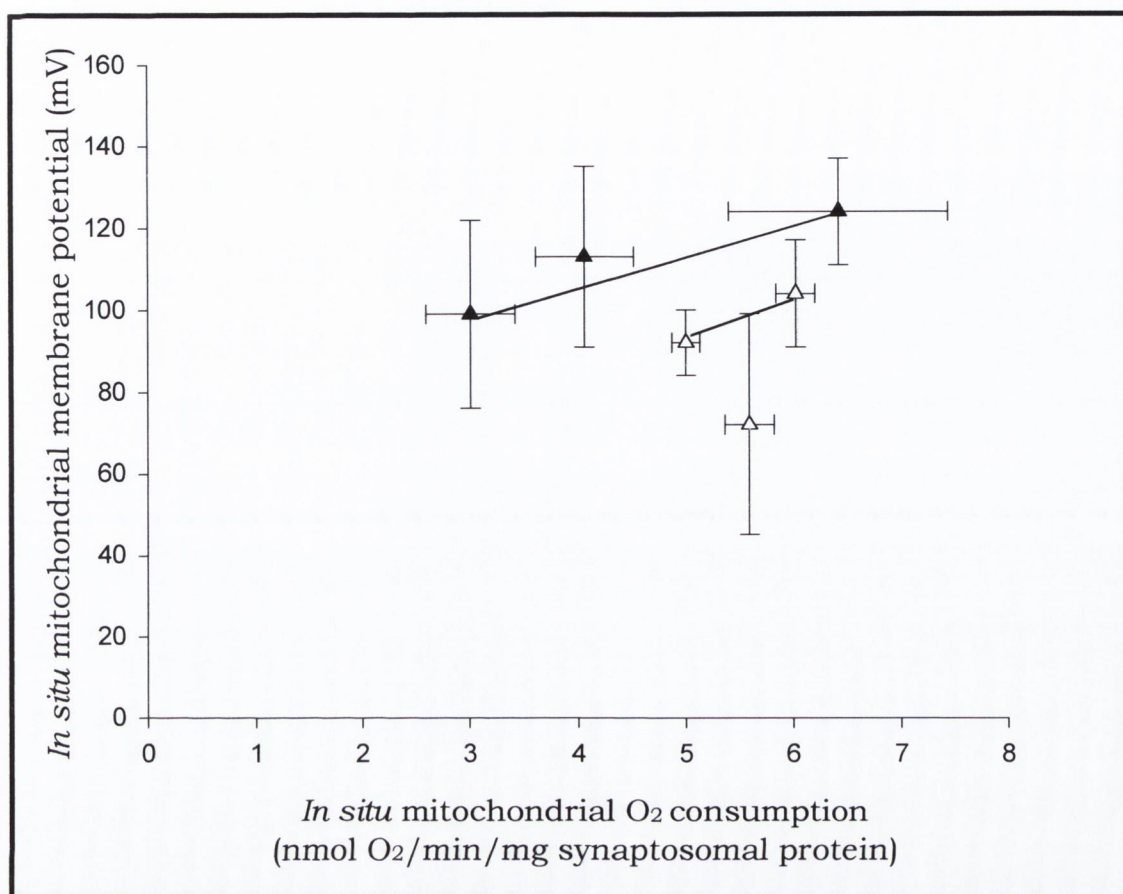


Figure 3.2.11. Comparison of the phosphorylation system titration in *in situ* mitochondria in synaptosomes isolated from young (3month) and (24 month) old rat brains. Synaptosomal *in situ* mitochondrial oxygen consumption and *in situ* mitochondrial membrane potential were determined as in Materials and Methods. Synaptosomes isolated from young (▲) and old (Δ) rats were preincubated in stoppered polycarbonate flasks for 10 min in a shaking water bath at 37°C before the titrations were carried out. For the phosphorylation system, the kinetics of mitochondrial membrane potential consumers were established by titrating the mitochondrial membrane potential in the presence of veratridine (100μM in young rat synaptosomes and 50μM in old rat synaptosomes) with myxothiazol (0.05, 0.1μM). Proton leak flux was subtracted from the mitochondrial membrane potential consumer flux to give phosphorylation kinetics. Oxygen consumption not due to oxidative phosphorylation was measured as dioxygen consumption in the presence of 2μM myxothiazol, oligomycin (0.4μg/ml incubation medium), 3μM valinomycin and 20μM FCCP and was subtracted from all mitochondrial fluxes. All data represent the mean $\pm$ sem of at least three experiments.

Table 3.2.1

Oxygen consumption rate (nmol O ₂ /min/mg synaptosomal protein)		
	<i>Young</i>	<i>Old</i>
Resting synaptosomal oxygen consumption	3.15±0.37(6)	3.22±0.19(6)
Resting mitochondrial oxygen consumption	2.61±0.37 (6)	2.75±0.21(5)
Non-phosphorylating conditions (+ oligomycin)	3.11±0.15 (8)*	2.04±0.16 (11)*
Maximal phosphorylating conditions (+ veratridine)	6.92±0.91 (5)	6.77±0.18 (4)
Uncoupled <i>in situ</i> mitochondria (+ FCCP)	7.87±0.48 (8) **	6.08±0.42 (11)**
Oxygen consumption not due to oxidative phosphorylation	0.54±0.05 (6)	0.47±0.10(6)

Table 3.2.1 Comparison of oxygen consumption values in young and old synaptosomes from whole brains. Values represent mean ± S.E.M. and values in parenthesis represent the number of determinations. *-Significant difference $p=0.0002$. **- Significant difference $P=0.0124$. Unpaired *Student's t*-test, two-tailed p value, comparing synaptosomes from young and old rats. [Oligomycin] = 0.4 µg/ml incubation medium, [Veratridine] = 50µM for old and 100µM for young, [FCCP] = 240nM.

Table 3.2.2

Membrane potential (mV)		
	Young	Old
Resting mitochondrial membrane potential	186±5 (6)	176±8 (6)
Resting plasma membrane potential	45±3 (3)	45±3 (3)
Non-phosphorylating conditions (+ oligomycin)	179±8 (8)	168±4 (12)
Maximal phosphorylating conditions (+ veratridine)	124±12 (8)	104±13 (4)
Uncoupled <i>in situ</i> mitochondria (+ FCCP).	61±14 (8)	70±17 (11)

Table 3.2.2 Comparison of the *in situ* mitochondrial membrane potentials and plasma membrane potentials in synaptosomes from young and old rats. Values represent the mean ± S.E.M. and values in parentheses represent the number of determinations. unc = uncoupled, [Oligomycin] = 0.4 µg/ml incubation medium, [Veratridine] = 50µM for old and 100µM for young, [FCCP] = 240nM.

synaptosomes, 50 μM for old synaptosomes) with myxothiazol (0.05, 0.1 μM). Oxygen consumption not due to oxidative phosphorylation was measured as oxygen consumption in the presence of 2 μM myxothiazol, oligomycin (0.4 $\mu\text{g/ml}$ incubation medium), 3 μM valinomycin and 20 μM FCCP and was subtracted from all mitochondrial fluxes. As some proton leak occurred in this phosphorylating state titration, one can only get a true measure of the phosphorylation system kinetics when proton leak, at any given Δp , is subtracted. Therefore, proton leak flux was subtracted from mitochondrial membrane potential consumer flux to give phosphorylation kinetics. All data represent the mean $\pm$ S.E.M. of at least three experiments. The lines defining the phosphorylation system were fitted using linear regression weighted to pass through the maximal oxygen consumption rate point (*in situ* state 3 point). The data show that the kinetic response of the phosphorylation systems for *in situ* mitochondria of synaptosomes isolated from young and old rat brains is similar, as deduced by an overlap in the error bars of the points defining the line.

Table 3.2.1 shows a comparison of oxygen consumption values in synaptosomes isolated from young and old rat brains.

Table 3.2.2 shows a comparison of the *in situ* mitochondrial membrane potentials and plasma membrane potentials in synaptosomes isolated from young and old rats.

3.3 Discussion

3.3.1 Comparison of oxygen consumption and *in situ* mitochondrial membrane potentials and plasma membrane potentials in synaptosomes from young and old rats

Synaptosomes isolated from young rats had a resting oxygen consumption rate of $3.15 \pm 0.37(6)$ [mean $\pm$ S.E.M(n)] nmol $\text{O}_2/\text{min}/\text{mg}$ synaptosomal protein (Table 3.2.1), a resting *in situ* mitochondrial membrane potential of 186 ± 5 mV (6) (Table 3.2.2) and a resting plasma membrane potential of 45 ± 3 mV (3) (Table 3.1.2). Isolated synaptosomes from old rats had a resting oxygen consumption rate of $3.22 \pm 0.19(6)$ nmol $\text{O}_2/\text{min}/\text{mg}$ synaptosomal protein (Table 3.2.1), a resting *in situ* mitochondrial membrane

potential of 176 ± 8 mV (6) (Table 3.2.2) and a resting plasma membrane potential of 45 ± 3 mV (3) (Table 3.1.2). The values for resting oxygen consumption rates and plasma membrane potentials obtained are consistent with those of other workers (Scott & Nicholls, 1980; Erecińska *et al.*, 1996). Despite this consistency, the plasma membrane potentials (as measured by potassium diffusion potentials) are lower than might be expected from knowledge of intra-neuronal concentrations of potassium. Microelectrode measurements show intra-neuronal potassium to be 80-90mM, but intra-synaptosomal potassium concentrations are reported to be between 45 and 50mM, using an equivalent synaptosomal preparation to that used in these experiments (McMahon & Nicholls, 1981; Erecińska *et al.*, 1996). So, clearly, potassium loss is inherent in the generation of synaptosomes.

In order to determine the oxygen consumption rate by synaptosomes not due to oxidative phosphorylation, it was necessary to inhibit oxygen consumption by the mitochondrial electron transport chain. This was achieved by the addition of excess myxothiazol ($3\mu\text{M}$). Simultaneously, in order to determine the accumulation of the lipophilic cation, TPMP⁺, at zero mitochondrial membrane potential, in addition to excess myxothiazol ($2\mu\text{M}$), excess oligomycin ($0.4\mu\text{g/ml}$), excess FCCP ($20\mu\text{M}$) and excess valinomycin ($3\mu\text{M}$) was added to the incubation medium containing synaptosomes. Figure 3.2.7 shows the amount of oxygen consumption not due to oxidative phosphorylation for synaptosomes isolated from young (3 month) rat brains and Fig. 3.2.8 shows the amount of oxygen consumption not due to oxidative phosphorylation for synaptosomes isolated from old (24 month) rat brains in conjunction with the measured overall kinetic responses of the (i) substrate oxidation system, (ii) the proton leak and (iii) the phosphorylation system (uncorrected for proton leak) to changes in $\Delta\Psi$.

The oxygen consumption rates not due to oxidative phosphorylation were found to be 0.54 ± 0.05 (6) and 0.47 ± 0.10 (6) nmol O₂/min/mg synaptosomal protein in synaptosomes from young and old rats, respectively. There was no significant difference between the oxygen consumption rates not due to oxidative phosphorylation in synaptosomes from young and old animals. Subtracting the oxygen consumption rates not due to oxidative

phosphorylation from the total oxygen consumption rates by the synaptosomes gives the oxygen consumption rate due solely to the *in situ* mitochondria (Table 3.2.1).

In order to determine the oxygen consumption rate due to the *in situ* mitochondria, the oxygen consumption rate not due to oxidative phosphorylation was subtracted from the total oxygen consumption rate of the synaptosomes. Synaptosomes isolated from young rats had a resting mitochondrial oxygen consumption rate of 3.61 ± 0.37 (6) [mean $\pm$ S.E.M. (n)] nmol O₂/min/mg synaptosomal protein (Table 3.1.1), whereas synaptosomes from old rats had a resting oxygen consumption rate of 2.75 ± 0.21 (6) nmol O₂/min/mg synaptosomal protein (Table 3.2.1). There was no significant difference between these two values.

The investigation found that there was no significant difference in the resting (non-stimulated) oxygen consumption rate, mitochondrial membrane potential and plasma membrane potential when comparing synaptosomes isolated from young and old rats (Tables 3.2.1 & 3.2.2; Fig. 3.2.7 & 3.2.8). Clearly, there was no difference in energy metabolism as manifest in these key parameters.

A further interesting observation was that there was no significant difference between the state 4 (non-phosphorylating) oxygen consumption rates or membrane potentials in synaptosomes from young and old rats when compared to their respective resting states (Tables 3.2.1 & 3.2.2; Fig. 3.2.7 & 3.2.8), an observation noted previously by Scott and Nicholls (1980) for guinea pig synaptosomes. Clearly, there is insignificant ATP turnover via the mitochondria in non-stimulated synaptosomes. Thus, just about all of the *in situ* mitochondrial oxygen consumption under resting conditions, in synaptosomes from young and old rats, is due to proton leak. Approximately 85% of the resting oxygen consumption by synaptosomes from young and old animals is due to mitochondrial proton leak.

3.3.2 Comparison of the kinetic response of the proton leak in *in situ* mitochondria in synaptosomes isolated from young (3 month) and old (24 month) rat brains

Figure 3.2.9 shows a comparison of the proton leak for mitochondria *in situ* in synaptosomes isolated from young and old rat brains, measured in the presence of optimal inhibitory amounts of oligomycin (0.4 $\mu\text{g/ml}$) and increasing concentrations of myxothiazol (0-0.5 μM). The data show the characteristic non-linear relationship between proton leak rate (or more correctly, the rate of oxygen consumption due to proton leak) and the driving force for that leak Δp . Curves fitted to data for synaptosomes from old and young rats practically superimpose, suggesting that the degree of mitochondrial proton leak is similar and not different, as deduced by an overlap in the error bars of the points defining the curve.

The *in situ* oxygen consumption rate due to proton leak in synaptosomes from young and old animals showed a typical non-ohmic relationship (Fig. 3.2.7 & 3.2.8). Similar non-ohmic relationships between proton leak and mitochondrial membrane potential have been observed in isolated mitochondria (Porter & Brand, 1993), mitochondria *in situ* in hepatocytes (Porter & Brand, 1995) and in thymocytes (Krauss *et al.*, 2002). The indistinguishable nature of the proton leak curves between the *in situ* mitochondria from young and old animals suggests that there is no difference between the proton permeability of the inner membrane of neuronal mitochondria in this comparison. The proton leak data are in good agreement with a report by Vitorica *et al.* (1985) who found no difference in the proton leak rate in a comparison of mitochondria isolated from whole brain of young (2-3months) and old (32 month) rats.

3.3.3 Comparison of the substrate oxidation system in *in situ* mitochondria in synaptosomes isolated from young (3 month) and old (24 month) rat brains

Figure 3.2.10 shows the kinetic response of the substrate oxidation system to changes in $\Delta\Psi_{\text{mito}}$ in *in situ* mitochondria isolated from young (3 month) and old (24 month) rat brains. The substrate oxidation kinetic response was achieved by firstly titrating the resting (non-stimulated, non-inhibited) oxygen consumption rate of synaptosomal mitochondria to a non-phosphorylating state (state 4) with oligomycin. It was calculated

that there was no significant difference between the resting oxygen consumption rates and the non-phosphorylating oxygen consumption rates for synaptosomes from young (2.61 ± 0.37 (6) and 3.11 ± 0.15 (6) nmol O₂/min/mg synaptosomal protein, respectively) and old (2.75 ± 0.21 (6) and 2.04 ± 0.16 (11) nmol O₂/min/mg synaptosomal protein, respectively), *i.e.* oligomycin had no significant effect on oxygen consumption rates (Table 3.2.1). Similarly, oligomycin had no significant effect on $\Delta\Psi_{\text{mito}}$ when comparing the resting state and the non-phosphorylating state for synaptosomes from young (186 ± 5 mV (6) and 179 ± 8 mV (8), respectively) and old (176 ± 8 mV (6) and 168 ± 4 mV (12) respectively) rats (Table 3.2.2).

In addition, the kinetic response of the substrate oxidation system was investigated using a titration to stimulate ATP turnover in the synaptosome and thus stimulate the *in situ* synaptosomal mitochondria to synthesize more ATP, thus driving the mitochondria to their maximal phosphorylation state (state 3). This was achieved by titration with a plasma membrane Na⁺-ionophore, veratridine, thus stimulating the plasma membrane Na⁺/K⁺-ATPase to work harder to re-establish the plasma membrane Na⁺-gradient and, by doing so, increasing the rate of ATP turnover. The *in situ* oxygen consumption rates by mitochondria due to maximal phosphorylation were significantly greater than the resting oxygen consumption rates for synaptosomes from both young (6.92 ± 0.91 (5) versus 2.61 ± 0.37 (6) nmol O₂/min/mg synaptosomal protein, $p=0.0011$, respectively) and old (6.77 ± 0.18 (4) versus 2.75 ± 0.21 (5) nmol O₂/min/mg synaptosomal protein, $p=0.0001$, respectively) animals. However, there was no significant difference in the rate of oxygen consumption in the non-phosphorylating oxygen consumption rates from young and old animals (3.11 ± 0.15 (8) versus 2.04 ± 0.16 (11) nmol O₂/min/mg synaptosomal protein, respectively) and maximal phosphorylating states when comparing synaptosomes from young and old animals (6.92 ± 0.91 (5) versus 6.77 ± 0.18 (4) nmol O₂/min/mg synaptosomal protein, respectively) (Table 3.2.1). In addition, there was no significant difference in the non-phosphorylating $\Delta\Psi_{\text{mito}}$ from young and old animals (179 ± 8 mV (6) versus 168 ± 4 mV (6), respectively) and maximal phosphorylating states when comparing synaptosomes from young and old animals (124 ± 12 mV (6) versus 104 ± 13 mV (4), respectively) (Table 3.2.2). In addition, maximal substrate oxidation flux (as opposed to

maximal phosphorylation flux) was measured by titration with carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP up to 240nM), an uncoupler of mitochondrial oxidative phosphorylation. Under these conditions, it was found that there was a significantly ($p=0.0124$) reduced oxygen consumption rate in synaptosomes from old rats (6.08 ± 0.42 , (11) nmol O₂/min/mg synaptosomal protein) when compared with synaptosomes from young rats (7.87 ± 0.48 (8) nmol O₂/min/mg synaptosomal protein Table 1). The $\Delta\Psi_{\text{mito}}$ at maximal substrate oxidation flux *i.e.*, in the presence of excess FCCP, was measured and found not to be significantly different between synaptosomes from young and old animals (61 ± 14 mV (8) versus 79 ± 17 mV (11), respectively Table 3.2.2).

It was found that the sensitivity of the substrate oxidation systems of the synaptosomes to changes in Δp of the *in situ* mitochondria was not different, as deduced by an overlap in the error bars of the points defining the line, when comparing synaptosomes from young and old animals (Fig. 3.2.7, 3.2.8 & 3.2.10). It can be seen that, at maximal phosphorylating capacity, the capacity of the substrate oxidation system to produce Δp for ATP synthesis is not different when comparing synaptosomes from young and older animals. Before the study, it was expected that the substrate oxidation system might have affected mitochondrial Δp producing capacity, as several investigators had found decreased specific activities of respiratory complexes in aged animals (Curti *et al.*, 1990; Bowling *et al.* 1993; Ferrandiz *et al.*, 1994). Clearly if there is a decreased specific activity of the enzyme complexes in synaptosomal *in situ* mitochondria, then the decreased activity is not manifest at the level of oxidative phosphorylation. A possible explanation for the lack of any such manifestation may be the existence of threshold activities within enzyme complexes *i.e.*, a certain amount of a protein complex activity needs to be lost before oxidative phosphorylation is affected, as has been observed for isolated mitochondria from a variety of sources (Davey *et al.*, 1998).

However, we did observe a significantly decreased flux through the substrate oxidation system in synaptosomes from old rats, compared to those from young rats, when *in situ* mitochondria were uncoupled with FCCP (Table 3.2.1). This suggests that there is a

difference in the substrate oxidation system, manifesting itself when the substrate oxidation system is forced to work hard in the absence of a substantial Δp (Table 2). The fact that differences in oxygen consumption rates were observed in synaptosomes from young and old rats in which mitochondria have been uncoupled and not in synaptosomes from young and old rats in which mitochondria have been pushed to maximally make ATP (in situ state 3), is not an inconsistent observation. In the former situation, the substrate oxidation system is working at maximum capacity, the electrochemical gradient is abolished and the oxygen consumption rate is not limited by the kinetics of the ATP synthase, adenine nucleotide transporter, and ATP usage. In the latter case, there is an electrochemical gradient and there are kinetic limitations associated with the ATP synthase and ATP usage. Thus, only when the limitations associated with the phosphorylating component and static head due to the proton electrochemical gradient are removed, does a difference in the substrate oxidation flux manifest itself. The source of this defect could be anywhere within the defined parameters of the substrate oxidation system *e.g.* substrate transport across the plasma membrane or the mitochondrial inner membrane, glycolysis, the tricarboxylate cycle and/or the electron transport chain of the mitochondrial inner membrane. One of the prime candidates for further investigation is the electron transport chain where, as already mentioned, there are reports of decreased electron transport chain activity with age in brain mitochondria (Curti *et al.*, 1990; Bowling *et al.* 1993; Ferrandiz *et al.*, 1994).

3.3.4 Comparison of the titration of the phosphorylation system titration in *in situ* mitochondria in synaptosomes isolated from young (3 month) and old (24 month) rat brains

Figure 3.2.11 shows the kinetic response of the phosphorylation system to changes in $\Delta\Psi_{\text{mito}}$ in *in situ* mitochondria in synaptosomes isolated from young (3 month) and old (24 month) rat brains at maximal phosphorylating capacity, corrected for proton leak. The phosphorylation kinetic response was achieved by titrating the *in situ* mitochondrial oxygen consumption with an electron transport chain inhibitor (myxothiazol) while the mitochondria were under maximal phosphorylating conditions. As some proton leak occurs in this phosphorylating state titration, one can only get a true measure of the

phosphorylation system kinetics when proton leak, at any given Δp , is subtracted. The data show that the kinetic response of the phosphorylation systems for *in situ* mitochondria of synaptosomes from young and old animals is similar, as deduced by an overlap in the error bars of the points defining the line.

There appears to be no significant difference between the kinetic response of the phosphorylation systems in a comparison of the synaptosomes from young and old animals (Fig. 3.2.7, 3.2.8 and 3.2.11). Again, before the study, one might have expected the capacity of the phosphorylation system of the synaptosomes from the older animal to differ from those from the younger animal. A report in the literature from Polosa & Attardi (1991) showed that there was a decrease in the level of the ATP synthase F_0 component, A8, following *in organello* translation of synaptosomal mitochondria from 24-month-old rats compared to synaptosomal mitochondria from 2-3-month-old rats.

3.4 Conclusions

In conclusion, it was demonstrated that 85% of the resting oxygen consumption of synaptosomes from young and old rats was due to mitochondrial proton leak (and possibly other ion cycling) across the mitochondrial inner membrane. There was no significant difference in the kinetic response of the substrate oxidation system, the mitochondrial proton leak and the phosphorylation system to changes in the proton electrochemical gradient when comparing synaptosomes from the young and old rats, although there was a significant reduction in the rate of oxygen consumption in synaptosomes from the old rats in the presence of uncoupler, when compared with synaptosomes from young rats. These results demonstrate an impaired flux through the substrate oxidation system in synaptosomes from old rats when compared to those from young animals.

Chapter 4

Metabolic control analysis of synaptosomal *in situ* mitochondria isolated from whole brain of young (3 month) and old (24 month) rats

4.1 Introduction

Top-down metabolic control analysis was used to acquire a general overview of the control structure of the energy pathways in synaptosomes isolated from young (3 month) and old (24 month) rat brains. Top-down metabolic control analysis simplifies the metabolic system under consideration to the blocks of reactions that produce a defined intermediate and those that consume it (Hafner *et al.*, 1990). In the case of respiration of synaptosomal *in situ* mitochondria isolated from whole rat brain, a suitable intermediate is the mitochondrial membrane potential. The intermediate is produced by the reactions of substrate oxidation and is consumed by two blocks of reactions, namely, the phosphorylating system and the proton leak system (see Fig 1.4.1).

In this study, an investigation was undertaken to establish the kinetic responses (elasticities) of the mitochondrial proton leak, substrate oxidation and phosphorylation to changes in the mitochondrial membrane potential in synaptosomes isolated from whole rat brain. Flux control coefficients were then calculated from these elasticities. In addition, in an endeavour to encompass all possible changes in synaptosomal energy metabolism due to the aging process, a comparison was made of the aforementioned kinetic responses to the mitochondrial membrane potential in synaptosomes isolated from young (3 month) and old rats (24 month).

4.2 Results

Table 4.2.1 shows the overall elasticities to Δp and the flux control coefficients for the substrate oxidation system, the phosphorylation system, and the proton leak over oxygen consumption at maximal phosphorylating capacity (state 3) for synaptosomes isolated from the whole brain of young (3 month) and old (24 month) rats. The control coefficients are described in terms of the schematic representation outlined in Fig. 1.4.1

and these data are calculated from the data in Figs. 3.2.9, 3.2.10 and 3.2.11 as described in Hafner *et al.* (1990) for a branched system.

Table 4.2.2 shows the flow ratio of the *in situ* mitochondria in synaptosomes isolated from young (3 month) and old (24 month) rat brains. The flow ratio, which is the effective P/O ratio as a fraction of the maximum P/O ratio, was calculated by dividing the oxygen consumption rate due to the phosphorylation system in state 3 by the oxygen consumption rate due to the substrate oxidation system in state 3.

Table 4.2.1

	Young	Old
$\epsilon_{\Delta\Psi_m}^S$	-1.09	-1.23
$\epsilon_{\Delta\Psi_m}^L$	4.65	1.93
$\epsilon_{\Delta\Psi_m}^P$	2.76	1.30
C_S^{JS}	0.71	0.53
C_P^{JS}	0.27	0.42
C_L^{JS}	0.02	0.05
C_S^{JP}	0.75	0.50
C_P^{JP}	0.30	0.56
C_L^{JP}	-0.05	-0.06
C_S^{JL}	1.16	0.74
C_P^{JL}	-1.08	-0.66
C_L^{JL}	0.92	0.92
$C_S^{\Delta\Psi_m}$	0.26	0.38
$C_P^{\Delta\Psi_m}$	-0.24	-0.34
$C_L^{\Delta\Psi_m}$	-0.02	-0.04
$C_S^{P/O}$	-0.02	-0.03
$C_P^{P/O}$	0.09	0.14
$C_L^{P/O}$	-0.07	-0.11

Table 4.2.1. Overall elasticities to $\Delta\Psi_m$ and control coefficients over the rates and over $\Delta\Psi_m$ in *in situ* mitochondria in state 3 in synaptosomes isolated from young and old rats. $\epsilon_{\Delta\Psi_m}^S$ refers to the elasticity of the substrate oxidation system. C_S^{JS} refers to the control the substrate oxidation system exerts over the flux through the substrate oxidation system. $C_S^{\Delta\Psi_m}$ refers to the control the substrate oxidation system exerts over the mitochondrial membrane potential. $C_S^{P/O}$ refers to the control the substrate oxidation system exerts over the P/O ratio. J_S refers to the flux through the substrate oxidation system and $\Delta\Psi_m$ refers to the mitochondrial membrane potential.

Table 4.2.2

	Young	Old
J_p/J_s	0.93	0.89

Table 4.2.2 Flow ratio of *in situ* mitochondria in synaptosomes isolated from young and old rat brains. J_p/J_s is the effective P/O ratio as a fraction of P/O max.

4.3 Discussion

4.3.1 Comparison of the kinetic responses (elasticities) of the mitochondrial proton leak, substrate oxidation and phosphorylation to changes in the mitochondrial membrane potential in synaptosomal *in situ* mitochondria isolated from young (3 month) and old (24 month) rat brains

Table 4.2.1 shows the overall elasticities to $\Delta\Psi_m$ and control coefficients over the rates and over $\Delta\Psi_m$ in *in situ* mitochondria in state 3 in synaptosomes isolated from young and old rats. The values of the control coefficients show that, in state 3, the flux through the substrate oxidation system is mainly controlled by the substrate oxidation system itself (0.71) in young rat brain synaptosomes, with little control by the phosphorylation system (0.27) and the proton leak (0.02) in *in situ* mitochondria isolated from young rat brains. A similar situation exists in synaptosomal *in situ* mitochondria isolated from old rat brains, although the quantity of control is different in old rats. In synaptosomal *in situ* mitochondria isolated from old rat brains in state 3, the flux through the substrate oxidation system is mainly controlled by the substrate oxidation system itself (0.53), with lesser control by the phosphorylation system (0.42) and little control by the proton leak (0.02).

The flux through the phosphorylation system is controlled mainly by the substrate oxidation system in young rats (0.75), with lesser control by the phosphorylating system (0.30). However, in old rats, the flux through the phosphorylation system is more evenly divided between the substrate oxidation system (0.50) and the phosphorylation system (0.56).

The change of emphasis in control of the flux through the substrate oxidation system and through the phosphorylating system in old rat synaptosomes compared with young rat synaptosomes could indicate a defect in the substrate oxidation system in old rat brain synaptosomes, as was indicated by the significant decrease in the state 3 uncoupled rate in old compared with young rats (Table 3.2.1). This is in good agreement with reports of decreased electron transport chain activity with age in brain mitochondria (Curti *et al.*, 1990; Bowling *et al.*, 1993; Ferrandiz *et al.*, 1994).

The negative flux control coefficient for the control of flux through the phosphorylation system (-0.05 in young and -0.06 in old) by the proton leak indicates that an increase in flux through proton leak would cause a decrease in the flux through the phosphorylation system, as would be expected.

The flux through proton leak is controlled mainly by the substrate oxidation system in young and old synaptosomal *in situ* mitochondria (1.16 in young and 0.74 in old), with some control by the leak itself (0.92 in young and old). The negative flux control coefficient of the phosphorylation system (-1.08 in young and -0.66 in old) with respect to the proton leak indicates that an increase in flux through the phosphorylation system would cause a decrease in the flux through the leak, as would be expected.

The control over the membrane potential lies with the substrate oxidation system in synaptosomal *in situ* mitochondria isolated from young (0.26) and old (0.38) rat brains. The negative flux values through the phosphorylation system and proton leak (-0.24 and -0.02 , respectively, in young, -0.34 and -0.04 in old) indicate that increased flux through the phosphorylation system or the proton leak would decrease the membrane potential. Clearly, in synaptosomal *in situ* mitochondria in state 3, the phosphorylating system has very little control over any events. The strong control by substrate oxidation over membrane potential (0.26 in young and 0.38 in old) is consistent with this.

The effective P/O ratio is the amount of ATP produced per total O consumed and so decreases if the leak reactions increase. In the presence of oligomycin, there is no

phosphorylation and the effective P/O ratio is zero. The flux control coefficients of substrate oxidation, phosphorylation and proton leak over the effective P/O ratio are all given in Table 4.2.1. Most of the control over the effective P/O ratio lies with the phosphorylation system (0.09 in young and 0.14 in old).

Table 4.2.2 shows the flow ratio, which is the effective P/O ratio as a fraction of the maximum P/O ratio. The flow ratio was calculated by dividing the oxygen consumption rate due to the phosphorylation system in state 3 by the oxygen consumption rate due to the substrate oxidation system in state 3. The value obtained was 0.93 in young rat brain synaptosomes and 0.89 in old rat brain synaptosomes. This implies that 93% of the maximum ATP output occurs in young rat brain synaptosomes and 89% of the maximum ATP output occurs in old rat brain synaptosomes. In other words, in state 3, 7% of the oxygen consumption is due to proton leak in young rat brain synaptosomes and 11% of the oxygen consumption is due to proton leak in old rat brain synaptosomes, with the remainder being used to drive ATP synthesis.

4.4 Conclusions

The pattern of control was similar in synaptosomes from young and old animals at maximal ATP-producing capacity. Control of flux through the substrate oxidation system was mainly by the substrate oxidation system in young and old rat brain synaptosomes. Control of the flux through the phosphorylation system was mainly by the substrate oxidation system in young rat brain synaptosomes, but mainly by the phosphorylating system itself in the old rat brain synaptosomes, however, the substrate oxidation system has a similar degree of control of flux through the phosphorylating system. Control of flux through the proton leak was mainly by the substrate oxidation system in young and old rat brain synaptosomes. Control of the mitochondrial membrane potential was mainly by the substrate oxidation system in young and old rat brain synaptosomes. The P/O ratio was controlled mainly by the phosphorylating system in young and old rat brain synaptosomes.

Chapter 5

The effect of nagarse on mitochondrial proteins

5.1 Introduction

Nagarse is a broad spectrum protease that is used in the isolation of muscle tissue mitochondria to release the mitochondria from the myofibrillar net (Souza-Pinto *et al.*, 1999). RCR and the P/O ratios are indicators of mitochondrial function; higher values generally indicate a better quality preparation. Muscle tissue mitochondria isolated using nagarse display higher RCRs and higher P/O ratios than muscle tissue mitochondria isolated in the absence of nagarse (Thakar and Ashmore, 1975; Rolfe *et al.*, 1994; Cunningham *et al.*, 2003). Even though the use of nagarse to isolate mitochondria gives rise to mitochondria with high RCR and P/O ratios, nagarse had a profound effect on the characteristic mitochondrial protein pattern when examined using blue native/tricine-SDS-PAGE two-dimensional electrophoresis. Complexes I, II, III, IV and V were identified by their characteristic polypeptide patterns in the second dimension, by comparison to the work of Bentlage *et al.* (1995) and of Schagger and Pfeiffer (2001). The effect nagarse had on mitochondrial proteins isolated from beef heart was determined here. Also, experiments were performed to find the protein range for which the staining intensity of selected complex subunits was linear, for the analysis of mitochondria isolated from young and old animals and for a comparison of tissue mitochondria using densitometry of second-dimension gels (see Chapter 6 and 7).

5.2 Results

Figure 5.2.1 A and B shows the resolution in the second dimension of the beef heart mitochondrial proteins isolated in the presence or absence of nagarse. The gels are Serva blue G stained. First-dimension gels had the in-gel native proteins denatured using 20mM DDT/1%SDS and the denatured proteins were resolved on a second dimension 10% tricine-SDS gel. The main difference between the beef heart mitochondria isolated without using nagarse (fig. 5.2.1 A) and the beef heart mitochondria isolated using nagarse (fig. 5.2.1 B) is that the proteins above 25kDa in the complex I, III and V region of the beef heart mitochondria isolated using nagarse appear to be missing. Any proteins below 25 kDa in the complex I, III, V region of

the beef heart mitochondria isolated using nagarse (fig. 5.2.1 B) do not match the corresponding pattern of complex I, III and V of the beef heart mitochondria isolated without using nagarse (fig. 5.2.1 A). On closer inspection, the area designated 'complex III' in the beef heart mitochondria isolated in the presence of nagarse (fig 5.2.1 B) could in fact be complex IV, as a number of proteins from 47.5kDa to 16.5kDa seem to match the pattern shown for complex IV in the beef heart mitochondria isolated without using nagarse (fig 5.2.1 A). The pattern of the complex IV in beef heart mitochondria isolated using nagarse largely matches that of the complex IV in the beef heart mitochondria isolated using nagarse.

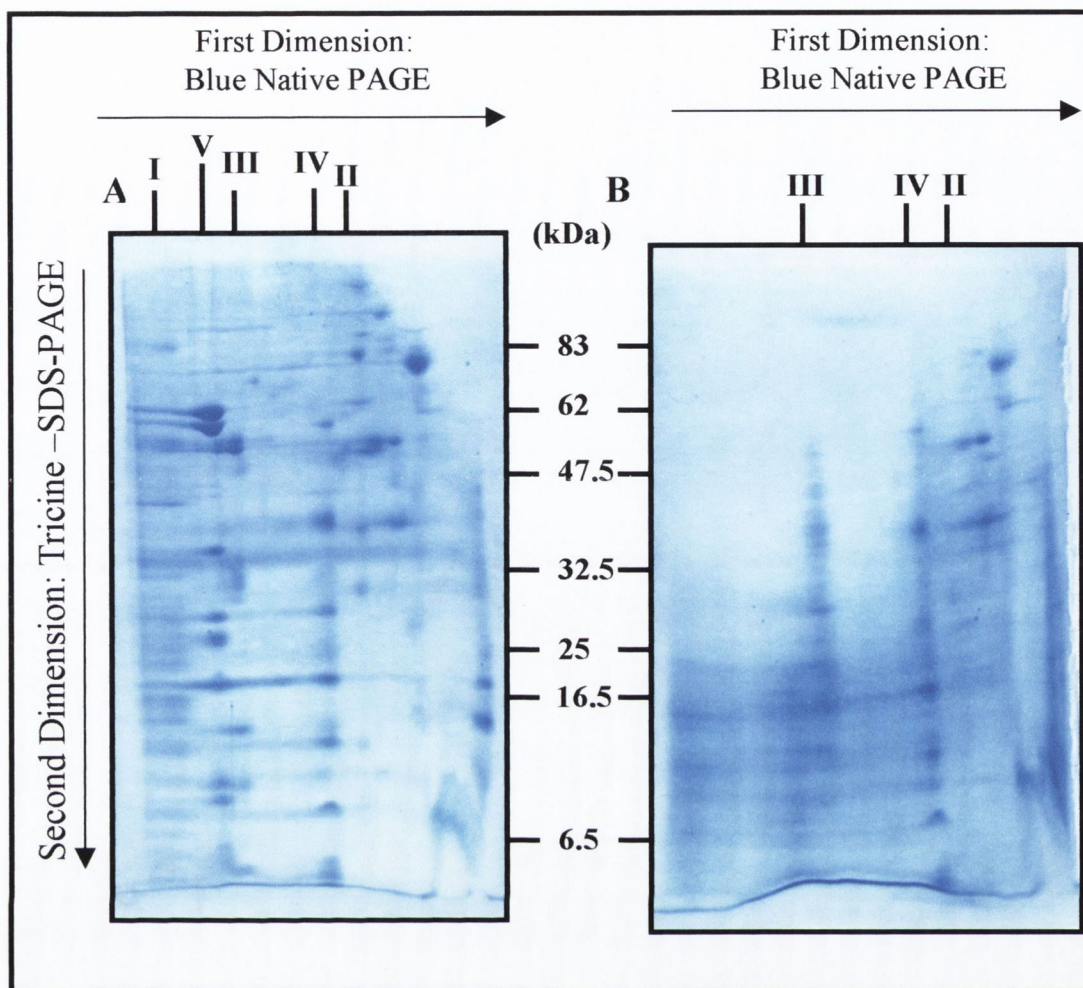


Figure 5.2.1. Two-dimensional resolution of mitochondrial proteins from beef heart mitochondria isolated in the presence or absence of nagarse using blue native PAGE in the first dimension and denaturing tricine-SDS-PAGE in the second dimension. The membrane protein complexes were separated in the native first dimension and identified by the characteristic polypeptide patterns in the denaturing second dimension. The gel was fixed, stained using Serva blue G and destained as in Materials and Methods. The positions of the complexes I, II, III, IV and V are indicated at the top of the gels. The molecular masses are indicated between the two gels. **(A)** A first dimension lane loaded with 25 μ l (91 μ g protein) of solubilized beef heart mitochondrial protein isolated without using nagarse was cut from the first dimension gel and the native in-gel proteins were denatured using 20mM DDT/1%SDS (see Materials and Methods). The mitochondrial proteins were resolved using a uniform 1mm 10% tricine-SDS gel. **(B)** A first-dimension lane loaded with 25 μ l (91 μ g protein) of solubilized beef heart mitochondrial protein isolated using nagarse was cut from the first dimension gel and the in-gel native proteins were denatured using 20mM DDT/1%SDS (see Materials and Methods). The mitochondrial proteins were resolved using a uniform 1mm 10% tricine-SDS gel.

Figure 5.2.2 is a graph showing the increasing absorbance of the 42kDa subunit of complex I in beef heart mitochondria isolated without using nagarse, with increasing protein loaded on the second-dimension tricine gel. Second-dimension tricine gels loaded with increasing amounts of beef heart mitochondrial protein (24 μ g to 720 μ g protein) were fixed, stained using Serva blue G and destained as in Materials and Methods. The absorbance of the 42kDa subunit of complex I was linear up to 0.454mg protein loaded on the second-dimension gel. All of the absorbances of the complex I subunits in the mitochondria used in the young and old comparison (see Chapter 6) and in the tissue comparison (see Chapter 7) fell within the absorbance range shown in fig 5.2.2.

Figure 5.2.3 is a graph showing the increasing absorbance of the 55kDa subunit of complex V in beef heart mitochondria isolated without using nagarse with increasing protein loaded on the second-dimension tricine gel. Second-dimension tricine gels loaded with increasing amounts of beef heart mitochondrial protein (24 μ g to 720 μ g protein) were fixed, stained using Serva blue G and destained as in Materials and Methods. The absorbance of the 55kDa subunit of complex V was linear up to 0.363 mg of protein loaded on the second-dimension gel. All of the absorbances of the complex V subunits in the mitochondria used in the young and old comparison (see Chapter 6) and in the tissue comparison (see Chapter 7) fell within the absorbance range shown in fig 5.2.3.

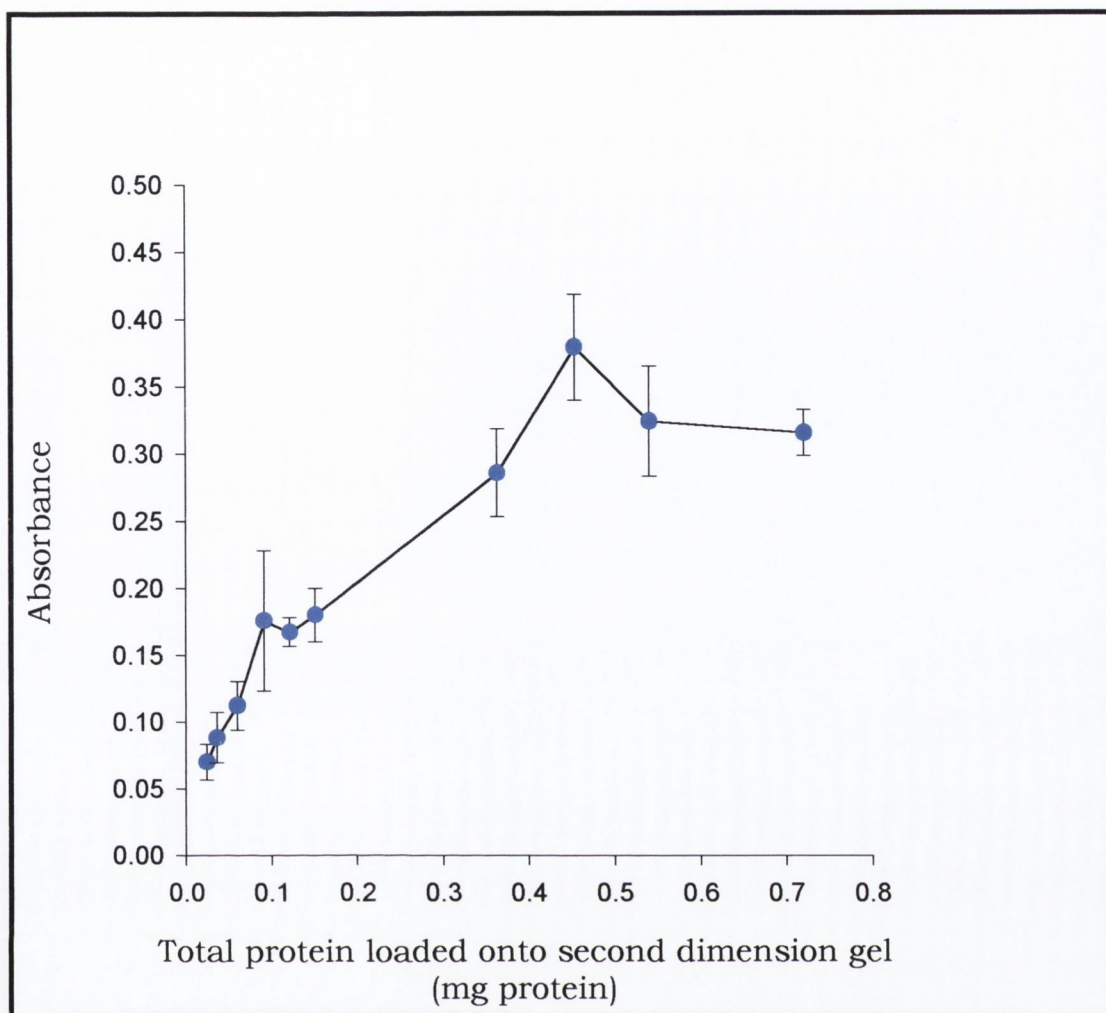


Figure 5.2.2 Increasing absorbance of complex I in beef heart mitochondria isolated without using nagarse with increasing protein loaded on a second-dimension tricine gel. Beef heart mitochondrial proteins were solubilized using a 3:1 ratio of maltoside to protein. First-dimension lanes loaded with increasing amounts (24 μ g to 720 μ g protein) of solubilized beef heart mitochondrial protein, isolated without using nagarse, were cut from the first-dimension gel and the native in-gel proteins were denatured using 20mM DDT/1%SDS (see Materials and Methods). The mitochondrial proteins were resolved using a uniform 1mm 10% tricine-SDS gel in the second dimension. The gel was fixed, stained using Serva blue G and destained as in Materials and Methods. The absorbance of the 42kDa subunit of complex I was measured at each protein concentration. All of the data represent the mean $\pm$ S.E.M. of three determinations.

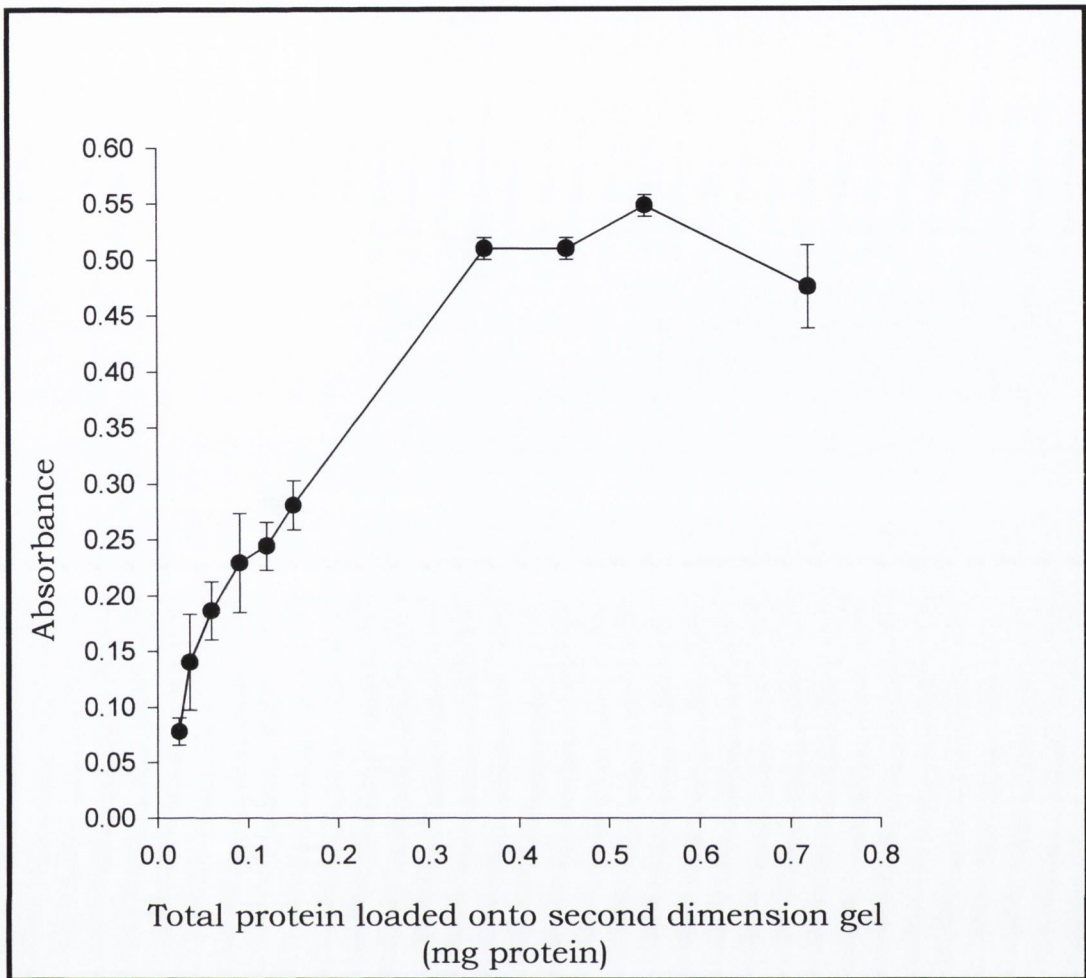


Figure 5.2.3 Increasing absorbance of complex V in beef heart mitochondria isolated without using nagarse with increasing protein loaded on a second-dimension tricine gel. Beef heart mitochondrial proteins were solubilized using a 3:1 ratio of maltoside to protein. First-dimension lanes loaded with increasing amounts (24 μ g to 720 μ g protein) of solubilized beef heart mitochondrial protein, isolated without using nagarse, were cut from the first-dimension gel and the native in-gel proteins were denatured using 20mM DDT/1%SDS (see Materials and Methods). The mitochondrial proteins were resolved using a uniform 1mm 10% tricine-SDS gel in the second dimension. The gel was fixed, stained using Serva blue G and destained as in Materials and Methods. The absorbance of the 42kDa subunit of complex I was measured at each protein concentration. All of the data represent the mean $\pm$ S.E.M. of three determinations.

Figure 5.2.4 is a graph showing the increasing absorbance of the 47kDa core protein II of complex III in beef heart mitochondria isolated without using nagarse with increasing protein loaded on the second-dimension tricine gel. Second-dimension tricine gels loaded with increasing amounts of beef heart mitochondrial protein (24 μ g to 720 μ g protein) were fixed, stained using Serva blue G and destained as in Materials and Methods. The absorbance of the 47kDa core protein II of complex III was linear up to 0.363 mg of protein loaded on the second-dimension gel. All of the absorbances of the complex III subunits in the mitochondria used in the young and old comparison (see Chapter 6) and in the tissue comparison (see Chapter 7) fell within the absorbance range shown in fig 5.2.4.

Figure 5.2.5 is a graph showing the increasing absorbance of the 26kDa subunit 2 of complex IV in beef heart mitochondria isolated without using nagarse with increasing protein loaded on the second-dimension tricine gel. Second-dimension tricine gels loaded with increasing amounts of beef heart mitochondrial protein (24 μ g to 720 μ g protein) were fixed, stained using Serva blue G and destained as in Materials and Methods. The absorbance of the 26kDa subunit 2 of complex IV was linear up to 0.363 mg protein loaded on the second-dimension gel. All of the absorbances of the complex I subunits in the mitochondria used in the young and old comparison (see Chapter 6) and in the tissue comparison (see Chapter 7) fell within the absorbance range shown in fig 5.2.5.

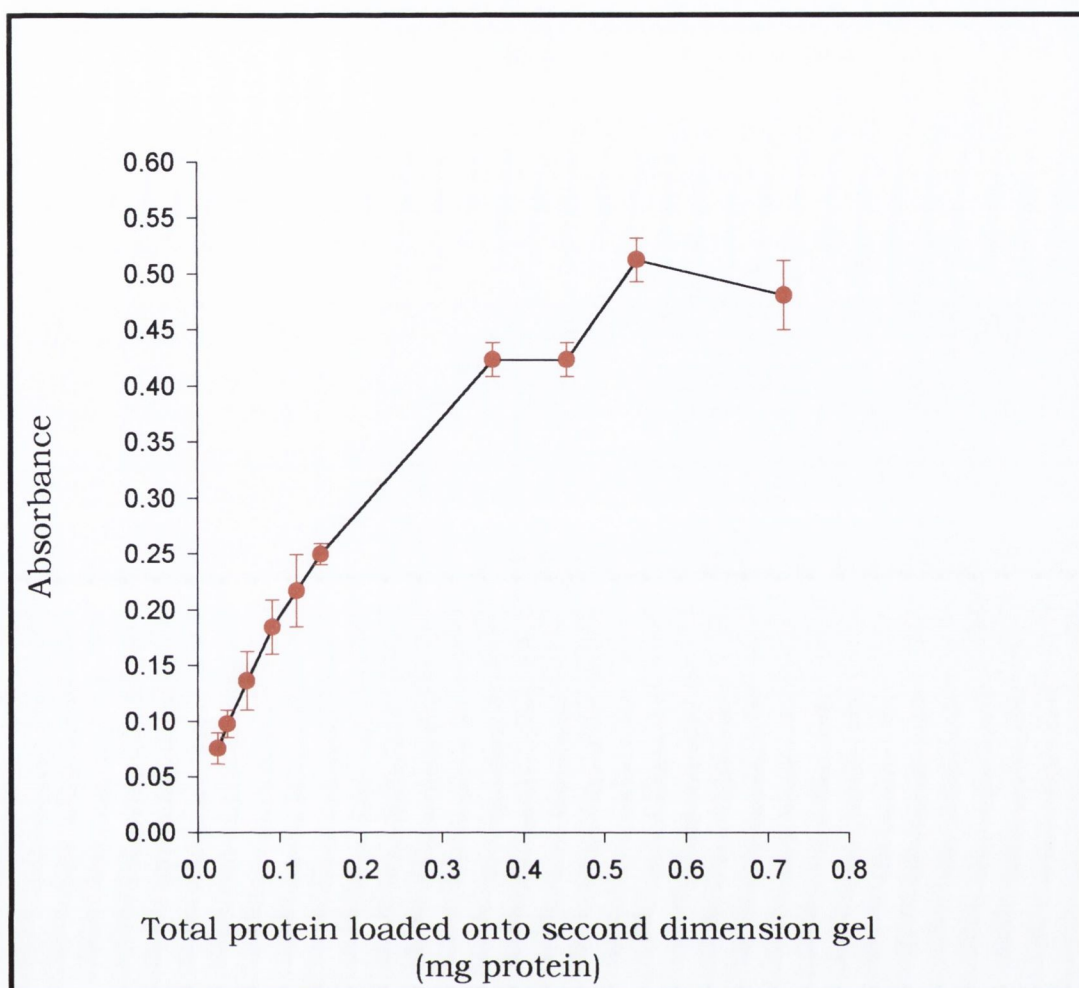


Figure 5.2.4 Increasing absorbance of complex III in beef heart mitochondria isolated without using nagarse with increasing protein loaded on a second-dimension tricine gel. Beef heart mitochondrial proteins were solubilized using a 3:1 ratio of maltoside to protein. First-dimension lanes loaded with increasing amounts (24 μ g to 720 μ g protein) of solubilized beef heart mitochondrial protein, isolated without using nagarse, were cut from the first-dimension gel and the native in-gel proteins were denatured using 20mM DDT/1%SDS (see Materials and Methods). The mitochondrial proteins were resolved using a uniform 1mm 10% tricine-SDS gel in the second dimension. The gel was fixed, stained using Serva blue G and destained as in Materials and Methods. The absorbance of the 42kDa subunit of complex I was measured at each protein concentration. All of the data represent the mean $\pm$ S.E.M. of three determinations.

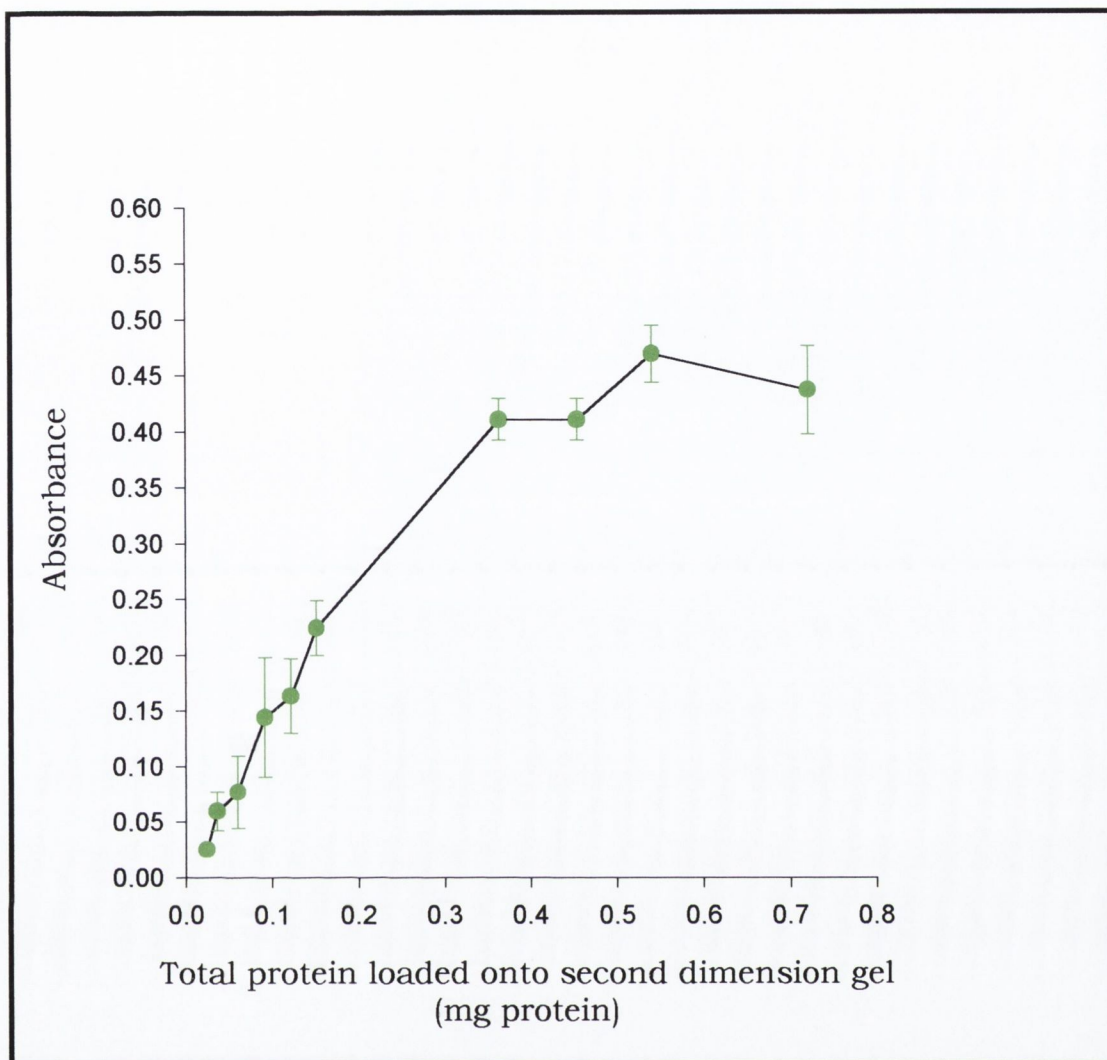


Figure 5.2.5 Increasing absorbance of complex IV in beef heart mitochondria isolated without using nagarse with increasing protein loaded on a second-dimension tricine gel. Beef heart mitochondrial proteins were solubilized using a 3:1 ratio of maltoside to protein. First-dimension lanes loaded with increasing amounts (24 μ g to 720 μ g protein) of solubilized beef heart mitochondrial protein, isolated without using nagarse, were cut from the first-dimension gel and the native in-gel proteins were denatured using 20mM DDT/1%SDS (see Materials and Methods). The mitochondrial proteins were resolved using a uniform 1mm 10% tricine-SDS gel in the second dimension. The gel was fixed, stained using Serva blue G and destained as in Materials and Methods. The absorbance of the 42kDa subunit of complex I was measured at each protein concentration. All of the data represent the mean $\pm$ S.E.M. of three determinations.

5.3 Conclusions

The protein pattern of beef heart mitochondria proteins isolated in the presence or absence of nagarse differs when the mitochondrial proteins are resolved on a second-dimension denaturing tricine gel. A Serva blue G stain of the second dimension tricine gel showed the lack of proteins above ~25kDa in complexes I, III and V in the beef heart mitochondria isolated in the presence of nagarse. Complex IV seemed to be largely unaffected by the use of nagarse to isolate beef heart mitochondria.

Any work on muscle tissue mitochondria isolated using nagarse will have to consider that mitochondrial proteins are digested so that the characteristic protein pattern of mitochondrial proteins resolved in two dimensions is absent when compared with mitochondria isolated using nagarse.

Once it was established that nagarse should not be used in the isolation of muscle tissue mitochondria, experiments were performed to find the protein range for which the staining intensity of selected complex subunits was linear, for the analysis of mitochondria isolated from young and old animals and for a comparison of tissue mitochondria using densitometry of second-dimension gels (see Chapter 6 and 7). The complex subunits whose absorbance was measured were: the 42kDa subunit of complex I, the 55kDa α subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. Using beef heart mitochondria, it was found that the staining of the above selected complex subunits was linear from 0.024mg to 0.363mg protein (see fig.5.2.2 to 5.2.5). All of the staining intensities of the mitochondria fell within this linear range.

Chapter 6

Comparison of mitochondria isolated from young and old rat tissue, including euthyroid and hyperthyroid rat liver mitochondria, using two dimensional electrophoresis

6.1 Introduction

The purpose of this chapter was to investigate if the amounts of assembled mitochondrial complexes I, III, IV and V were different in mitochondria isolated from young (3 month) and old (24 month) rat heart, liver, synaptosomes and in liver mitochondria isolated from euthyroid and hyperthyroid rats. The amounts of the mitochondrial complexes were analyzed using a two-dimensional electrophoresis technique. The mitochondrial protein complexes were solubilized using maltoside and were separated in the first dimension using blue native PAGE. The in-gel native proteins were denatured and the complex subunits were further resolved in the second dimension using tricine-SDS-PAGE. All of the mitochondrial protein samples were solubilized using a maltoside:protein ratio of between 3:1 and 1:1, as recommended by Schägger (1994). The second-dimension tricine gels were stained using Serva blue G and were used for densitometric analysis. The staining intensity or absorbance of selected complex subunits was measured and the absorbances of complex subunits were taken as a relative measure of the amount of complex in that tissue. Reports found that where there was a decline in the level of a complex, the change was reflected in a uniform decline in all of the subunits of that complex (Reinheckel *et al.*, 1995; Bentlage *et al.*, 1995). The subunits whose absorbances were measured were the 42kDa subunit of complex I, the 55kDa α subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The subunits used in the comparison were identified according to the work of Bentlage *et al.* (1995) and of Schägger and Pfeiffer (2001). However, N-terminal sequencing would be necessary to verify the identity of these proteins. The amount of complex II was not measured because there were either too many unidentified proteins in the same region of the gel or complex II could not be detected using a Coomassie stain. The individual subunits of the complexes were quantified after a second dimension tricine gel rather than the first-dimension blue native gel to avoid including overlaps with

contaminating proteins (Schägger & Ohm, 1995). For example, for rat heart mitochondrial proteins on the second dimension tricine-SDS gel (fig 6.1 A and B), it can clearly be seen that complex I is contaminated by complex V.

The electrophoretic technique was optimized using beef heart, rat heart, liver and synaptic mitochondria. Using beef heart mitochondria, it was found that the Coomassie staining intensity of the subunits of the complexes correlated linearly with the protein load over the range used for analysis of rat mitochondria (see chapter 5, figs 5.2.24 to 5.2.27).

Three gels were used for each comparison of tissues from young and old rats. The absorbances of the selected subunits of the complexes were measured and the absorbances are presented in three ways. Firstly, the average absorbance of the subunits of the complexes was normalised per mg (milligram) of protein loaded onto the first-dimension gel.

Secondly, the average absorbance of the subunits of the complexes were normalised per μmol of mitochondrial citrate synthase activity. It has been suggested that the mitochondrial population isolated from old animals can be more heterogeneous than those isolated from young animals since it is possible for loss of damaged mitochondria to occur during isolation, especially when isolating mitochondria from old animals (Wilson and Franks, 1975). Therefore, to take into consideration possible differences in the mitochondrial content of young and old preparations, the average absorbance of the subunits of the complexes were normalised to citrate synthase activity (Souza-Pinto *et al.*, 1999). Citrate synthase activity has been widely used as marker of mitochondrial content since citrate synthase activity (Hansford and Castro, 1982) and citrate synthase message levels do not change with age (Marin-Garcia *et al.*, 1997).

Finally, the average absorbance of the selected subunits of complexes I, III and V were normalised relative to the in-gel complex IV subunit on a per gel basis. The absorbance of the complex IV subunit on the gel was set to 1 and the other complex subunits were

expressed relative to this. The absorbances of the complex subunits relative to the complex IV subunit from the three gels were then pooled. Using the complex IV subunit as an internal standard means that the analysis becomes independent of the absolute amount of mitochondria (Bentlage *et al.*, 1995). Using complex IV as an internal standard also means that comparing staining ratios of complexes within individual gels eliminates experimental deviations that result from variations in mitochondrial content and in protein load (Schägger and Ohm, 1995). Complex IV was chosen as the internal standard because it is used as a marker for mitochondria activity and content (Hudson *et al.*, 1998).

6.2 Results

Figure 6.2.1 A and B show the resolution in the second dimension of rat heart mitochondrial proteins isolated from young (3 month) and old (24 month) rats. The gels were stained with Serva blue G. First-dimension gel lanes loaded with 227µg of young and old rat heart mitochondrial protein had the in-gel proteins denatured using 20mM DDT/1%SDS and the denatured proteins were resolved on a second dimension 10%-tricine-SDS gel. Complexes I, III, IV and V were identified on the basis of their characteristic patterns in the second dimension in heart mitochondria isolated from young (fig. 6.2.1 A) and old (fig. 6.2.1 B) rats. Complex I was quite diffuse in young and old rat heart mitochondria and complex II could not be identified in either the young or old rat heart mitochondria because of a large number of unidentified proteins in the same region of the gel. Arrows on the gels mark the protein subunits whose staining intensity was measured for the quantification of second dimension gels. The entire pattern of proteins looks the same in the young rat heart and the old rat heart, however, some proteins outside of complexes I, III, IV and V are of less intensity in the rat heart mitochondria isolated from old rats (fig. 6.2.1 B) when compared with young rat heart mitochondria (fig. 6.2.1A).

Figure 6.2.2 shows the absorbance normalised per mg of protein loaded on the first-dimension gel of complexes I, III, IV and V in mitochondria isolated from young and old rat heart mitochondria. The absorbances are also listed in Table 6.2.1. The absorbances

of complex I were 0.52 ± 0.06 and 0.50 ± 0.12 in young and old rat heart mitochondria, respectively. The absorbances of complex V were 0.81 ± 0.03 and 0.81 ± 0.12 in young and old rat heart mitochondria, respectively. The absorbances of complex III were 0.68 ± 0.03 and 0.61 ± 0.10 in young and old rat heart mitochondria respectively. The absorbances of complex IV were 0.64 ± 0.03 and 0.64 ± 0.05 , respectively, in heart mitochondria isolated from young and old rats. There were no significant differences in the absorbances of the complexes between young and old rat heart mitochondria (*Student's* unpaired *t*-test) when normalised to mg of protein loaded on the first-dimension gel.

Figure 6.2.3 shows the absorbances normalised per μmol of citrate synthase activity of complexes I, III, IV and V in mitochondria isolated from young and old rat heart mitochondria. The absorbances are also listed in Table 6.2.2. The absorbances of complex I were 0.12 ± 0.01 and 0.10 ± 0.02 in young and old rat heart mitochondria, respectively. The absorbances of complex V were 0.18 ± 0.01 and 0.15 ± 0.02 in young and old rat heart mitochondria, respectively. The absorbances of complex III were 0.15 ± 0.01 and 0.11 ± 0.02 in young and old rat heart mitochondria, respectively. The absorbances of complex IV were 0.14 ± 0.01 and 0.12 ± 0.01 , respectively, in heart mitochondria isolated from young and old rats. There were no significant differences in the absorbances of the complexes between young and old rat heart mitochondria (*Student's* unpaired *t*-test) when normalised to citrate synthase activity.

Figure 6.2.4 shows the absorbances of complexes I, III and V normalised to the absorbance of complex IV in mitochondria isolated from young and old rat heart mitochondria. The absorbances are also listed in Table 6.2.3. The absorbances of complex I were 0.82 ± 0.12 and 0.80 ± 0.21 in young and old rat heart mitochondria, respectively. The absorbances of complex V were 1.27 ± 0.09 and 1.27 ± 0.18 in young and old rat heart mitochondria, respectively. The absorbances of complex III were 1.05 ± 0.08 and 0.95 ± 0.14 in young and old rat heart mitochondria, respectively. There were

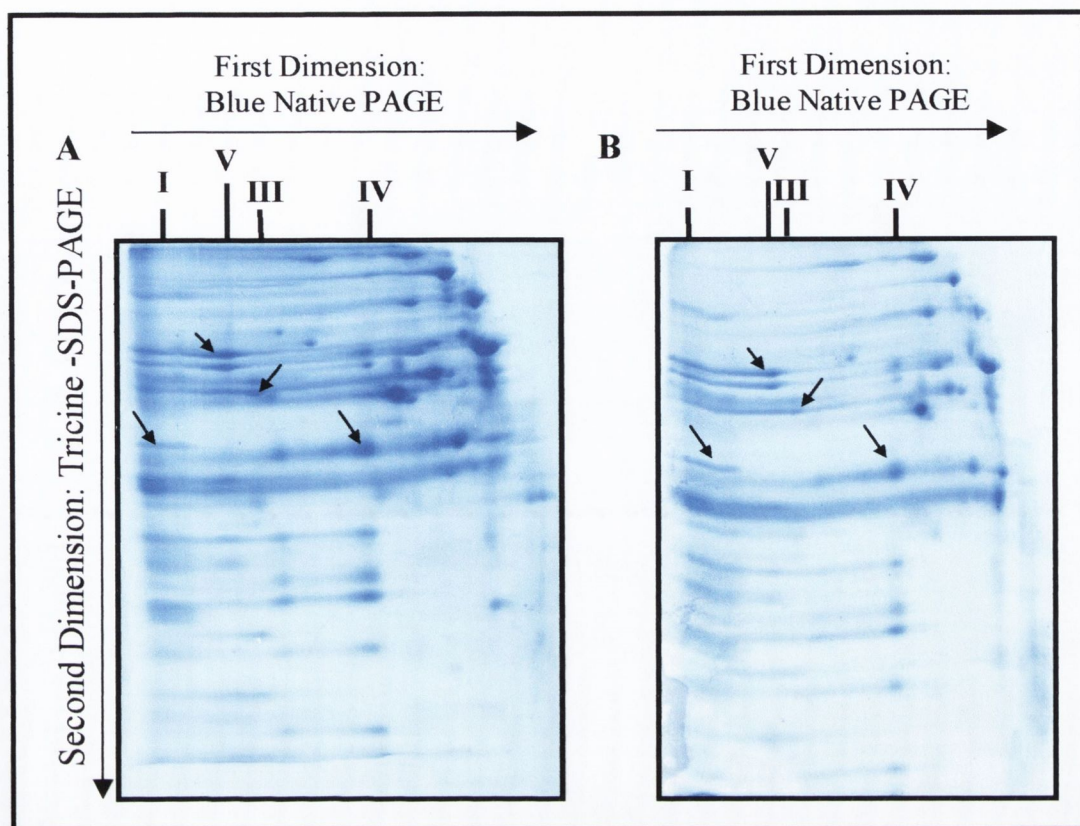


Figure 6.2.1. Two-dimensional resolution of mitochondrial proteins from rat heart mitochondria isolated from young (3 month) and old (24 month) rats using blue native PAGE in the first dimension and denaturing tricine-SDS-PAGE in the second dimension. The membrane protein complexes were separated in the native first dimension and identified by their characteristic polypeptide patterns in the denaturing second dimension. The gel was fixed, stained using Serva blue G and destained as in Materials and Methods. The positions of the complexes I, III, IV and V are indicated at the top of the gels. Arrows on the gels mark the protein subunits whose staining intensity or absorbance was measured for the quantification of second-dimension gels. The complex subunits whose absorbances were measured were: the 42kDa subunit of complex I, the 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The young rat heart mitochondrial proteins (**A**) and the old rat heart mitochondrial proteins (**B**) were solubilized using a 2:1 ratio of maltoside to protein and were resolved using blue native PAGE in the first dimension (1mm gel, 5.5-13% non-linear gradient gel). A first-dimension lane loaded with 25 μ l (227 μ g mitochondrial protein) of solubilized young or old rat heart mitochondrial protein was cut from the first-dimension gel and the in-gel native proteins were denatured using 20mM DDT/1%SDS (see Materials and Methods). The mitochondrial proteins were resolved using a uniform 1mm 10% tricine-SDS gel in the second dimension. The gels shown in A and B are representative of three gels that were used for quantification of second-dimension gels.

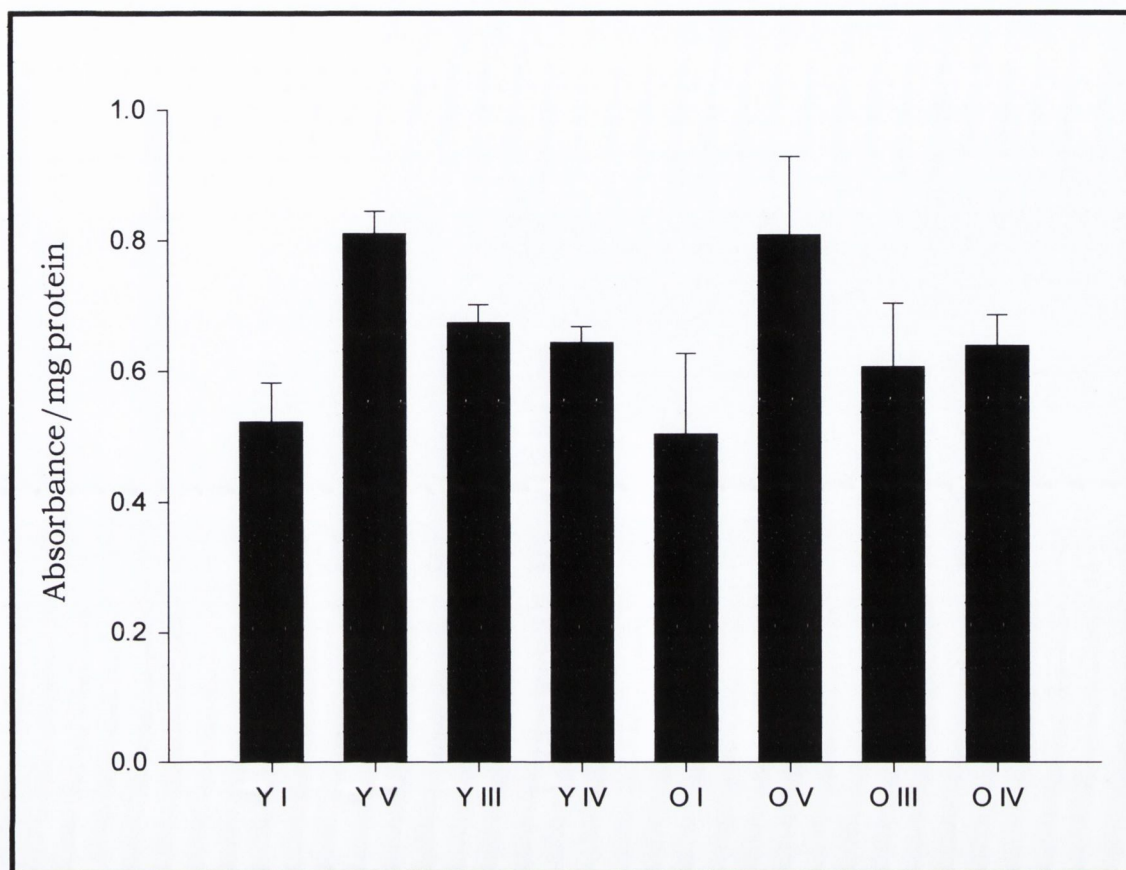


Figure 6.2.2. Comparison of the absorbances of complexes I, V, III and IV normalised per mg protein in mitochondria isolated from young (3 month) and old (24 month) rat heart. All mitochondrial proteins were solubilized using maltoside. The membrane protein complexes I, III, IV and V were separated using blue native PAGE in the first dimension and were identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second dimension. The second-dimension gel, which was fixed, stained using Serva blue G and destained as in Materials and Methods, was used for densitometric analysis. The subunits used for densitometric analysis are indicated by arrows on the gel shown in fig. 6.2.1. The subunits whose absorbances were measured were: the 42kDa subunit of complex I, the 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The graph labels are as follows: Y I: young complex I; Y V: young complex V; Y III: young complex III; Y IV: young complex IV; O I: old complex I; O V: old complex V; O III: old complex III; O IV: old complex IV. A 227 μ g amount of rat heart mitochondria was loaded on the first-dimension gel in the case of young and old mitochondria. All of the data represent the mean $\pm$ S.E.M. of three determinations. The data are also represented in Table 6.2.1.

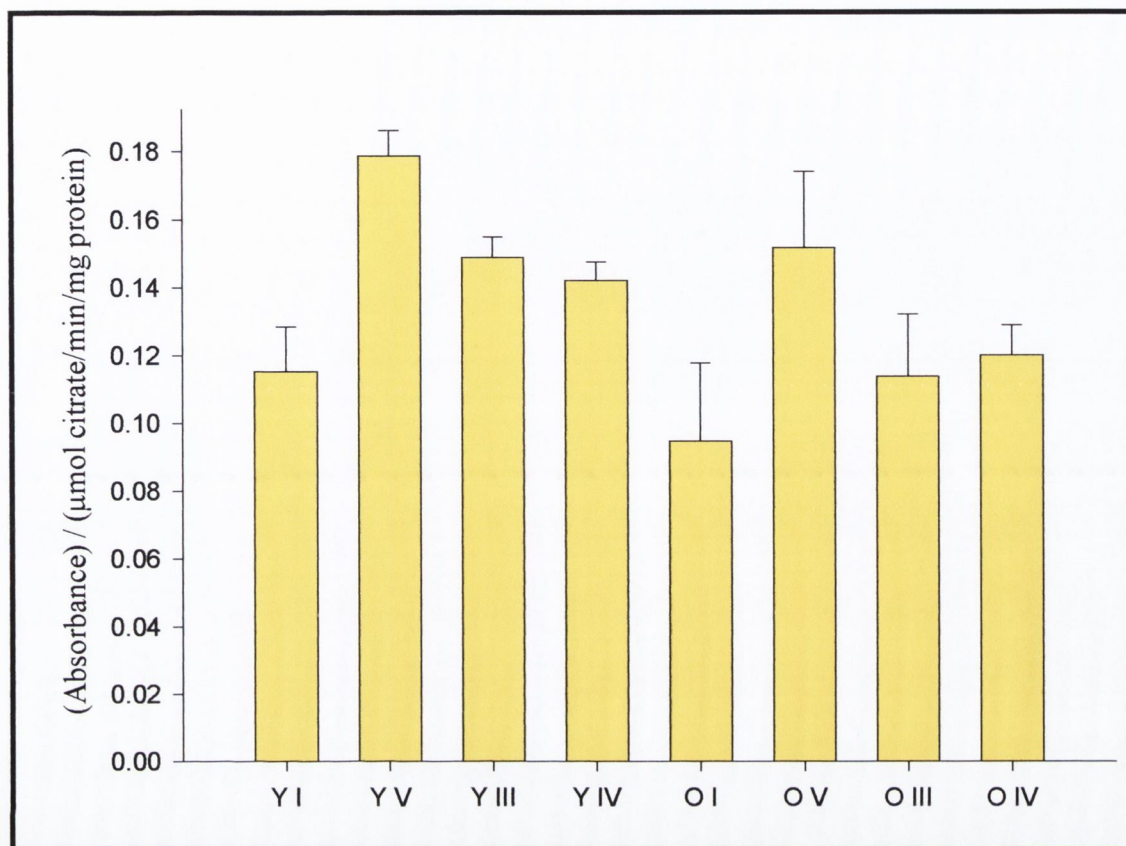


Figure 6.2.3. Comparison of the absorbances of complexes I, V, III and IV per μmol citrate synthase activity in mitochondria isolated from young (3 month) and old (24 month) rat heart. All mitochondrial proteins were solubilized using maltoside. The membrane protein complexes I, III, IV and V were separated using blue native PAGE in the first dimension and were identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second dimension. The second-dimension gel, which was fixed, stained using Serva blue G and destained as in Materials and Methods, was used for densitometric analysis. The subunits used for densitometric analysis are indicated by arrows on the gel shown in fig. 6.2.1. The subunits whose absorbances were measured were: the 42kDa subunit of complex I, the 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The graph labels are as follows: Y I: young complex I; Y V: young complex V; Y III: young complex III; Y IV: young complex IV; O I: old complex I; O V: old complex V; O III: old complex III; O IV: old complex IV. A 227 μg amount of rat heart mitochondria was loaded on the first-dimension gel in the case of young and old mitochondria. All of the data represent the mean $\pm$ S.E.M. of three determinations. The data are also represented in Table 6.2.2.

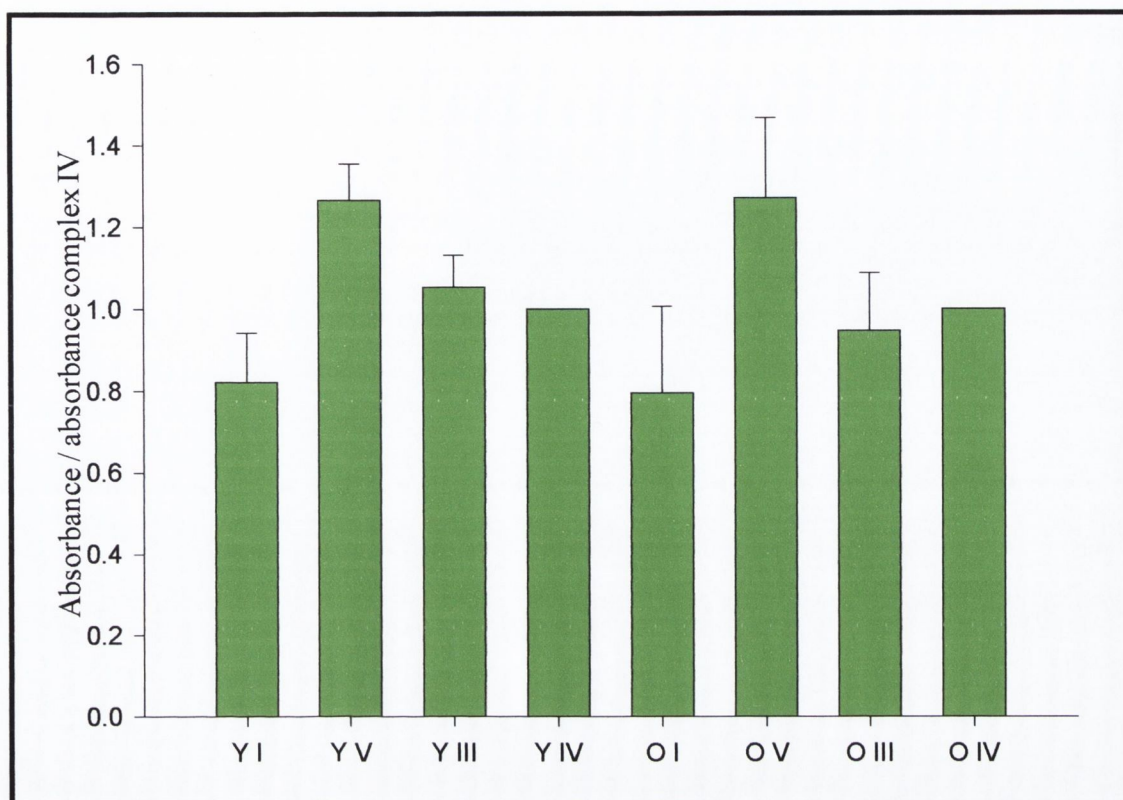


Figure 6.2.4. Comparison of the absorbances of complexes I, V and III normalised to the absorbance of complex IV in mitochondria isolated from young (3 month) and old (24 month) rat heart. All mitochondrial proteins were solubilized using maltoside. The membrane protein complexes I, III, IV and V were separated using blue native PAGE in the first dimension and were identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second dimension. The second-dimension gel, which was fixed, stained using Serva blue G and destained as in Materials and Methods, was used for densitometric analysis. The subunits used for densitometric analysis are indicated by arrows on the gel shown in fig. 6.2.1. The subunits whose absorbances were measured were: the 42kDa subunit of complex I, the 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The graph labels are as follows: Y I: young complex I; Y V: young complex V; Y III: young complex III; Y IV: young complex IV; O I: old complex I; O V: old complex V; O III: old complex III; O IV: old complex IV. A 227 μ g amount of rat heart mitochondria was loaded on the first-dimension gel in the case of young and old mitochondria. All of the data represent the mean $\pm$ S.E.M. of three determinations. The data are also represented in Table 6.2.3.

no significant differences in the absorbances of the complexes between heart mitochondria isolated from young and old rats (*Student's* unpaired *t*-test) when normalised to the in-gel absorbance of complex IV.

Figure 6.2.5 A and B show the resolution in the second dimension of rat liver mitochondrial proteins isolated from young (3 month) and old (24 month) rats. The gels were stained with Serva blue G. First-dimension gel lanes loaded with 454 µg of young and old protein had the in-gel proteins denatured using 20mM DDT/1%SDS and the denatured proteins were resolved on a second dimension 10%-tricine-SDS gel. Complexes I, III, IV and V were identified on the basis of their characteristic patterns in the second dimension in liver mitochondria isolated from young (fig. 6.2.5 A) and old (fig. 6.2.5 B) rats. Complex II could not be identified in either the young or old rat liver mitochondria because of a large number of unidentified proteins in the same region of the gel. Arrows on the gels mark the protein subunits whose staining intensity was measured for the quantification of second-dimension gels. The complete pattern of proteins appears to be the same in the young and old rat liver mitochondria.

Figure 6.2.6 shows the absorbances normalised per mg of protein loaded on the first-dimension gel of complexes I, III, IV and V in mitochondria isolated from young and old rat liver mitochondria. The absorbances are also listed in Table 6.2.1. The absorbances of complex I were 0.39 ± 0.06 and 0.07 ± 0.01 in young and old rat heart mitochondria, respectively. The difference in absorbance between complex I in mitochondria from young and old rat heart was significant ($p=0.0063$, $n=3$, *Student's* unpaired *t*-test). The absorbances of complex V were 0.66 ± 0.06 and 0.25 ± 0.03 in young and old rat heart mitochondria, respectively. The difference in absorbance between complex V in mitochondria from young and old rat liver was significant ($p=0.0036$, $n=3$, *Student's* unpaired *t*-test). The absorbances of complex III were 0.36 ± 0.03 and 0.07 ± 0.01 in young and old rat heart mitochondria, respectively. The difference in absorbance between complex III in mitochondria from rat heart isolated from young and old animals was significant ($p=0.0008$, $n=3$, *Student's* unpaired *t*-test). The absorbances of complex IV

were 0.38 ± 0.05 and 0.11 ± 0.01 respectively, in young and old rat heart mitochondria. The difference in absorbance between complex IV in mitochondria from young and old rat heart was significant ($p=0.0061$, $n=3$, *Student's* unpaired *t*-test).

Figure 6.2.7 shows the absorbances normalised per μmol of citrate synthase activity of complexes I, III, IV and V in mitochondria isolated from young and old rat liver mitochondria. The absorbances are also listed in Table 6.2.2. The absorbances of complex I were 4.65 ± 0.74 and 1.09 ± 0.19 in rat liver mitochondria from young and old animals, respectively. The difference in absorbance between complex I in mitochondria from young and old rat liver was significant ($p=0.0096$, $n=3$, *Student's* unpaired *t*-test). The absorbances of complex V were 7.84 ± 0.79 and 3.70 ± 0.45 in young and old rat liver mitochondria, respectively. The difference in absorbance between complex V in mitochondria from young and old rat liver was significant ($p=0.0104$, $n=3$, *Student's* unpaired *t*-test). The absorbances of complex III were 4.35 ± 0.44 and 1.13 ± 0.21 in young and old rat liver mitochondria, respectively. The difference in absorbance between complex III in mitochondria from young and old rat liver was significant ($p=0.0027$, $n=3$, *Student's* unpaired *t*-test). The absorbances of complex IV were 4.55 ± 0.65 and 1.59 ± 0.11 , respectively in liver mitochondria isolated from young and old rats. The difference in absorbance between complex IV in mitochondria from young and old rat liver was significant ($p=0.0109$, $n=3$, *Student's* unpaired *t*-test).

Figure 6.2.8 shows the absorbances of complexes I, III and V normalised to the absorbance of complex IV in mitochondria isolated from young and old rat liver. The absorbances are also listed in Table 6.2.3. The absorbances of complex I were 1.06 ± 0.02 and 0.68 ± 0.09 in young and old rat liver mitochondria, respectively. The absorbances of complex V were 1.75 ± 0.11 and 2.31 ± 0.16 in liver mitochondria isolated from young and old rats, respectively. The difference in absorbance between complex V in mitochondria from young and old rat liver was significant ($p=0.0448$, $n=3$, *Student's* unpaired *t*-test). The apparent increase in the amount of complex V in old rat

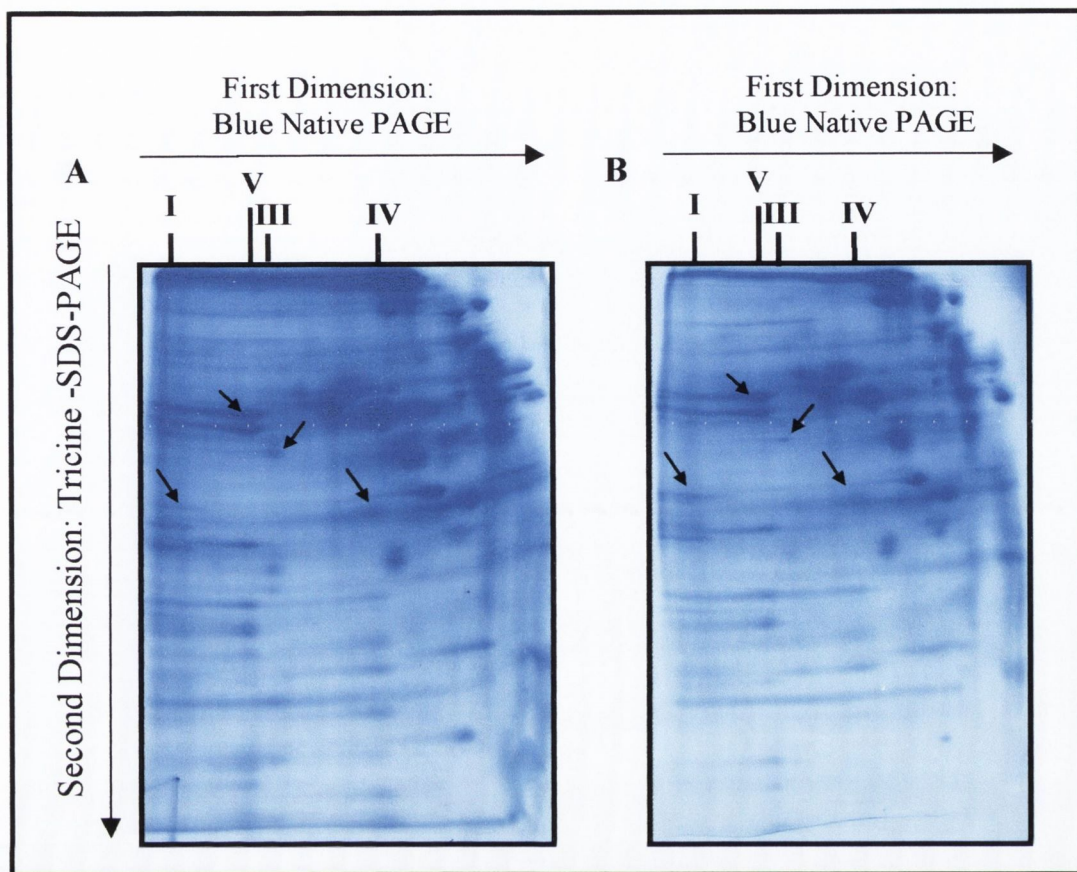


Figure 6.2.5. Two-dimensional resolution of mitochondrial proteins from rat liver mitochondria isolated from young (3 month) and old (24 month) rats using blue native PAGE in the first dimension and denaturing tricine-SDS-PAGE in the second dimension. The membrane protein complexes were separated in the native first dimension and identified by their characteristic polypeptide patterns in the denaturing second dimension. The gel was fixed, stained using Serva blue G and destained as described in Materials and Methods. The positions of the complexes I, III, IV and V are indicated at the top of the gels. Arrows on the gels mark the protein subunits whose staining intensity or absorbance was measured for the quantification of second-dimension gels. The subunits whose absorbances were measured were: the 42kDa subunit of complex I, the 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The young rat liver mitochondrial proteins (**A**) and the old rat liver mitochondrial proteins (**B**) were solubilized using a 2:1 ratio of maltoside to protein and were resolved using blue native PAGE in the first dimension (1mm gel, 5.5-13% non-linear gradient gel). A first-dimension lane loaded with 25 μ l (227 μ g mitochondrial protein) of solubilized young or old rat liver mitochondrial protein was cut from the first-dimension gel and the in-gel native proteins were denatured using 20mM DDT/1%SDS (see Materials and Methods). The mitochondrial proteins were resolved using a uniform 1mm 10% tricine-SDS gel in the second dimension. The gels shown in A and B are representative of three gels that were used for the quantification of second-dimension gels.

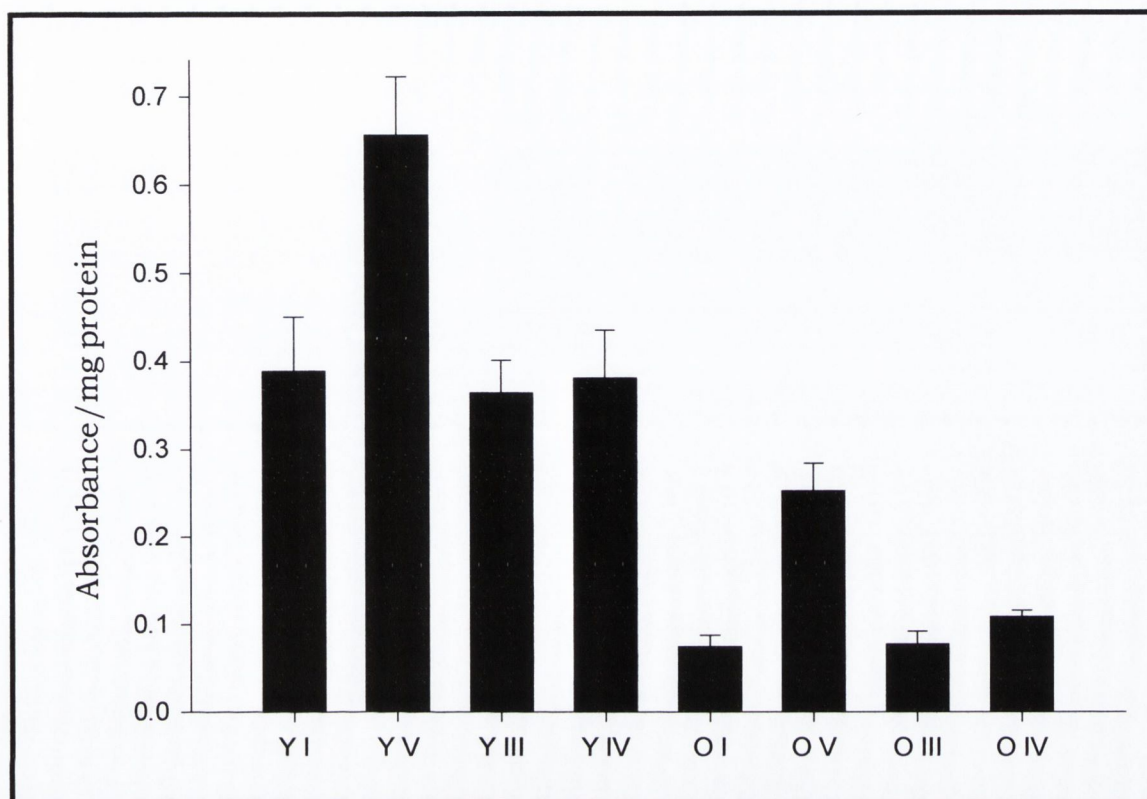


Figure 6.2.6. Comparison of the absorbances of complexes I, V, III and IV normalised per mg protein in mitochondria isolated from young (3 month) and old (24 month) rat liver. All mitochondrial proteins were solubilized using maltoside. The membrane protein complexes I, III, IV and V were separated using blue native PAGE in the first dimension and identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second dimension. The second dimension gel, which was fixed, stained using Serva blue G and destained as described in Materials and Methods, was used for densitometric analysis. The subunits used for densitometric analysis are indicated by arrows on the gel shown in fig. 6.2.5. The subunits whose staining intensity was measured were: the 42kDa subunit of complex I, the 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The graph labels are as follows Y I: young complex I; Y V: young complex V; Y III: young complex III; Y IV: young complex IV; O I: old complex I; O V: old complex V; O III: old complex III and O IV: old complex IV. A 227 μ g amount of rat liver mitochondria was loaded on the first-dimension gel in the case of young and old mitochondria. All of the data represent the mean $\pm$ S.E.M. of three determinations. The data are also represented in table 6.2.1.

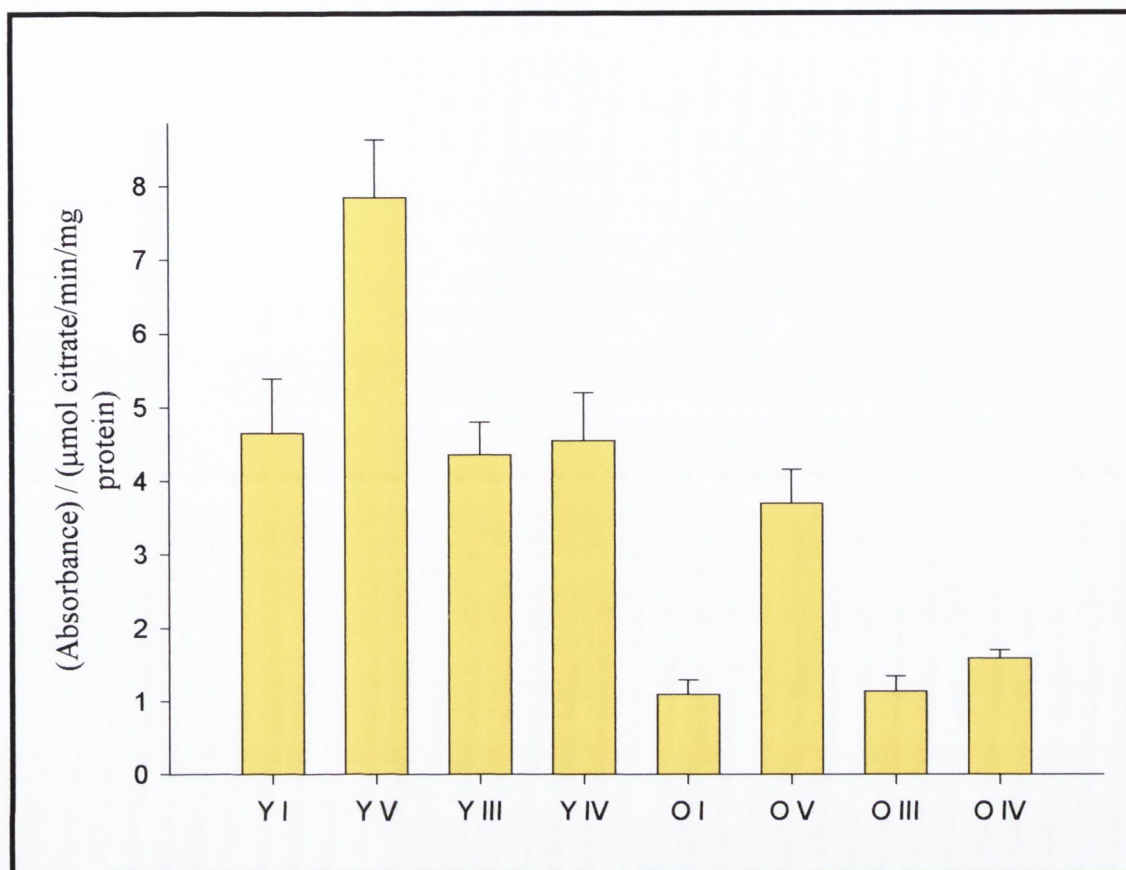


Figure 6.2.7. Comparison of the absorbances of complexes I, V, III and IV per μmol citrate synthase activity in mitochondria isolated from young (3 month) and old (24 month) rat liver. All mitochondrial proteins were solubilized using maltoside. The membrane protein complexes I, III, IV and V were separated using blue native PAGE in the first dimension and identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second dimension. The second dimension gel, which was fixed, stained using Serva blue G and destained as described in Materials and Methods, was used for densitometric analysis. The subunits used for densitometric analysis are indicated by arrows on the gel shown in fig. 6.2.5. The complex subunits whose staining intensity was measured were: the 42kDa subunit of complex I, the 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The graph labels are as follows Y I: young complex I; Y V: young complex V; Y III: young complex III; Y IV: young complex IV; O I: old complex I; O V: old complex V; O III: old complex III and O IV: old complex IV. A 454 μg amount of rat liver mitochondria was loaded on the first-dimension gel in the case of young and old mitochondria. All of the data represent the mean $\pm$ S.E.M. of three determinations. The data are also represented in table 6.2.2.

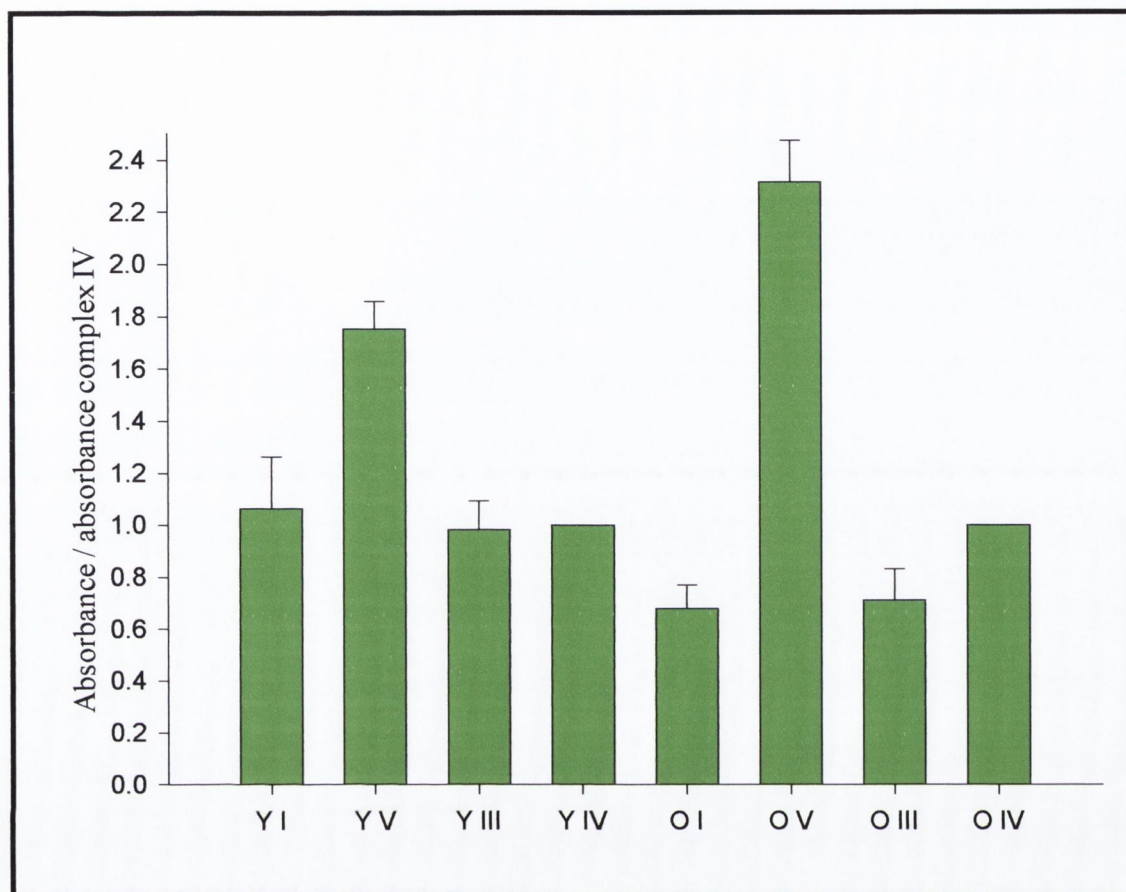


Figure 6.2.8. Comparison of the absorbances of complexes I, V and III normalised to the absorbance of complex IV in mitochondria isolated from young (3 month) and old (24 month) rat liver. All mitochondrial proteins were solubilized using maltoside. The membrane protein complexes I, III, IV and V were separated using blue native PAGE in the first dimension and identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second dimension. The second dimension gel, which was fixed, stained using Serva blue G and destained as described in Materials and Methods, was used for densitometric analysis. The subunits used for densitometric analysis are indicated by arrows on the gels shown in fig. 6.2.2. The subunits whose absorbance was measured were: : the 42kDa subunit of complex I, the 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The graph labels are as follows Y I: young complex I; Y V: young complex V; Y III: young complex III; Y IV: young complex IV; O I: old complex I; O V: old complex V; O III: old complex III and O IV: old complex IV. A 227 μ g amount of rat liver mitochondria was loaded on the first-dimension gel in the case of young and old mitochondria. All of the data represent the mean $\pm$ S.E.M. of three determinations. The data is also represented in table 6.2.3.

liver mitochondria compared with young is due to a decrease in the amount of complex IV. When the absorbance of complex IV is normalised to the in-gel absorbance of complex V in young and old rat liver mitochondria, the amount of complex IV decreases from 0.58 ± 0.03 in young rat liver mitochondria to 0.44 ± 0.03 ($p=0.0299$, $n=3$, *Student's* unpaired *t*-test). The absorbances of complex III were 0.98 ± 0.11 and 0.71 ± 0.12 in liver mitochondria isolated from young and old rats, respectively.

Figure 6.2.9 A and B show the resolution in the second dimension of rat liver mitochondrial proteins isolated from euthyroid and hyperthyroid rats. The gels were stained with Serva blue G. First-dimension gel lanes loaded with $454\mu\text{g}$ of euthyroid and hyperthyroid mitochondrial protein had the in-gel proteins denatured using 20mM DDT/1%SDS and the denatured proteins were resolved on a second dimension 10%-tricine-SDS gel. Complexes I, III, IV and V were identified on the basis of their characteristic patterns in the second dimension in the euthyroid (fig. 6.2.9 A) and hyperthyroid (fig. 6.2.9 B) rat liver mitochondria. Complex II could not be identified in either euthyroid or hyperthyroid rat liver mitochondria because of a large number of unidentified proteins in the same region of the gel. Arrows on the gels mark the protein subunits whose staining intensity was measured for the quantification of second-dimension gels.

Figure 6.2.10 shows the absorbance normalised per mg of protein loaded on the first dimension gel of complexes I, III, IV and V in mitochondria isolated from euthyroid and hyperthyroid rat liver mitochondria. The absorbances are also listed in Table 6.2.1. The absorbances of complex I were 0.42 ± 0.03 and 0.53 ± 0.09 in euthyroid and hyperthyroid rat liver mitochondria, respectively. The absorbances of complex V were 0.69 ± 0.01 and 0.80 ± 0.06 in euthyroid and hyperthyroid rat liver mitochondria, respectively. The absorbances of complex III were 0.46 ± 0.01 and 0.54 ± 0.05 in euthyroid and hyperthyroid rat liver mitochondria, respectively. The absorbances of complex IV were 0.49 ± 0.03 and 0.57 ± 0.04 in euthyroid and hyperthyroid rat liver mitochondria, respectively. There were no significant differences in the absorbances of

the complexes between liver mitochondria isolated from euthyroid and hyperthyroid rats (*Student's* unpaired *t*-test).

Figure 6.2.11 shows the absorbance normalised per μmol of citrate synthase activity of complexes I, III, IV and V in mitochondria isolated from euthyroid and hyperthyroid rat liver mitochondria. The absorbances are also listed in Table 6.2.2. The absorbances of complex I were 4.14 ± 0.33 and 4.47 ± 0.82 in euthyroid and hyperthyroid rat liver mitochondria, respectively. The absorbances of complex V were 6.86 ± 0.14 and 6.76 ± 0.49 in euthyroid and hyperthyroid rat liver mitochondria, respectively. The absorbances of complex III were 4.57 ± 0.09 and 4.55 ± 0.46 in euthyroid and hyperthyroid rat liver mitochondria, respectively. The absorbances of complex IV were 4.92 ± 0.26 and 4.79 ± 0.34 in liver mitochondria isolated from euthyroid and hyperthyroid rats, respectively. There were no significant differences in the absorbances of the complexes between euthyroid and hyperthyroid rat liver mitochondria (*Student's* unpaired *t*-test).

Figure 6.2.12 shows the absorbance of complexes I, III and V normalised to the absorbance of complex IV in mitochondria isolated from euthyroid and hyperthyroid rat liver mitochondria. The absorbances are also listed in Table 6.2.3. The absorbances of complex I were 0.85 ± 0.09 and 0.92 ± 0.12 in euthyroid and hyperthyroid rat liver mitochondria, respectively. The absorbances of complex V were 1.40 ± 0.04 and 1.42 ± 0.04 in euthyroid and hyperthyroid rat liver mitochondria, respectively. The absorbances of complex III were 0.93 ± 0.03 and 0.95 ± 0.06 in euthyroid and hyperthyroid rat liver mitochondria, respectively. There were no significant differences in the absorbances of the complexes between liver mitochondria isolated from euthyroid and hyperthyroid rats (*Student's* unpaired *t*-test).

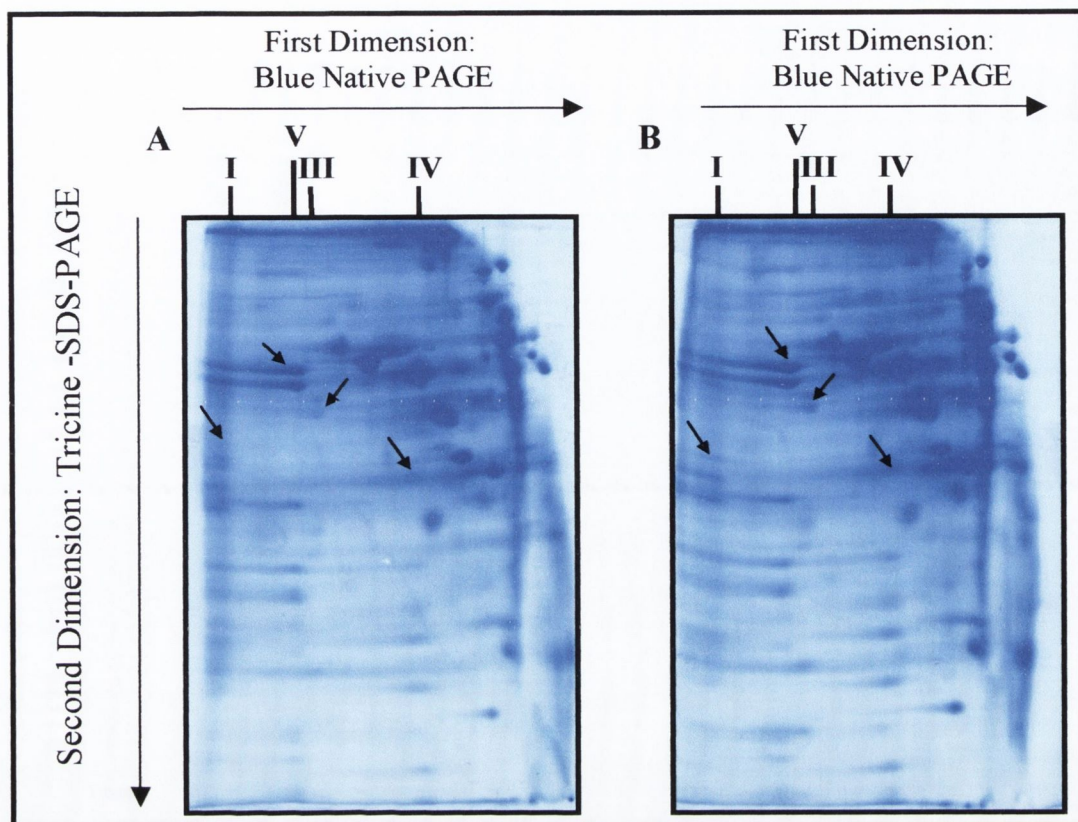


Figure 6.2.9. Two dimensional resolution of mitochondrial proteins from rat liver mitochondria isolated from euthyroid and hyperthyroid rats using blue native PAGE in the first dimension and denaturing tricine-SDS-PAGE in the second dimension. The membrane protein complexes were separated in the native first dimension and identified by the characteristic polypeptide patterns in the denaturing second dimension. The gel was fixed, stained using Serva blue G and destained as described in Materials and Methods. The positions of the complexes I, III, IV and V are indicated at the top of the gels. Arrows on the gels mark the protein subunits whose staining intensity was measured for the quantification of second-dimension gels. The subunits whose staining intensity was measured were the 42kDa subunit of complex I, the 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The euthyroid rat liver mitochondrial proteins (**A**) and the hyperthyroid rat liver mitochondrial proteins (**B**) were solubilized using a 1:1 ratio of maltoside to protein and were resolved using blue native PAGE in the first dimension (1mm gel, 5.5-13% non-linear gradient gel). A first-dimension lane loaded with 25 μ l (454 μ g mitochondrial protein) of solubilized euthyroid or hyperthyroid rat liver mitochondrial protein was cut from the first dimension gel and the in-gel native proteins were denatured using 20 mM DDT/1%SDS (see Materials and Methods). The mitochondrial proteins were resolved using a uniform 1mm 10% tricine-SDS gel in the second dimension. The gels shown in A and B are representative of three gels that were used for the quantification of second dimension gels.

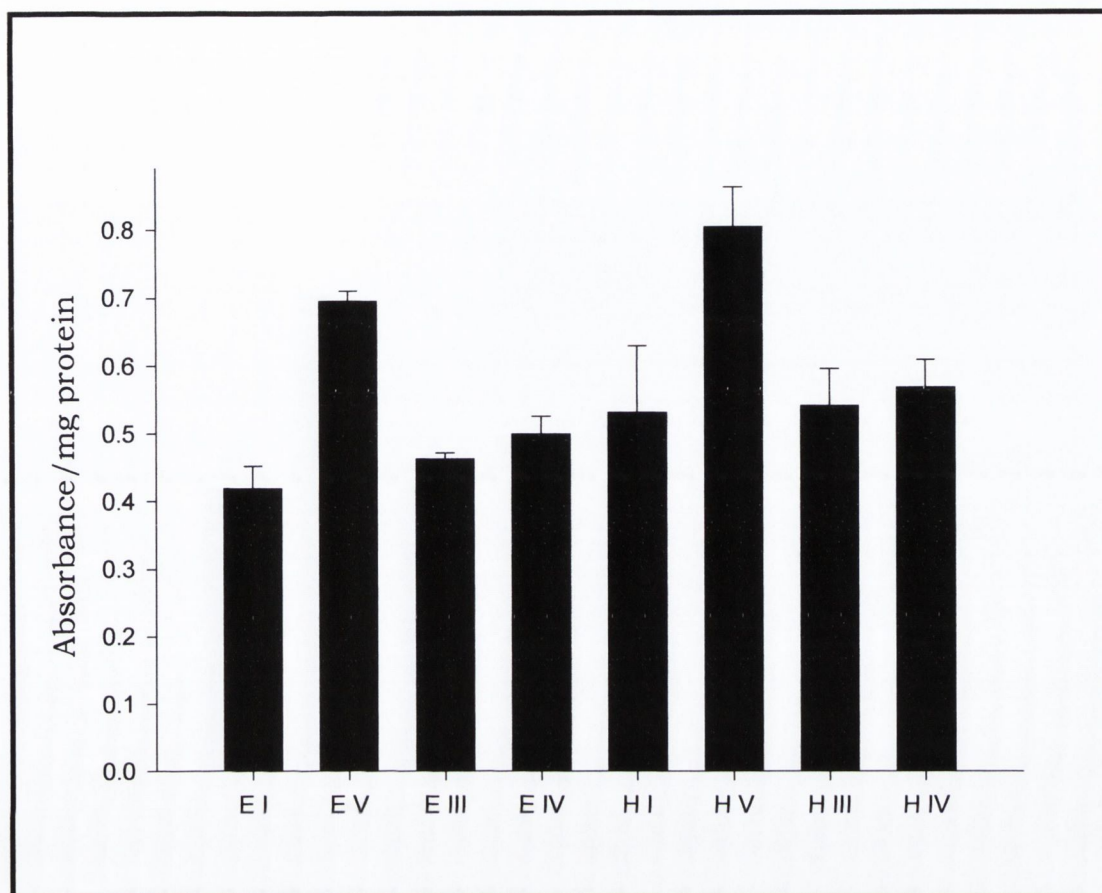


Figure 6.2.10. Comparison of the absorbances of complexes I, V, III and IV normalised per mg protein in mitochondria isolated from euthyroid and hyperthyroid rat liver. All mitochondrial proteins were solubilized using maltoside. The membrane protein complexes I, III, IV and V were separated using blue native PAGE in the first dimension and were identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second dimension. The second-dimension gel, which was fixed, stained using Serva blue G and destained as described in Materials and Methods, was used for densitometric analysis. The subunits used for densitometric analysis are indicated by arrows on the gel shown in fig. 6.2.9. The complex subunits whose staining intensity was measured were: the 42kDa subunit of complex I, the 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The graph labels are as follows E I: euthyroid complex I; E V: euthyroid complex V; E III: euthyroid complex III; E IV: euthyroid complex IV; H I: hyperthyroid complex I; H V: hyperthyroid complex V; H III: hyperthyroid complex III and E IV: hyperthyroid complex IV. A 454 μ g amount of rat liver mitochondria was loaded on the first-dimension gel in the case of young and old mitochondria. All of the data represent the mean $\pm$ S.E.M. of three determinations. The data are also represented in Table 6.2.1.

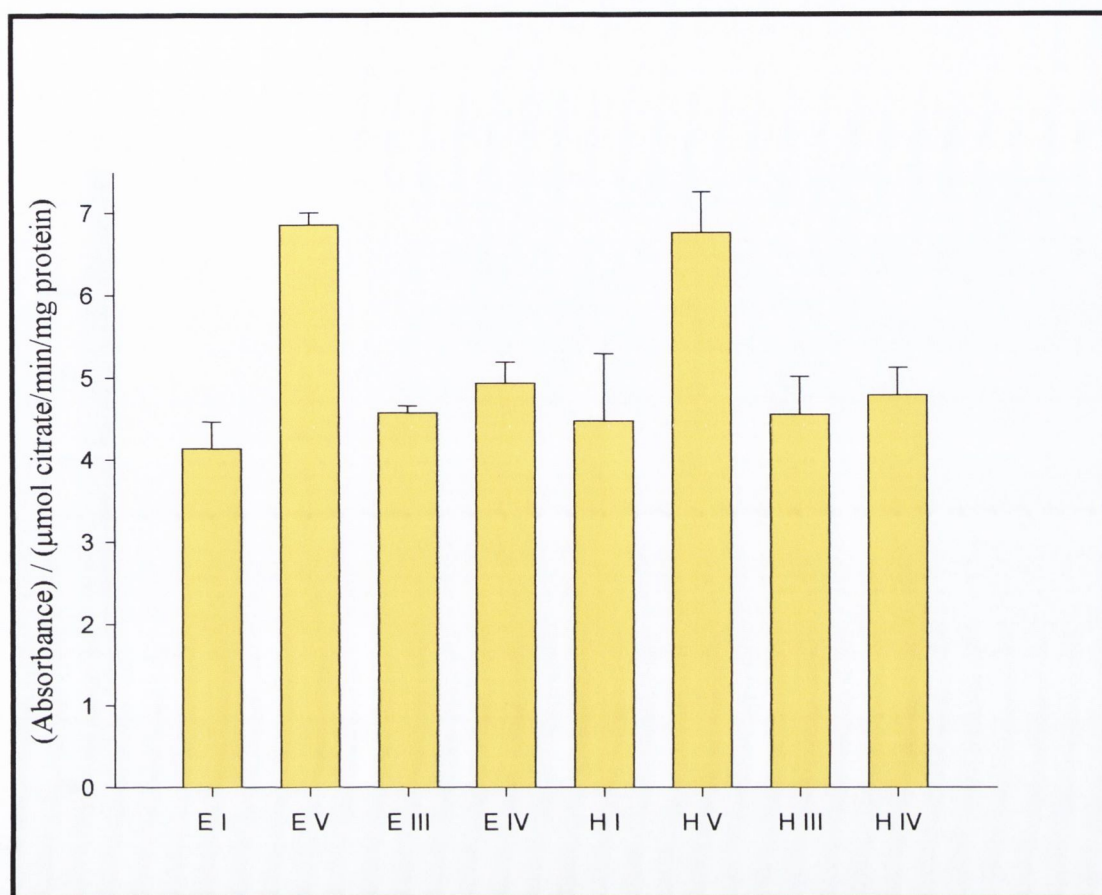


Figure 6.2.11. Comparison of the absorbances of complexes I, V, III and IV per μmol citrate synthase activity in mitochondria isolated from euthyroid and hyperthyroid rat liver. All mitochondrial proteins were solubilized using maltoside. The membrane protein complexes I, III, IV and V were separated using blue native PAGE in the first dimension and were identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second dimension. The second-dimension gel, which was fixed, stained using Serva blue G and destained as described in Materials and Methods, was used for densitometric analysis. The subunits used for densitometric analysis are indicated by arrows on the gel shown in fig. 6.2.9. The subunits whose staining intensity was measured were: the 42kDa subunit of complex I, the 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The graph labels are as follows E I: euthyroid complex I; E V: euthyroid complex V; E III: euthyroid complex III; E IV: euthyroid complex IV; H I: hyperthyroid complex I; H V: hyperthyroid complex V; H III: hyperthyroid complex III and H IV: hyperthyroid complex IV. A 454 μg amount of rat liver mitochondria was loaded on the first-dimension gel in the case of young and old mitochondria. All of the data represent the mean $\pm$ S.E.M. of three determinations. The data are also represented in Table 6.2.2.

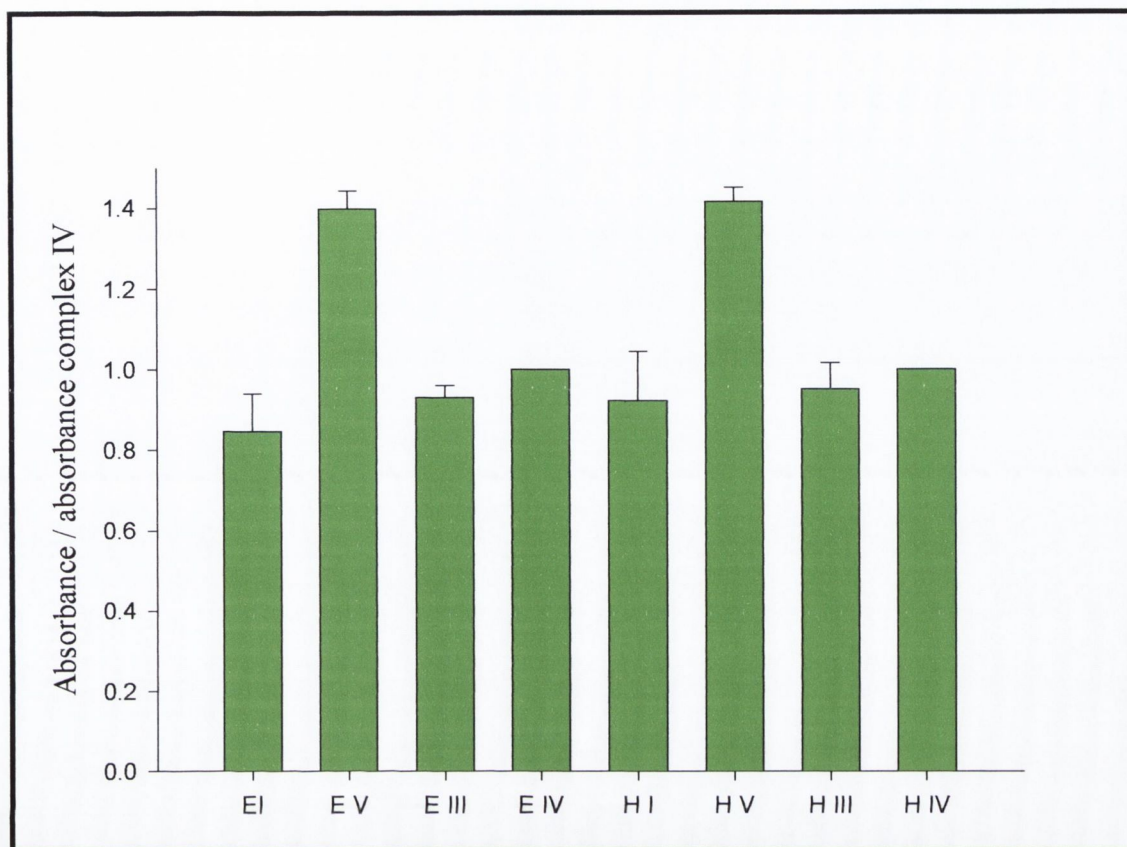


Figure 6.2.12. Comparison of the absorbances of complexes I, V and III normalised to the absorbance of complex IV in mitochondria isolated from euthyroid and hyperthyroid rat liver. All mitochondrial proteins were solubilized using maltoside. The membrane protein complexes I, III, IV and V were separated using blue native PAGE in the first dimension and identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second dimension. The second-dimension gel, which was fixed, stained using Serva blue G and destained as described in Materials and Methods, was used for densitometric analysis. The subunits used for densitometric analysis are indicated by arrows on the gel shown in fig. 6.2.9. The subunits whose staining intensity was measured were: the 42kDa subunit of complex I, the 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The graph labels are as follows E I: euthyroid complex I; E V: euthyroid complex V; E III: euthyroid complex III; E IV: euthyroid complex IV; H I: hyperthyroid complex I; H V: hyperthyroid complex V; H III: hyperthyroid complex III and E IV: hyperthyroid complex IV. A 454 μ g amount of rat liver mitochondria was loaded on the first-dimension gel in the case of young and old mitochondria. All of the data represent the mean $\pm$ S.E.M. of three determinations. The data are also represented in Table 6.2.2.

Figure 6.2.13 A and B show the resolution in the second dimension of rat synaptic mitochondrial proteins isolated from young (3 month) and old (24 month) rats. The gels were stained with Serva blue G. First-dimension gel lanes loaded with 227µg of young and old synaptic mitochondrial protein had the in-gel proteins denatured using 20mM DDT/1%SDS and the denatured proteins were resolved on a second dimension 10%-tricine-SDS gel. Complexes I, III, IV and V were identified on the basis of their characteristic patterns in the second dimension in the young (fig. 6.2.13 A) and old (fig. 6.2.13 B) rat synaptic mitochondria. Complex II could not be identified in either the young or old rat synaptic mitochondria because of a large number of unidentified proteins in the same region of the gel. Arrows on the gels mark the protein subunits whose staining intensity was measured for the quantification of second-dimension gels. The pattern of proteins appears to be the same in the young (fig. 6.2.13 A) and old (fig. 6.2.13 B) rat synaptic mitochondria.

Figure 6.2.14 shows the absorbance normalised per mg of protein loaded on the first-dimension gel of complexes I, III, IV and V in mitochondria isolated from young and old rat synaptic mitochondria. The absorbances are also listed in Table 6.2.1. The absorbances of complex I were 0.54 ± 0.09 and 0.83 ± 0.15 in young and old rat synaptic mitochondria, respectively. The absorbances of complex V were 0.63 ± 0.06 and 0.97 ± 0.15 in young and old rat synaptic mitochondria, respectively. The absorbances of complex III were 0.52 ± 0.03 and 0.77 ± 0.12 in young and old rat synaptic mitochondria, respectively. The absorbances of complex IV were 0.54 ± 0.04 and 0.74 ± 0.08 in young and old rat synaptic mitochondria, respectively. There were no significant differences in the absorbances of the complexes between synaptic mitochondria isolated from young and old rats (*Student's* unpaired *t*-test).

Figure 6.2.15 shows the absorbance normalised per µmol of citrate synthase activity of complexes I, III, IV and V in mitochondria isolated from young and old rat synaptic mitochondria. The absorbances are also listed in Table 6.2.3. The absorbances of complex I were 0.25 ± 0.04 and 0.67 ± 0.12 in young and old rat synaptic mitochondria,

respectively. The difference in absorbance of complex I in mitochondria from young and old rat synaptic mitochondria was significant ($p=0.0294$, *Student's* unpaired *t*-test). The absorbances of complex V were 0.30 ± 0.03 and 0.79 ± 0.12 in young and old rat synaptic mitochondria, respectively. The difference in absorbance of complex V in mitochondria from young and old rat synaptic mitochondria was significant ($p=0.0167$, *Student's* unpaired *t*-test). The absorbances of complex III were 0.24 ± 0.01 and 0.63 ± 0.10 in young and old rat synaptic mitochondria, respectively. The difference in absorbance of complex III in mitochondria from young and old rat synaptic mitochondria was significant ($p=0.0178$, *Student's* unpaired *t*-test). The absorbances of complex IV were 0.25 ± 0.02 and 0.60 ± 0.07 in young and old rat synaptic mitochondria, respectively. The difference in absorbance of complex IV in mitochondria isolated from young and old rat synaptosomes was significant ($p=0.0086$, *Student's* unpaired *t*-test).

Figure 6.2.16 shows the absorbance of complexes I, III and V normalised to the absorbance of complex IV in mitochondria isolated from young and old rat synaptic mitochondria. The absorbances are also listed in Table 6.2.3. The absorbances of complex I were 1.05 ± 0.26 and 1.13 ± 0.18 in young and old rat synaptic mitochondria, respectively. The absorbances of complex V were 1.21 ± 0.21 and 1.34 ± 0.19 in young and old rat synaptic mitochondria, respectively. The absorbances of complex III were 0.99 ± 0.12 and 1.06 ± 0.13 in young and old rat synaptic mitochondria, respectively. There were no significant differences in the absorbances of the complexes between young and old rat synaptic mitochondria (*Student's* unpaired *t*-test).

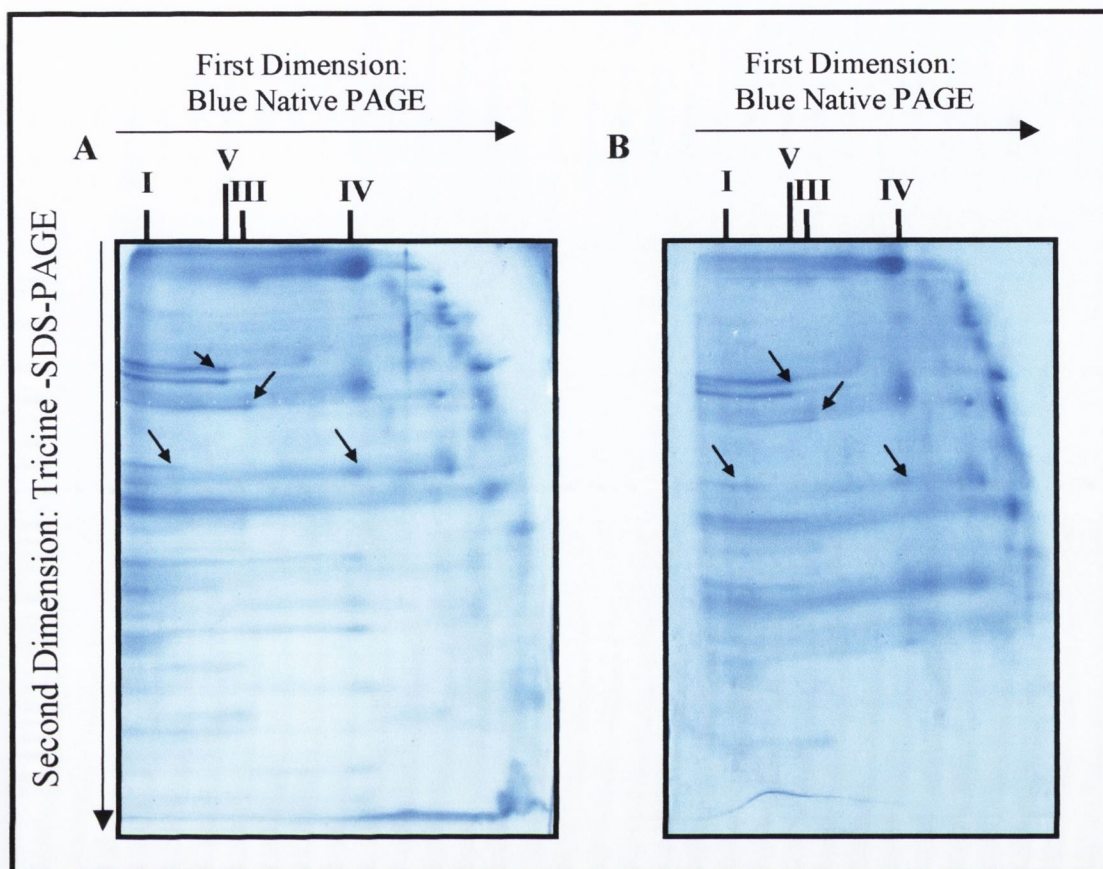


Figure 6.2.13. Two dimensional resolution of mitochondrial proteins from rat synaptic mitochondria isolated from young (3 month) and old (24 month) rats using blue native PAGE in the first dimension and denaturing tricine-SDS-PAGE in the second dimension. The membrane protein complexes were separated in the native first dimension and identified by the characteristic polypeptide patterns in the denaturing second dimension. The gel was fixed, stained using Serva blue G and destained as in Materials and Methods. The positions of the complexes I, III, IV and V are indicated at the top of the gels. Arrows on the gels mark the protein subunits whose staining intensity was measured for the quantification of second-dimension gels. The subunits whose staining intensity was measured were the 42kDa subunit of complex I, the 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The young rat synaptic mitochondrial proteins (**A**) and the old rat synaptic mitochondrial proteins (**B**) were solubilized using a 2:1 ratio of maltoside to mitochondrial protein and resolved using blue native PAGE in the first dimension (1mm gel, 5.5-13% non-linear gradient gel). A first-dimension lane loaded with 25 μ l (227 μ g mitochondrial protein) of solubilized young and old rat synaptic mitochondrial protein was cut from the first-dimension gel and the in-gel native proteins were denatured using 20mM DDT/1%SDS (see Materials and Methods). The mitochondrial proteins were resolved using a uniform 1mm 10% tricine-SDS gel in the second dimension. The gels shown in A and B are representative of three gels that were used for quantification of second dimension gels.

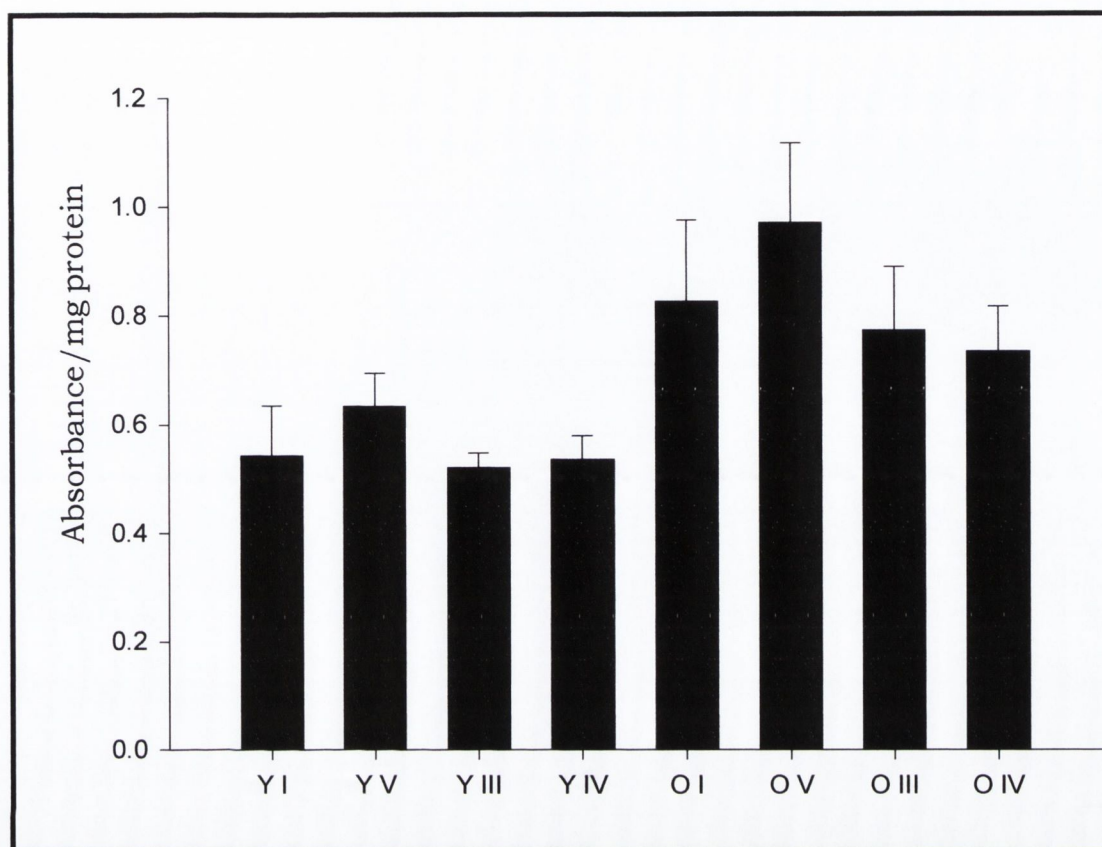


Figure 6.2.14. Comparison of the absorbances of complexes I, V, III and IV normalised per mg protein in mitochondria isolated from young (3 month) and old (24 month) rat synaptosomes. All mitochondrial proteins were solubilized using maltoside. The membrane protein complexes I, III, IV and V were separated using blue native PAGE in the first dimension and identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second dimension. The second-dimension gel, which was fixed, stained using Serva blue G and destained as described in Materials and Methods, was used for densitometric analysis. The subunits used for densitometric analysis are indicated by arrows on the relevant fig. 6.2.13. The subunits whose staining intensity was measured were the 42kDa subunit of complex I, the 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The graph labels are as follows Y I: young complex I; Y V: young complex V; Y III: young complex III; Y IV: young complex IV; O I: old complex I; O V: old complex V; O III: old complex III and O IV: old complex IV. A 227 μ g amount of rat synaptic mitochondria was loaded on the first dimension gel in the case of young and old mitochondria. All of the data represents the mean $\pm$ S.E.M. of three determinations. The data are also represented in Table 6.2.1.

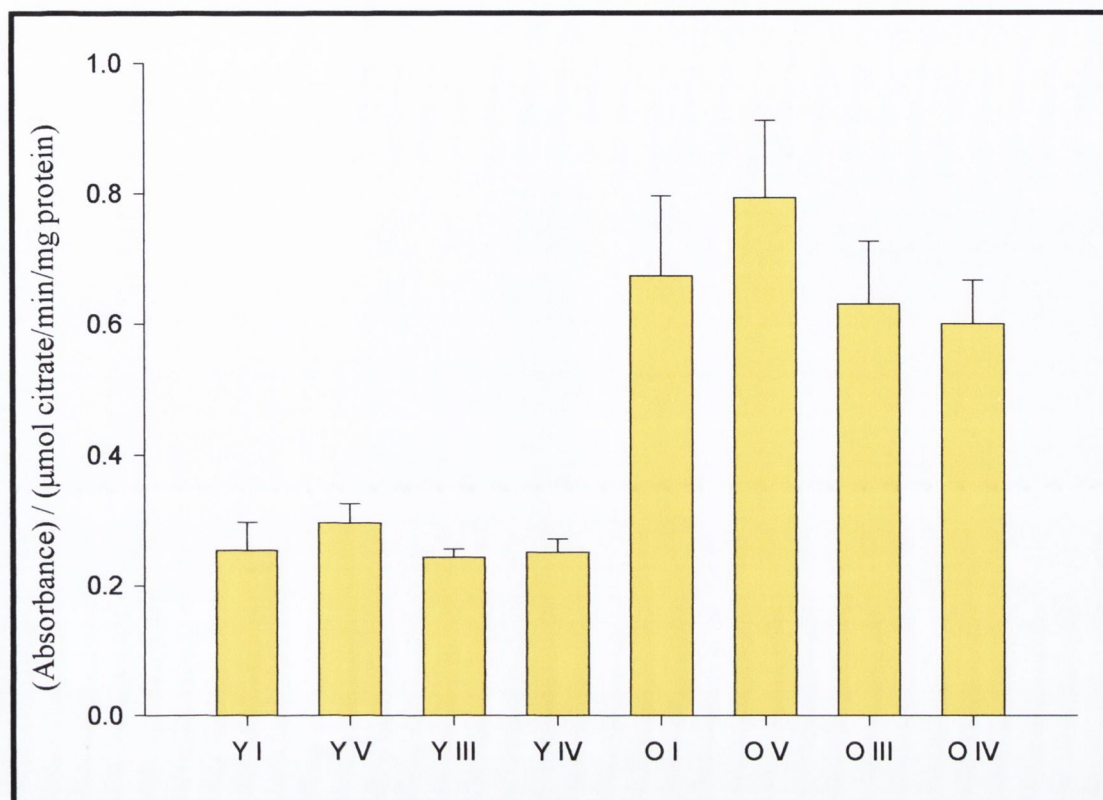


Figure 6.2.15. Comparison of the absorbance of complex I, V, III and IV per μmol citrate synthase activity in mitochondria isolated from young (3 month) and old (24 month) rat synaptosomes. All mitochondrial proteins were solubilized using maltoside. The membrane protein complexes I, III, IV and V were separated using blue native PAGE in the first dimension and identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second dimension. The second dimension gel, which was fixed, stained using Serva blue G and destained as described in Materials and Methods, was used for densitometric analysis. The subunits used for densitometric analysis are indicated by arrows on the gel shown in fig. 6.2.13. The subunits whose staining intensity was measured were the 42kDa subunit of complex I, the 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The graph labels are as follows Y I: young complex I; Y V: young complex V; Y III: young complex III; Y IV: young complex IV; O I: old complex I; O V: old complex V; O III: old complex III and O IV: old complex IV. A 227 μg amount of rat synaptic mitochondria was loaded on the first dimension gel in the case of young and old mitochondria. All of the data represents the mean $\pm$ S.E.M. of three determinations. The data are also represented in Table 6.2.2.

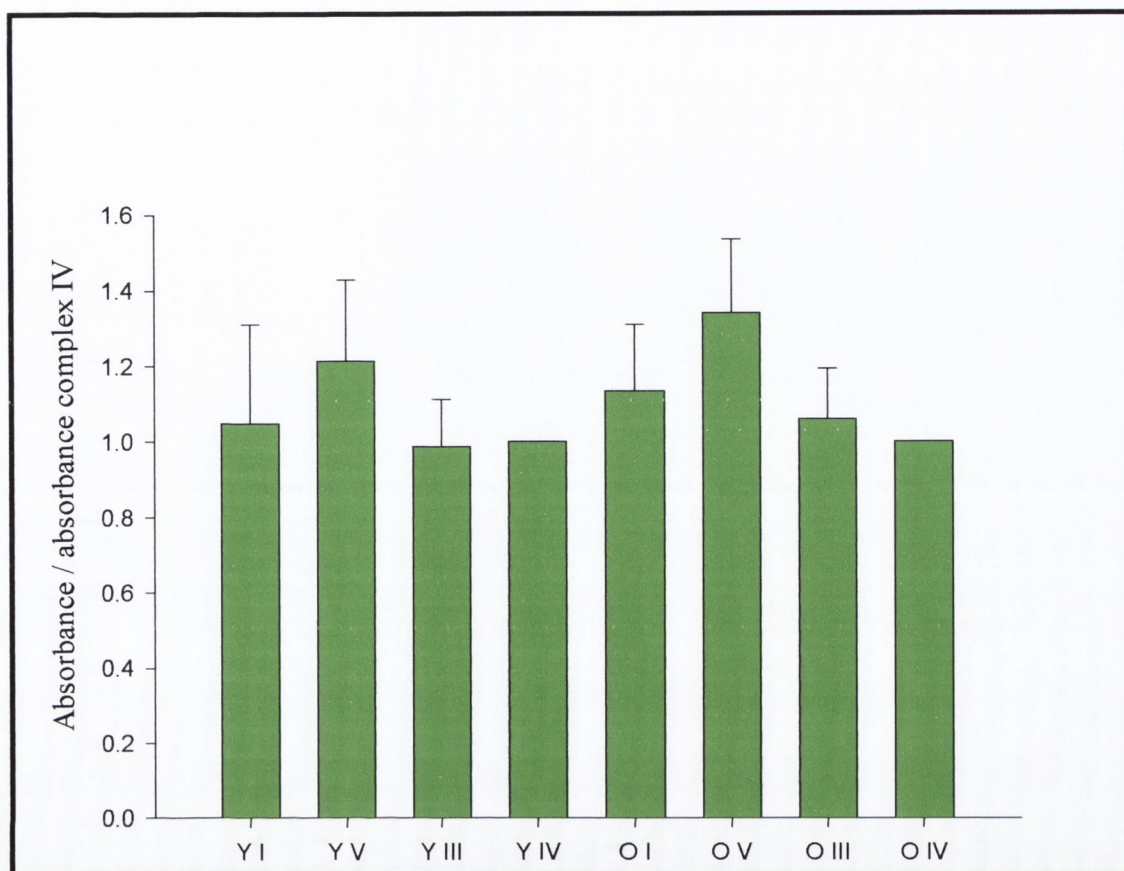


Figure 6.2.16. Comparison of the absorbances of complexes I, V, III normalised relative to the absorbance of complex IV in mitochondria isolated from young (3 month) and old (24 month) rat synaptosomes. All mitochondrial proteins were solubilized using maltoside. The membrane protein complexes I, III, IV and V were separated using blue native PAGE in the first dimension and identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second dimension. The second-dimension gel, which was fixed, stained using Serva blue G and destained as described in Materials and Methods, was used for densitometric analysis. The subunits used for densitometric analysis are indicated by arrows on the gel shown in fig. 6.2.13. The subunits whose staining intensity was measured were the 42kDa subunit of complex I, the 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The graph labels are as follows Y I: young complex I; Y V: young complex V; Y III: young complex III; Y IV: young complex IV; O I: old complex I; O V: old complex V; O III: old complex III and O IV: old complex IV. A 227 μ g amount of rat synaptic mitochondria was loaded on the first dimension gel in the case of young and old mitochondria. All of the data represents the mean $\pm$ S.E.M. of three determinations. The data are also represented in Table 6.2.2.

Table 6.2.1 shows a comparison of the absorbance/mg protein of complexes I, III, IV and V in mitochondria isolated from young and old rat heart, rat liver, rat synaptosomes and in rat liver mitochondria isolated from euthyroid and hyperthyroid rats.

Table 6.2.2 shows a comparison of the absorbance per mmol citrate/min/mg protein (citrate synthase activity) of complexes I, III, IV and V in mitochondria isolated from rat heart, rat liver, rat synaptosomes and in rat liver mitochondria isolated from euthyroid and hyperthyroid rats.

Table 6.2.3 shows a comparison of the absorbance of complexes I, III and V normalised to the absorbance of complex IV in mitochondria isolated from rat heart, rat liver, rat synaptosomes and in rat liver mitochondria isolated from euthyroid and hyperthyroid rats.

Table 6.2.1

	I	V	III	IV
Young rat heart mitochondria	0.52 ± 0.06	0.81 ± 0.03	0.68 ± 0.03	0.64 ± 0.03
Old rat heart mitochondria	0.50 ± 0.12	0.81 ± 0.12	0.61 ± 0.10	0.64 ± 0.05
Young rat liver mitochondria	0.39 ± 0.06 a	0.66 ± 0.06 b	0.36 ± 0.03 c	0.38 ± 0.05 d
Old rat liver mitochondria	0.07 ± 0.01 <i>p</i> =0.0063 a	0.25 ± 0.03 <i>p</i> =0.0036 b	0.07 ± 0.01 <i>p</i> =0.001 c	0.11 ± 0.01 <i>p</i> =0.0061 d
Euthyroid rat liver mitochondria	0.42 ± 0.03	0.69 ± 0.01	0.46 ± 0.01	0.49 ± 0.03
Hyperthyroid rat liver mitochondria	0.53 ± 0.09	0.80 ± 0.06	0.54 ± 0.05	0.57 ± 0.04
Young rat synaptic mitochondria	0.54 ± 0.09	0.63 ± 0.06	0.52 ± 0.03	0.54 ± 0.04
Old rat synaptic mitochondria	0.83 ± 0.15	0.97 ± 0.15	0.77 ± 0.12	0.74 ± 0.08

Table 6.2.1. Comparison of the absorbance/mg protein of complexes I, V, III and IV in mitochondria isolated from rat heart, rat liver, rat synaptosomes and in rat liver mitochondria isolated from euthyroid and hyperthyroid rats. The membrane protein complexes I, III, IV and IV were separated using blue native PAGE in the first dimension and identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second dimension. The second dimension gel, which was fixed, stained using Serva blue G and destained as described in Materials and Methods, was used for densitometric analysis. The subunits used for densitometric analysis are indicated by arrows on the relevant gels (figs. 6.2.1, 6.2.5, 6.2.9, 6.2.13). All of the data represent the mean ± S.E.M. of three determinations. The labels a,b,c and d highlight the fact that an unpaired *Student's t*-test, two-tailed *p* value indicated a significant difference between young and old rat liver mitochondria. *p* < 0.05 was considered significant.

Table 6.2.2

	I	V	III	IV
Young rat heart mitochondria	0.12 ± 0.01	0.18 ± 0.01	0.15 ± 0.01	0.14 ± 0.01
Old rat heart mitochondria	0.10 ± 0.02	0.15 ± 0.02	0.11 ± 0.02	0.12 ± 0.01
Young rat liver mitochondria	4.65 ± 0.74 e	7.84 ± 0.79 f	4.35 ± 0.44 g	4.55 ± 0.65 h
Old rat liver mitochondria	1.09 ± 0.19 <i>p</i> =0.0096 e	3.70 ± 0.45 <i>p</i> =0.0104 f	1.13 ± 0.21 <i>p</i> =0.0027 g	1.59 ± 0.11 <i>p</i> =0.0108 h
Euthyroid rat liver mitochondria	4.14 ± 0.33	6.86 ± 0.14	4.57 ± 0.09	4.92 ± 0.26
Hyperthyroid rat liver mitochondria	4.47 ± 0.82	6.76 ± 0.49	4.55 ± 0.46	4.79 ± 0.34
Young rat synaptic mitochondria	0.25 ± 0.04 i	0.30 ± 0.03 j	0.24 ± 0.01 k	0.25 ± 0.02 l
Old rat synaptic mitochondria	0.67 ± 0.12 <i>p</i> =0.0294 i	0.79 ± 0.12 <i>p</i> =0.0167 j	0.63 ± 0.10 <i>p</i> =0.0178 k	0.60 ± 0.07 <i>p</i> =0.0086 l

Table 6.2.2. Comparison of the absorbance per mmol citrate/min/mg protein (citrate synthase activity) of complexes I, V, III and IV in mitochondria isolated from rat heart, rat liver, rat synaptosomes and in rat liver mitochondria isolated from euthyroid and hyperthyroid rats. The membrane protein complexes I, III, IV and IV were separated using blue native PAGE in the first dimension and identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second dimension. The second dimension gel, which was fixed, stained using Serva blue G and destained as described in Materials and Methods, was used for densitometric analysis. The subunits used for densitometric analysis are indicated by arrows on the relevant gels (figs. 6.2.1, 6.2.5, 6.2.9, 6.2.13). All of the data represent the mean ± S.E.M. of three determinations. The labels e,f,g,h and i,j,k,l highlight the fact that an unpaired *Student's t*-test, two-tailed *p* value indicated a significant difference between young and old rat liver mitochondria and between young and old rat synaptic mitochondria, respectively. *p* < 0.05 was considered significant.

Table 6.2.3

	I	V	III	IV
Young rat heart mitochondria	0.82 ± 0.12	1.27 ± 0.09	1.05 ± 0.08	1.00
Old rat heart mitochondria	0.80 ± 0.21	1.27 ± 0.20	0.95 ± 0.14	1.00
Young rat liver mitochondria	1.06 ± 0.20	1.75 ± 0.11 m	0.98 ± 0.11	1.00
Old rat liver mitochondria	0.68 ± 0.09	2.31 ± 0.16 p=0.0448 m	0.71 ± 0.12	1.00
Euthyroid rat liver mitochondria	0.85 ± 0.09	1.40 ± 0.04	0.93 ± 0.03	1.00
Hyperthyroid rat liver mitochondria	0.92 ± 0.12	1.42 ± 0.04	0.95 ± 0.06	1.00
Young rat synaptic mitochondria	1.05 ± 0.26	1.21 ± 0.21	0.99 ± 0.12	1.00
Old rat synaptic mitochondria	1.13 ± 0.18	1.34 ± 0.19	1.06 ± 0.13	1.00

Table 6.2.3. Comparison of the absorbances of complexes I, V and III normalised to the absorbance of complex IV in mitochondria isolated from rat heart, rat liver, rat synaptosomes and in rat liver mitochondria isolated from euthyroid and hyperthyroid rats. The membrane protein complexes I, III, IV and IV were separated using blue native PAGE in the first dimension and identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second dimension. The second dimension gel, which was fixed, stained using Serva blue G and destained as described in Materials and Methods, was used for densitometric analysis. The subunits used for densitometric analysis are indicated by arrows on the relevant gels (figs. 6.2.1, 6.2.5, 6.2.9, 6.2.13). All of the data represent the mean ± S.E.M. of three determinations. The label m highlights the fact that an unpaired *Student's t*-test, two-tailed *p* value indicated a significant difference between young and old rat liver mitochondria. *p* < 0.05 was considered significant.

6.3 Discussion

6.3.1 Young and old rat heart mitochondria

Mitochondrial proteins from young (3 month) and old (24 month) rat hearts were resolved in two dimensions using blue native PAGE in the first dimension followed by tricine-SDS-PAGE in the second dimension. After Serva blue G staining, the pattern of the protein subunits of the complexes was found to be the same in rat heart mitochondria isolated from young (3 month) and old (24 month) rats when resolved on a second-dimension tricine gel (fig 6.2.1 A and B). Four complexes could be identified on the second-dimension gel after Serva blue G staining, namely complexes I, V, III and IV. Complex II could not be detected using a Coomassie stain. The global polypeptide pattern of proteins looks the same, however, some of these proteins look to be present in lesser amounts in old when compared with young mitochondria. The significance of the apparent difference is not known as the identity of these proteins is not known.

The second-dimension tricine gels were used for densitometric analysis. The absorbance of selected protein subunits of the complexes was determined in the young and old rat heart mitochondria. When the absorbances were normalised to protein loaded on the gel (fig 6.2.2 and Table 6.2.1), all of the protein subunits of the complexes were decreased in old rat heart mitochondria when compared with young, however, the differences were not significant. When the absorbances of the selected protein subunits of the complexes were normalised to mitochondrial citrate synthase activity (fig 6.2.3 and table 6.2.2), all of the complex amounts were decreased in old rat heart mitochondria when compared with young, however, the differences were not significant. When the absorbances of the selected protein subunits of the complexes were normalised to the in gel absorbance of complex IV (fig 6.2.4 and table 6.2.3), all of the protein subunits of the complexes were decreased in old rat heart mitochondria when compared with young, however, the differences were not significant.

There have been many reports of age-related changes in rat heart mitochondria. A significant decrease in the activity of complex I has been shown in rat heart mitochondria

isolated from 24-month-old rats (Genova *et al.*, 1995). The state 3 rate in rat heart mitochondria has been shown to decrease with age (Capozza *et al.*, 1994, Guerrieri *et al.*, 1996). The rate of ATP synthesis was found to be decreased in mitochondria isolated from old (24 month) rat heart mitochondria compared with ATP synthesis in mitochondria isolated from young rat heart mitochondria (Guerrieri *et al.*, 1996). Paradies *et al.* (1997) showed a decrease in the activity of complex IV in heart mitochondria isolated from 26-month-old rats compared with rat heart mitochondria isolated from 5-month-old rats. Immunoblot analysis using an antibody to the $F_1\beta$ part of the ATP synthase showed a decrease in the amount of $F_1\beta$ in mitochondria isolated from 24-month-old rat heart compared with that from 3-month-old rats (Guerrieri *et al.*, 1992 a). Hudson *et al.* (1998) showed a 30% decrease in complex IV activity in old (24 month) rat heart mitochondria compared with young (6 month), with no change in citrate synthase activity. Hudson *et al.* (1998) also showed that there was a 35% decrease in the rate of mitochondrial protein synthesis, which corresponded to a 30-50% reduction in the levels of most mitochondrially encoded proteins and, by Western blot, a 23% decrease in COX I protein. No change was detected by Western blot in the levels of COX IV subunit with age in old rat heart mitochondria. Hudson *et al.* (1998) reasoned that since the mitochondrially encoded subunits of complex IV make up the catalytic core of the enzyme, the decrease in the mitochondrial protein synthesis is likely to contribute to the decline in complex IV activity.

The results of Guerrieri *et al.* (1992) and Hudson *et al.* (1998) are in contrast with the results presented for the young and old rat heart mitochondria in this thesis. Guerrieri *et al.* (1996) found a decrease in the $F_1\beta$ subunit of complex V whereas no difference was found in the level of complex V by comparison of the levels of $F_1\alpha$ with age in the results presented here. Hudson *et al.* (1998) found a decrease in the level of COX I protein by Western blot and no difference in the level of complex IV by Western blot with age in old rat heart mitochondria. The difference in the level of COX I with age and the lack of difference in the level of COX IV is in contrast to other reports that found, using two-dimensional electrophoresis, that when a complex is decreased in amount, all of the protein subunits of the complexes show a decrease in a uniform fashion (Reinheckel *et*

al., 1995, Bentlage *et al.*, 1995). Furthermore, Paradies *et al.* (1997) showed that the activity of complex IV decreased in rat heart mitochondria isolated from old (26 month) rat heart mitochondria compared with young (5 month) rat heart mitochondria. Paradies *et al.* (1997) found that by adding exogenous cardiolipin to old (26 month) rat heart mitochondria using liposomes, the activity of complex IV was restored to the levels observed in young rat heart mitochondria (5 month). Therefore, any differences in the activity of complex IV in rat heart mitochondria with age may not be due to a decrease in the amount of complex IV in old rat heart mitochondria.

It should be noted that, in many of the reports of changes in rat heart mitochondria with age, the mitochondria may have been isolated using the protease nagarse. Nagarse, as has been shown in this chapter 5 has a deleterious effect on mitochondrial proteins when used to isolate heart mitochondria and may affect results in a comparison study of young and old rat heart mitochondria. Nagarse was not used in the isolation of rat heart mitochondria for the comparison of young and old presented here.

In contrast, there are reports that are in good agreement with the results presented here. Manzelmann and Harmon (1987) examined the heart mitochondria of rats that had shown significant differences with age in their synaptic brain mitochondria. They found that cytochrome content and cytochrome absorbance wavelength maxima do not change with age in heart mitochondria. Also, complex activities, RCR, P:O and H^+/O ratios did not change with increasing age. Castelluccio *et al.* (1994) investigated ubiquinone-9 and cytochrome content in rats of different ages between 2 and 26 months. No major changes in the content of ubiquinone-9 and cytochrome were observed in old rats compared with young, indicating that the relative ratio of the respiratory chain components is unchanged with age. However, the activity of the complexes with age was investigated: there was no significant difference in the activity of complex V. Complexes I, III and IV all showed decreases in activity between 18 and 26 months.

It would seem, based on the results presented here and by comparison with the results of others, that any decreases in the activity of rat heart mitochondrial complexes with age is

not due to a decrease in the amount of the complexes in rat heart mitochondria. When rat heart mitochondrial proteins are isolated from young (3 month) and old (24 month) rats and separated on a blue native gel and further resolved on a tricine gel, there is no difference in the pattern of mitochondrial proteins, and the levels of complexes I, V, III and IV do not change with age.

6.3.2 Young and old rat liver mitochondria

Mitochondrial proteins from young (3 month) and old (24 month) rat livers were resolved in two dimensions using blue native PAGE in the first dimension followed by tricine-SDS-PAGE in the second dimension. After staining with Serva blue G, the pattern of protein subunits of the complexes was found to be the same in rat liver mitochondria isolated from young (3 month) and old (24 month) rats when resolved on a second dimension tricine gel (fig 6.2.5 A and B). Four complexes could be identified on the second-dimension gel after Serva blue G staining *i.e.* complexes I, V, III and IV. Complex II could not be detected using a Serva blue G stain. The pattern of proteins appeared to be the same excluding the protein subunits of the complexes, however, some of these proteins seem to be present in lesser amounts in old when compared with young mitochondria. The significance of the apparent difference is not known as the identity of these proteins is not known.

The second-dimension tricine gels were used for densitometric analysis. The absorbances of selected protein subunits of the complexes were determined in young and old rat liver mitochondria. When the absorbances of the complexes from the young and old rat liver mitochondria were normalised to protein loaded on the gel, it was found that the amounts of complexes I, V, III and IV were significantly less in old mitochondria compared with young (see fig 6.2.6 and table 6.2.1). If the amount of complexes I, V, III and IV is defined as 1:1:1:1 in young rat liver mitochondria, the values for old rat liver mitochondria would be 0.18:0.54:0.19:0.29. Complex I was decreased from 0.39 ± 0.06 in rat liver mitochondria isolated from young rats to 0.07 ± 0.01 in rat liver mitochondria isolated from old rats ($p=0.0063$, $n=3$, unpaired *Student's t*-test). Complex V was decreased from 0.66 ± 0.06 in young rat liver mitochondria to 0.25 ± 0.03 in old rat liver

mitochondria ($p=0.0036$, $n=3$, unpaired *Student's t*-test). Complex III was decreased from 0.36 ± 0.03 in young rat liver mitochondria to 0.07 ± 0.01 in old rat liver mitochondria ($p=0.0008$, $n=3$, unpaired *Student's t*-test). Complex IV was decreased from 0.38 ± 0.05 in young rat liver mitochondria to 0.11 ± 0.01 in old rat liver mitochondria ($p=0.0061$, $n=3$, unpaired *Student's t*-test) in old rats compared with young.

Similarly, when the absorbances of the complexes from the young and old rat liver mitochondria were normalised to the activity of citrate synthase, it was found that the amounts of complexes I, V, III and IV decreased in old rats compared with young (see Fig 6.2.7 and table 6.2.2). If the amount of complexes I, V, III and IV is defined as 1:1:1:1 in young rat liver mitochondria, the values for old rat liver mitochondria would be 0.23:0.47:0.26:0.35. Complex I was decreased from 4.65 ± 0.74 in young rat liver mitochondria to 1.09 ± 0.19 in old rat liver mitochondria ($p=0.0096$, $n=3$, unpaired *Student's t*-test). Complex V was decreased from 7.84 ± 0.79 in young rat liver mitochondria to 3.70 ± 0.45 in old rat liver mitochondria ($p=0.0104$, $n=3$, unpaired *Student's t*-test). Complex III was decreased from 4.35 ± 0.44 in young rat liver mitochondria to 1.13 ± 0.21 in old rat liver mitochondria ($p=0.0027$, $n=3$, unpaired *Student's t*-test). Complex IV was decreased from 4.55 ± 0.65 in young rat liver mitochondria to 1.59 ± 0.11 in old rat liver mitochondria ($p=0.0109$, $n=3$, unpaired *Student's t*-test). The reduction in the amounts of complexes I, V, III and IV in old rat liver compared with young rat liver shown by normalisation to citrate synthase is in good agreement with the reduction seen when the data are normalised to protein loaded on the gel.

In contrast to the decreases observed when the absorbances are normalised to protein loaded on the gel and citrate synthase activity, when the absorbances are normalised to the in-gel complex IV absorbance, the results are different. The decrease in the amount of complex I in old rat liver mitochondria is not significant compared with the amount of complex I in young rat liver mitochondria when the absorbance is normalised to the

amount of in-gel complex IV. The decrease in the amount of complex III in old rat liver mitochondria is not significant compared with the amount of complex III in young rat liver mitochondria when the absorbance is normalised to the amount of in-gel complex IV. The amount of complex V apparently increases from 1.75 ± 0.11 in young rat liver mitochondria to 2.31 ± 0.16 in old rat liver mitochondria ($p=0.048$, $n=3$, unpaired *Student's t*-test) when the absorbances are normalised to the amount of in-gel complex IV. The apparent increase in complex V in old rat liver mitochondria compared to young rat liver mitochondria is in direct contrast to the decrease seen in old rat liver compared with young rat liver when the absorbance is normalised to protein loaded or citrate synthase activity. When the amount of complex IV is normalised to the in-gel complex V, it can be seen that complex IV actually decreases in old rat liver mitochondria compared with young. The amount of complex IV decreases from 0.58 ± 0.03 in young rat liver mitochondria to 0.44 ± 0.03 ($p=0.0299$, $n=3$, unpaired *Student's t*-test) when the absorbance of complex IV is normalised to the in-gel absorbance of complex V. The decrease in complex IV is the reason why there is an apparent increase in complex V in old rat liver mitochondria compared with young.

The above results suggest that the absolute amounts of complexes I, V, III and IV are decreased in old rat liver mitochondria compared with young rat liver mitochondria. However, relative to each other, the amounts of complexes I, III and V remain the same in old rat liver mitochondria compared with young, only the relative amount of complex IV changes with age.

There have been many reports of age-related changes in rat liver mitochondria. The activity of complex I has been reported to decrease with age in mitochondria isolated from rat liver (Torres-Mendoza *et al.* 1999). Genova *et al.* (1995) also showed that there was a significant decrease in the activity of complex I in liver mitochondria isolated from old rats (24 month). Ventura *et al.* (2002) compared the activity of complex I in old (30 month) rat liver mitochondria with the activity of complex I in young (4 month) rat liver mitochondria and found that the activity complex I decreased 25% in old rat liver

mitochondria. Ventura *et al.* (2002) also found that the flux control coefficients of complex I increased with age, meaning that complex I becomes more rate-limiting with age. They also found that state 3 and state 4 respiration decreased significantly with age. The state 3 rate in rat liver mitochondria has been shown to decrease with age (Capozza *et al.*, 1994; Guerrieri *et al.*, 1996; Ventura *et al.*, 2002) and the state 4 rate has also been reported to decrease with age in rat liver mitochondria (Ventura *et al.*, 2002). The rate of ATP synthesis was found to be decreased in mitochondria isolated from old (24 month) rat liver compared with the rate of ATP synthesis in mitochondria isolated from young rat liver (Guerrieri *et al.*, 1996).

The decline in rat liver mitochondrial function with age from the various reports above could be due to mtDNA damage. Hudson *et al.*, (1998) showed there was an age-associated change in the level of damage to mitochondrial DNA in liver mitochondria. They measured the level of 8-oxo-2-deoxyguanosine in liver mitochondrial DNA and found it increased 2.5-fold in old rats (23 months) compared with young rats (6 months). 8-oxo-2-deoxyguanosine is a lesion that is mutagenic and has been shown to increase in cancer and aging.

The age-related decline in rat liver mitochondrial activity (complex I activity, state 3 and state 4 rates) could be due to a decrease in the amount of mitochondrial complexes with age, which may, in turn, be due to damage to mtDNA. Guerrieri *et al.* (1992) showed a decrease in the amount of $F_1\beta$ of the ATP synthase in mitochondria isolated from 24-month-old rat liver compared with that from 3-month-old rats using a Western blot. The report of a decrease in the amount of the $F_1\beta$ of the ATP synthase in old rat liver mitochondria is in good agreement with the results presented here. When the absorbances of the selected protein subunits of the complexes of the young and old rat liver mitochondria were normalised to protein loaded on the gel and normalised to citrate synthase activity, there were significant decreases in the levels of complexes I, V, III and IV in old rats. The decrease in the absorbances of the selected protein subunits of the complexes with age normalised to protein loaded on the gel and normalised to citrate synthase activity seem to suggest that the amounts of complexes I, V, III and IV decrease

with age in rat liver mitochondria. When the absorbances of the selected protein subunits of the complexes were normalised to the in gel complex IV absorbance, the only significant difference was in the level of complex V, which seemed to increase with age. When the absorbance of complex IV was normalised to complex V, it was found that complex IV actually decreased with age, meaning that the relative ratios of complexes I, III and V do not change with age in rat liver mitochondria, but the relative ratio of complex IV does.

6.3.3 Mitochondria isolated from euthyroid and hyperthyroid rat liver

Mitochondrial proteins from euthyroid rat livers and hyperthyroid rat livers were resolved in two dimensions using blue native PAGE in the first dimension followed by tricine-SDS-PAGE in the second dimension. After Serva blue G staining, the pattern of complex subunits was found to be the same in rat liver mitochondria isolated from euthyroid and hyperthyroid rats when resolved on a second-dimension tricine gel (fig 6.2.9 A and B). Four complexes could be identified on the second-dimension gel after Serva blue G staining *i.e.* complexes I, V, III and IV. Complex II could not be detected using a Coomassie stain. The pattern of proteins looks the same excluding protein subunits of the complexes in euthyroid and hyperthyroid rat liver mitochondria.

The second-dimension tricine gels were used for densitometric analysis. The absorbances of selected protein subunits of the complexes were determined in euthyroid and hyperthyroid rat liver mitochondria. When the absorbances were normalised to protein loaded on the gel (fig 6.2.10 and table 6.2.1), there were no significant differences in the levels of complexes I, V, III and IV in hyperthyroid rat liver mitochondria compared with euthyroid rat liver mitochondria. When the absorbances were normalised to citrate synthase activity (fig 6.2.11 and table 6.2.2), there were no significant differences in the levels of complexes I, III and V in hyperthyroid rat liver mitochondria compared with euthyroid rat liver mitochondria. When the absorbances of the selected protein subunits of the complexes were normalised to the in-gel absorbance of complex IV (fig 6.2.12 and table 6.2.3), there were no significant differences in the levels of

complexes I, V, III and IV in hyperthyroid rat liver mitochondria compared with euthyroid rat liver mitochondria.

There are a number of reports of the effects on different thyroid status on mitochondria. It has been reported that rats made hyperthyroid have increased liver mitochondrial proton leak rates at any given membrane potential when compared to sham-treated controls, but have decreased proton leak compare to euthyroid controls (Harper & Brand, 1993). It has also been shown that hyperthyroid rat liver mitochondria have greater state 3 rates than euthyroid rat liver mitochondria. Cote and Boulet (1985) found that there was an increase in the rate of mitochondrial protein synthesis in heart mitochondria isolated from hyperthyroid rats. Guerrieri *et al.* (1997) found that the decrease observed in catalytic activity of the ATP synthase in rat liver mitochondria isolated from hypothyroid rats was accompanied by a decrease in the amount of subunits $F_1\beta$, F_o OSCP of the ATP synthase.

Based on the above reports, one might expect to see an increase in the level of complexes I, V, III and IV in rat liver mitochondria isolated from hyperthyroid rats compared with rat liver mitochondria isolated from euthyroid rats. However, the results presented here show no significant difference in the levels of complexes I, V, III and IV in hyperthyroid and euthyroid rat liver mitochondria when the levels are normalised to protein loaded on the gel, citrate synthase activity and in-gel complex IV.

Cardiolipin may be involved in the different rates of respiration observed in rat mitochondria of different status. Cardiolipin is a major component of the mitochondrial inner membrane and plays an important role in the function of cytochrome oxidase and the adenine nucleotide carrier (Hoch, 1992; Paradies *et al.*, 1998). Paradies *et al.* (1997) showed that cardiolipin content decreased with age in rat heart mitochondria, as did the activity of complex IV. By adding cardiolipin back to the old rat heart mitochondria using liposomes, the old rat heart complex IV activity was returned to the level observed in the young rat heart mitochondria. Cardiolipin synthase has been shown to be regulated

by the action of thyroid hormones (Hostetler, 1991). Perhaps this regulation is responsible for the increased rates of respiration observed in hyperthyroid mitochondria.

6.3.4 Young and old rat synaptic mitochondria

Mitochondrial proteins from young (3 month) and old (24 month) rat synaptosomes were resolved in two dimensions using blue native PAGE in the first dimension followed by tricine-SDS-PAGE in the second dimension. After Serva blue G staining, the pattern of complex subunits was found to be the same in rat synaptic mitochondria isolated from young and old rats when resolved on a second dimension tricine gel (fig 6.2.9 A and B). Four complexes could be identified on the second dimension gel after Serva blue G staining namely complexes I, V, III and IV. Complex II could not be detected using a Coomassie stain. The pattern of proteins looks the same excluding protein subunits of the complexes in young and old rat synaptic mitochondria.

The second-dimension tricine gels were used for densitometric analysis. The absorbance of selected complex subunits was determined in the young and old rat synaptic mitochondria. When the absorbances were normalised to protein loaded on the gel (fig 6.2.14 and table 6.2.1) there were no significant differences in the amounts of complexes I, V, III and IV in old rat synaptic mitochondria compared with young rat synaptic mitochondria. However, when the absorbances of the complexes were normalised to citrate synthase activity, the amounts of complexes I, V, III and IV were increased in old rat synaptic mitochondria compared with young rat synaptic mitochondria (see fig 6.2.15 and table 6.2.2). If the amount of complexes I, V, III and IV is defined as 1:1:1:1 in young rat synaptic mitochondria, the values for old rat synaptic mitochondria would be 2.68:2.63:2.62:2.40. Complex I was increased from 0.25 ± 0.04 in young rat synaptic mitochondria to 0.67 ± 0.12 old rat synaptic mitochondria ($p=0.0294$, $n=3$, unpaired Student's *t*-test). Complex V was increased from 0.30 ± 0.03 in young rat synaptic mitochondria to 0.79 ± 0.12 in old rat synaptic mitochondria ($p=0.0167$, $n=3$, unpaired Student's *t*-test). Complex III was increased from 0.24 ± 0.01 in young rat synaptic mitochondria to 0.63 ± 0.10 in old rat synaptic mitochondria ($p=0.0178$, $n=3$, unpaired

Student's t-test). Complex IV was increased from 0.25 ± 0.02 in young rat synaptic mitochondria to 0.60 ± 0.07 in old rat synaptic mitochondria ($p=0.0086$, $n=3$, unpaired *Student's t-test*) in old rats compared with young. When the absorbances of the selected protein subunits of the complexes was normalised to the in gel absorbance of complex IV (fig 6.2.16 and table 6.2.3), there were no significant differences in the levels of complexes I, III, IV and V in old rat synaptic mitochondria compared with young rat synaptic mitochondria.

There have been a number of reports of age-associated changes in rat brain mitochondria. The 4834 bp deletion in rat mtDNA has been shown to increase in rat brain with age (Gadaleta *et al.*, 1999). Mitochondrial protein synthesis was shown to be reduced in aged rat brain (Gadaleta *et al.*, 1994). Fernandez-Silva (1991) showed that the transcription of mitochondrial DNA decreased in rat brain mitochondria with age. The levels of the complex V A6 and A8 mRNA subunits were 20% that of young rats. Mitochondrial protein synthesis has been reported to decrease in rat brain synaptosomes isolated from 27-month-old rats (Gadaleta *et al.*, 1994).

The report by Nicoletti *et al.* (1995) is in good agreement with the results normalised to citrate synthase activity presented here. Nicoletti *et al.* (1995) studied at the levels of the subunits I, II, III and IV of complex IV by Western blot analysis in mitochondria isolated from rat cerebral cortex. All of the complex IV subunits were increased in amount in old (24 month) rats compared with young (2 month) rats. The α , β and γ (all part of F_0) subunits of complex V were also analysed by Western blot and the complex V subunits also increased in amount in old (24 month) rats compared with young (2 month) rats. The synaptic mitochondria analyzed in the study presented in this thesis were isolated from synaptosomes using hypotonic lysis. As the mitochondria isolated from the synaptosomes were not further purified using a percoll gradient, there were probably some synaptic membrane proteins associated with the synaptic mitochondria. Normalising the absorbances of the complex subunits to citrate synthase activity means that the young and old comparison was corrected for differences in the isolation young and old mitochondria. The fact that there was no significant difference in the

absorbances of the selected protein subunits of the complexes when normalised to the in gel complex IV absorbance means that the relative amount of the complexes does not change with age in young and old rat synaptic mitochondria.

6.4 Conclusions

This is the first time blue native/tricine-SDS-PAGE two-dimensional electrophoresis was used in an aging comparison or for a comparison of mitochondria isolated from euthyroid and hyperthyroid animals. No significant difference was found in the levels of complexes I, III, IV and V in a comparison of young and old rat heart mitochondria or in a comparison of euthyroid and hyperthyroid rat liver mitochondria. In the age comparison, significant differences were found in a comparison of young rat liver mitochondria with old rat liver mitochondria. The levels of complexes I, V, III and IV, when normalised per mg protein and when normalised to citrate synthase activity, were shown to be significantly less in liver mitochondria isolated from old rats compared with liver mitochondria isolated from young rats. However, only complex IV showed a significant decrease when normalised to the in gel complex V in liver mitochondria isolated from old rats compared with liver mitochondria isolated from young rats. The results found in the rat liver mitochondria comparison were surprising given that liver is a mitotic tissue and aging effects are hypothesized to be manifest greater in post mitotic tissues. The levels of complexes I, III, IV and V were shown to have increased in synaptic mitochondria isolated from old rats compared with synaptic mitochondria isolated from young rats when the absorbances were normalised to citrate synthase activity. When the levels of complexes I, III, V were normalised to the in gel complex IV, there were no significant differences in mitochondria isolated from young and old rat brain synaptosomes. The results obtained for synaptic mitochondria were novel because this was the first time proteins from synaptic mitochondria isolated from whole brain were resolved using blue native/tricine-SDS-PAGE two-dimensional electrophoresis. Previously, Schagger and Ohm (1995) used blue native/tricine-SDS-PAGE two-dimensional electrophoresis to compare hippocampal mitochondrial proteins of control and Alzheimer's brain, that is to say, there was no distinction between synaptic and non-synaptic mitochondria.

Chapter 7

Comparison of the relative amounts of complexes I, III, IV and V in mitochondria isolated from rat heart, rat kidney, rat liver, rat brown adipose tissue, rat skeletal muscle, rat synaptosomes and beef heart.

7.1 Introduction

The purpose of this work was to isolate mitochondria from rat heart, rat kidney, rat liver, rat brown adipose tissue, rat skeletal muscle, rat brain synaptosomes and beef heart, to use blue native/tricine-SDS-PAGE two-dimensional electrophoresis to resolve the mitochondrial proteins, to determine if there were any differences in the polypeptide pattern in the second dimension and in the levels of complexes I, V, III and IV. The mitochondrial protein complexes were solubilized using dodecyl maltoside and separated in the first dimension using blue native PAGE. The in-gel native proteins were denatured and the complex subunits were further resolved in the second dimension using tricine-SDS-PAGE. All of the mitochondrial protein samples were solubilized using a dodecyl maltoside:protein ratio of between 3:1 and 1:1, as recommended by Schägger (1994). The second-dimension tricine gels were stained using Serva blue G and were used for densitometric analysis. The staining intensity or absorbances of selected subunits of the complexes were measured and the complex subunit absorbances were taken as a relative measure of the amount of complex in that tissue. Reports found that where there was a decline in the level of a complex, the change was reflected in a uniform decline in all of the subunits of that complex (Reinheckel *et al.*, 1995; Bentlage *et al.*, 1995). The subunits of the complex whose absorbances were measured were: the 42-kDa subunit of complex I, the 55-kDa α -subunit of complex V, the 47-kDa core protein II of complex III and the 26-kDa subunit 2 of complex IV. The subunits of the complexes used in the comparison were identified according to the work of Bentlage *et al.* (1995) and Schägger and Pfeiffer (2001). However, N-terminal sequencing would be necessary to verify the identity of these proteins. The amount of complex II was not measured because either there were too many unidentified proteins in the same region of the gel or complex II could not be detected using a Coomassie stain. The individual subunits of the complexes were quantified after a second-dimension tricine gel rather than quantifying the complexes on the first-dimension blue native gel, to avoid including overlaps with

contaminating proteins (Schägger & Ohm, 1995). For example, in rat heart mitochondrial proteins on the second-dimension tricine-SDS gel (fig 7.2.1 A and B), it can be seen that complex I is contaminated by complex V.

The electrophoretic technique was optimized using beef heart mitochondria. Using beef heart mitochondria, it was found that the Coomassie staining intensity of the subunits of the complexes correlated linearly with the protein load over a broader range than that used for analysis of rat mitochondria (see chapter 5, figs 5.2.24 to 5.2.27).

Three gels were used for each mitochondrial analysis. The absorbances of the selected subunits of the complexes were measured and the absorbances were presented in three ways. Firstly, the average absorbances of the complexes subunits were normalised per mg (milligram) of protein loaded onto the first dimension gel.

Secondly, the average absorbance of the subunits of the complexes were normalised per μmol of mitochondrial citrate synthase activity. Citrate synthase activity has been widely used as a marker of mitochondrial content, especially in comparisons of mitochondrial aging since citrate synthase activity (Hansford and Castro, 1982) and the message level of citrate synthase do not change with age (Marin-Garcia *et al.*, 1997).

Finally, the average absorbance of the selected subunits of complexes I, V and III were normalised relative to the in gel complex IV subunit on a per gel basis. The absorbance of the complex IV subunit on the gel was set to 1 and the other subunits of the complexes were expressed relative to this. The absorbances of the complex subunits relative to the in gel complex IV subunit from the three gels were then pooled. Using the complex IV subunit as an internal standard means that the analysis becomes independent of the absolute amount of mitochondria (Bentlage *et al.*, 1995). Using complex IV as an internal standard also means that comparing staining ratios of complexes within individual gels eliminates experimental deviations that result from variations in mitochondrial content and in protein load (Schägger and Ohm, 1995). Complex IV was

chosen as the internal standard because it is used as a marker for mitochondria activity and content (Hudson *et al.*, 1998).

It was interesting to note that different amounts of dodecyl maltoside were required to solubilize different mitochondria. Mitochondrial membrane proteins were solubilized with dodecyl maltoside by a modification of a method by Schägger *et al.* (1994). The fact that different amounts of dodecyl maltoside were required to solubilize the various mitochondria could be due to different lipid compositions of the mitochondrial inner membranes. Indeed, Rolfe *et al.* (1994) examined the lipid:protein ratio (mg/mg) of mitochondria isolated from rat liver, kidney, brain and skeletal muscle. The lipid:protein ratios of liver and kidney mitochondria were quite similar, being 0.19 and 0.21, respectively. A dodecyl maltoside:protein ratio of 1:1 was required to solubilize both rat liver and kidney mitochondrial proteins. The lipid:protein (mg/mg) ratio of brain mitochondria was 0.35 and a dodecyl maltoside:protein ratio of 2:1 was required to solubilize rat synaptic mitochondrial proteins. The lipid:protein (mg/mg) ratio of skeletal muscle mitochondria was 0.46 and a dodecyl maltoside:protein (mg/mg) ratio of 3:1 was required to solubilize rat skeletal muscle mitochondrial proteins. The results listed here seem to suggest that the higher the lipid:protein ratio of mitochondria, the more dodecyl maltoside is required to solubilize mitochondrial membrane proteins.

The pattern of the selected protein subunits of the complexes was the same for all of the mitochondria examined. However, excluding the subunits of the complexes, the remaining polypeptide pattern varied considerably between the various mitochondria when the mitochondrial proteins were resolved on a second-dimension tricine gel. The significance of these differences is not known as the identity of the proteins outside of those of the complex is not known at this time.

Occasionally, in some of the mitochondria examined, a second band with the same subunit composition as complex IV was detected close to complex III. According to Schägger and Von Jagow (1991), the second band with the same subunit composition as complex IV might represent the dimeric form of complex IV. As complex III is reported to have a molecular mass of ~500 kDa and the monomeric form of complex IV a

molecular mass of ~200 kDa (Schägger and Von Jagow, 1991), a dimeric complex IV could have a molecular mass of ~400 kDa, which would place it close to complex III on the second-dimension tricine gel. However, the second band with the same subunit composition as complex IV was not considered in the analysis of the mitochondria for two reasons. The second band did not occur in all of the mitochondria examined and further investigation would be required to verify that the second band was in fact a complex IV dimer (e.g. Western blotting).

7.2 Results

Figure 7.2.1 shows the resolution in the second dimension of rat heart mitochondrial proteins. The gel was stained using Serva blue G. First-dimension gel lanes loaded with 227µg of protein had the in-gel proteins denatured using 20mM DDT/1%SDS and the denatured proteins were resolved on a second-dimension denatured 10%-tricine-SDS gel. Complexes I, V, III and IV were identified on the basis of their characteristic polypeptide patterns in the second dimension. Complex I was quite diffuse and complex II could not be identified in rat heart mitochondria because of a large number of unidentified proteins in the same region of the gel. Arrows on the gel mark the protein subunits whose staining intensities were measured for the quantification of second-dimension gels. The gel shown in fig 7.2.1 is representative of three gels that were used for the analysis of rat heart mitochondria.

Figure 7.2.2, shows the absorbance normalised per mg of protein loaded on the first-dimension gel of complexes I, V, III and IV in mitochondria isolated from rat heart. The absorbances are also listed in Table 7.2.1a. The absorbances of complexes I, V, III and IV were 0.52 ± 0.06 , 0.81 ± 0.03 , 0.68 ± 0.03 and 0.64 ± 0.02 , respectively, when normalised per mg of protein. When the data shown in figure 7.2.2 are normalised to IV=1, the I:V:III:IV ratio is 0.81:1.26:1.06:1.00 (Table 7.2.1b).

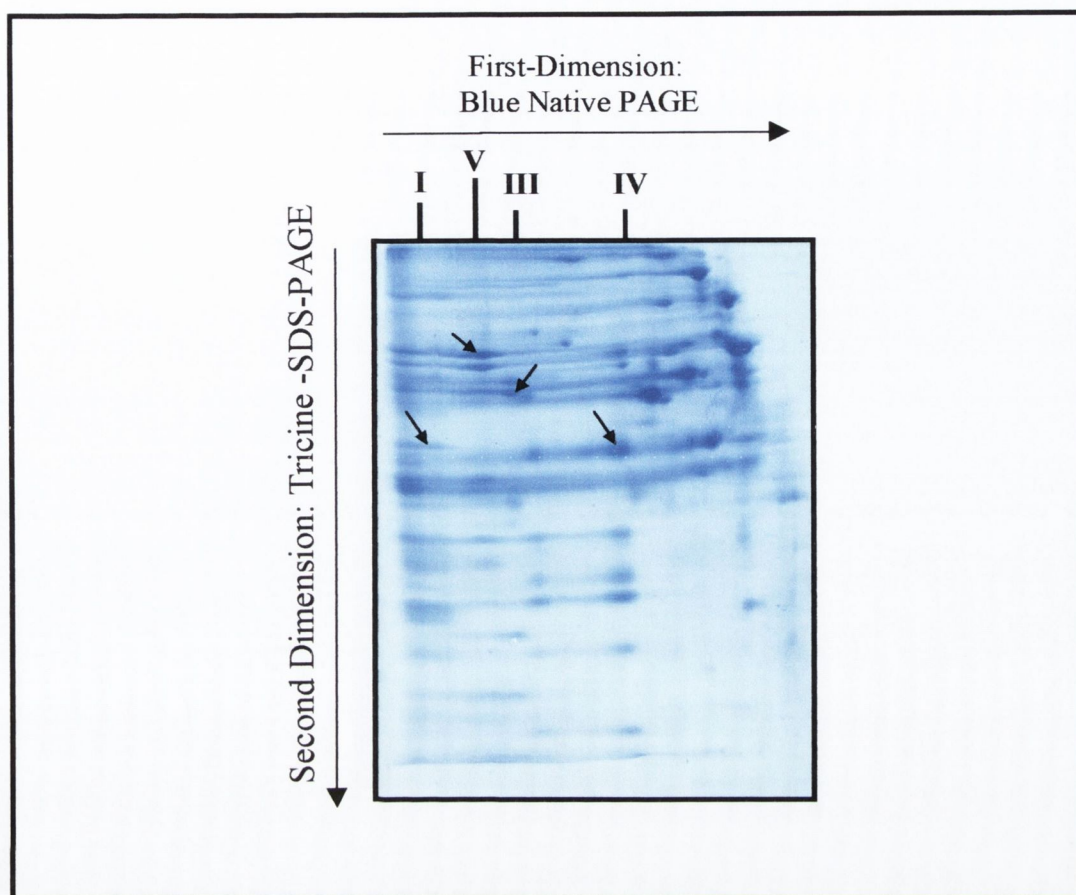


Figure 7.2.1. Two-dimensional resolution of mitochondrial proteins from rat heart mitochondria using blue native PAGE in the first dimension and denaturing tricine-SDS-PAGE in the second dimension. The membrane protein complexes were separated in the native first dimension and identified by their characteristic polypeptide patterns in the denaturing second dimension. The gel was fixed, stained using Serva blue G and destained as described in Materials and Methods. The positions of the complexes I, V, III and IV are indicated at the top of the gel. Arrows on the gel mark the protein subunits whose staining intensity or absorbance was measured for the quantification of second-dimension gels. The complex subunits whose staining intensity was measured were as follows: 42kDa subunit of complex I, 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The rat heart mitochondrial proteins were solubilized using a 2:1 ratio of dodecyl maltoside to protein and were resolved using blue native PAGE in the first-dimension (1mm gel, 5.5-13% non-linear gradient gel). A first-dimension lane loaded with 25 μ l (227 μ g protein) of solubilized rat heart mitochondrial protein was cut from the first-dimension gel and the in-gel native proteins were denatured using 20mM DDT/1%SDS (see Materials and Methods). The mitochondrial proteins were resolved using a uniform 1mm 10% tricine-SDS gel in the second-dimension. The gel shown is representative of three gels that were used for the quantification of heart mitochondria.

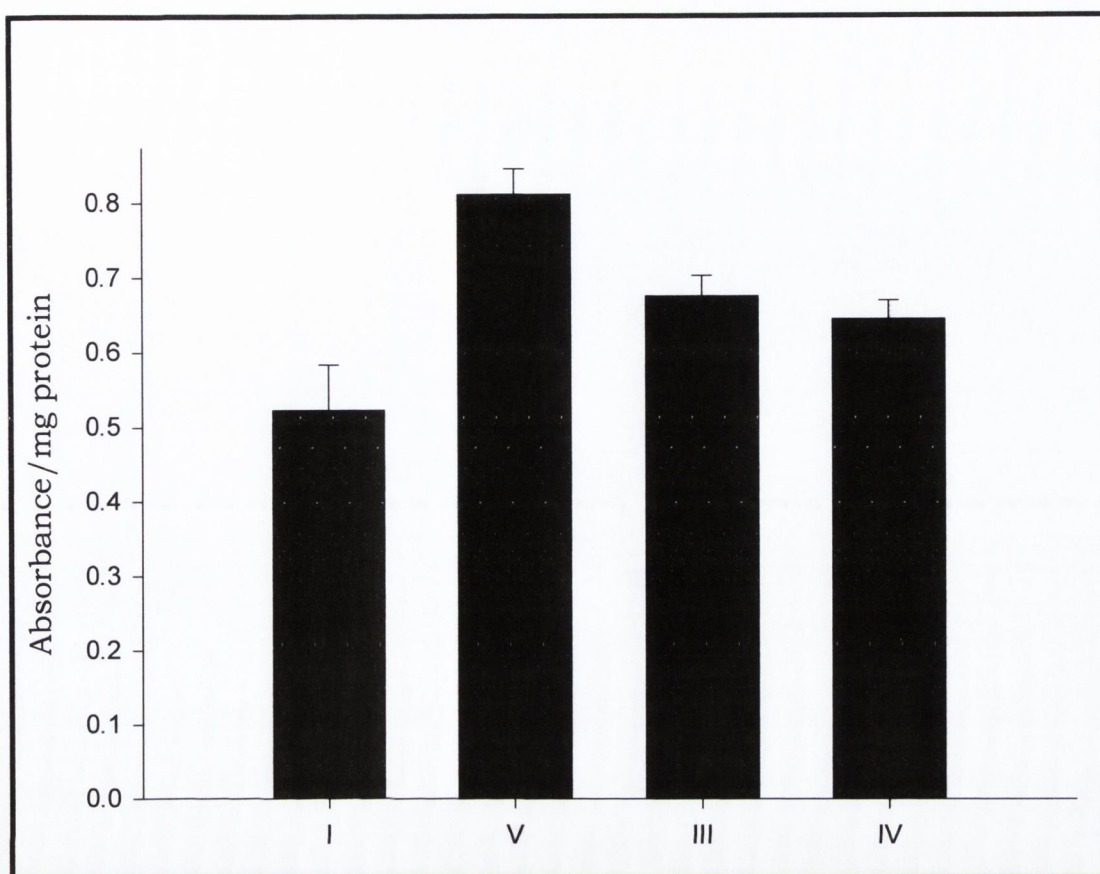


Figure 7.2.2. Comparison of the absorbances of complexes I, V, III and IV normalised per mg protein in mitochondria isolated from rat heart. All mitochondrial proteins were solubilized using dodecyl maltoside. The membrane protein complexes I, V, III and IV were separated using blue native PAGE in the first dimension and identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second-dimension. The second-dimension gel, which was fixed, stained using Serva blue G and destained as described in Materials and Methods, was used for densitometric analysis. The subunits used for densitometric analysis are indicated by arrows on the gel shown in fig. 7.2.1. The subunits of the complexes whose absorbances were measured were as follows: 42kDa subunit of complex I, 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The graph labels are as follows: I: complex I, V: complex V, III: complex III, IV: complex IV. 227 μ g rat heart mitochondria was loaded on the first-dimension gel. All of the data represent the mean $\pm$ SEM of three determinations. The data are also represented in Table 7.2.1a and 7.2.1b.

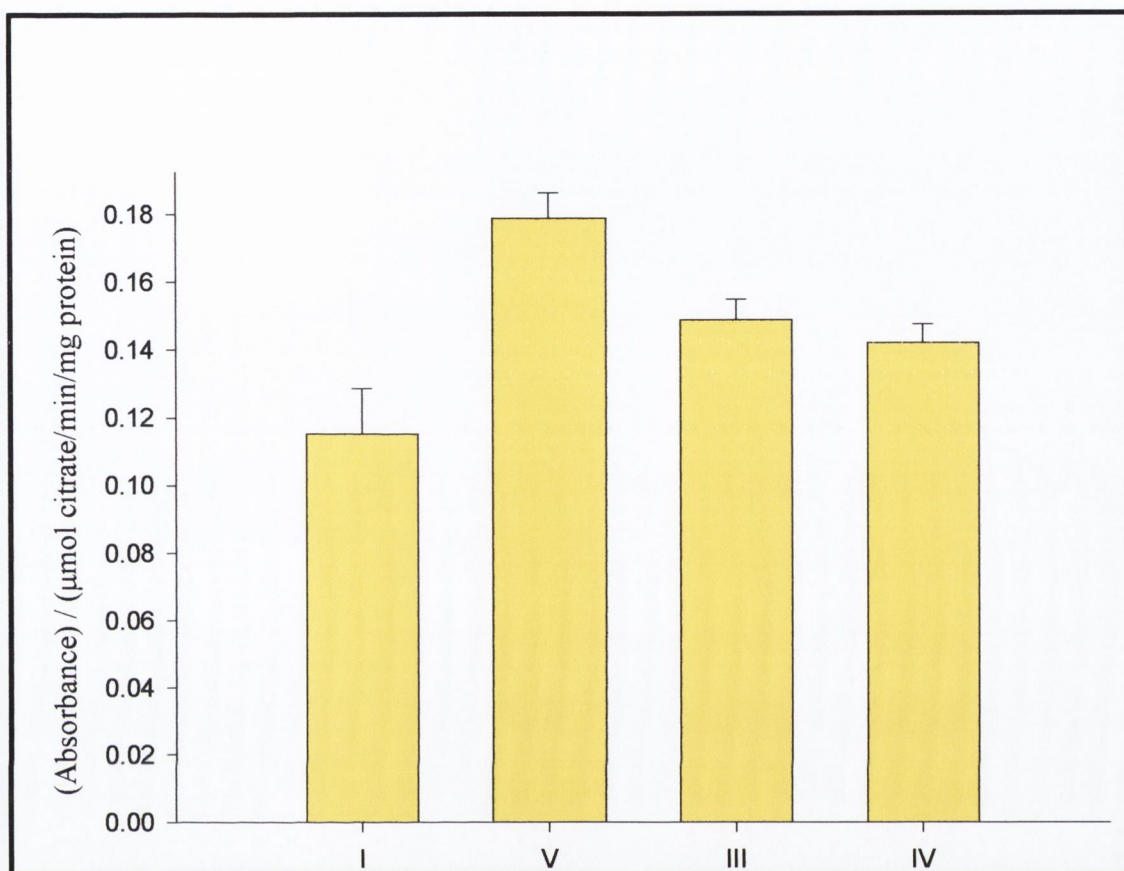


Figure 7.2.3. Comparison of the absorbances of complexes I, V, III and IV per μmol citrate synthase activity in mitochondria isolated from rat heart. All mitochondrial proteins were solubilized using dodecyl maltoside. The membrane protein complexes I, V, III and IV were separated using blue native PAGE in the first-dimension and identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second dimension. The second-dimension gel, which was fixed, stained using Serva blue G and destained as described in Materials and Methods, was used for densitometric analysis. The subunits used for densitometric analysis are indicated by arrows on the gel shown in fig. 7.2.1. The subunits whose absorbances was measured were as follows: 42kDa subunit of complex I, 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The graph labels are as follows: I: complex I, V: complex V, III: complex III, IV: complex IV. 227 μg rat heart mitochondria was loaded on the first-dimension gel. All of the data represent the mean $\pm$ SEM of three determinations. The data are also represented in Table 7.2.2a and 7.2.2b.

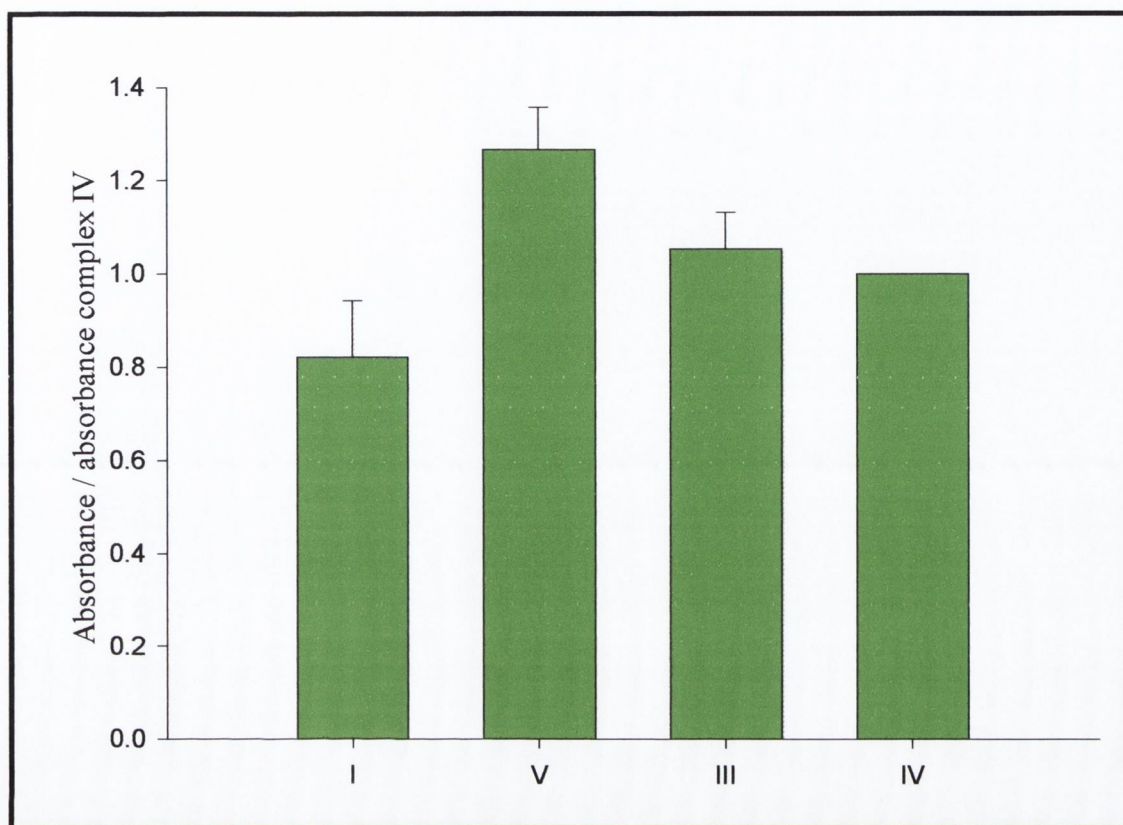


Figure 7.2.4. Comparison of the absorbances of complexes I, V, III normalised to the absorbance of complex IV in mitochondria isolated from rat heart. All mitochondrial proteins were solubilized using dodecyl maltoside. The membrane protein complexes I, V, III and IV were separated using blue native PAGE in the first dimension and identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second-dimension. The second-dimension gel, which was fixed, stained using Serva blue G and destained as described in Materials and Methods, was used for densitometric analysis. The subunits used for densitometric analysis are indicated by arrows on the gel shown in fig. 7.2.1. The complex subunits whose absorbance was measured were as follows: 42kDa subunit of complex I, 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The graph labels are as follows: I: complex I, V: complex V, III: complex III, IV: complex IV. 227 μ g rat heart mitochondria was loaded on the first-dimension gel. All of the data represent the mean $\pm$ SEM of three determinations. The data are also represented in Table 7.2.3.

Figure 7.2.3 shows the absorbance normalised per μmol of citrate synthase activity of complexes I, V, III and IV in mitochondria isolated from rat heart. The absorbances are also listed in Table 7.2.2a. The absorbances of complexes I, V, III and IV were 0.12 ± 0.01 , 0.18 ± 0.01 , 0.16 ± 0.01 and 0.14 ± 0.01 , respectively, when normalised to citrate synthase activity. When the data shown in figure 7.2.3 were normalised to IV=1, the I:V:III:IV ratio was found to be 0.86:1.29:1.14:1.00 (Table 7.2.2b).

Figure 7.2.4, shows the absorbances of complexes I, V and III normalised to the absorbance of complex IV in mitochondria isolated from rat heart. The absorbances are also listed in Table 7.1.3. The absorbances of complexes I, V and III were 0.82 ± 0.12 , 1.27 ± 0.09 , 1.05 ± 0.08 , respectively, when normalised to the absorbance of complex IV.

Figure 7.2.5 shows the resolution in the second dimension of rat kidney mitochondrial proteins. The gel was stained using Serva blue G. First-dimension gel lanes loaded with $454\mu\text{g}$ of protein had the in-gel proteins denatured using 20mM DDT/1%SDS and the denatured proteins were resolved on a second-dimension denatured 10%-tricine-SDS gel. Complexes I, V, III and IV were identified on the basis of their characteristic patterns in the second dimension. Complex I was quite diffuse and complex II could not be identified in the rat heart mitochondria because of a large number of unidentified proteins in the same region of the gel. Arrows on the gel mark the protein subunits whose staining intensity was measured for the quantification of second dimension gels. The gel shown in Fig 7.2.5 is representative of three gels that were used for the analysis of rat kidney mitochondria.

Figure 7.2.6, shows the absorbances normalised per mg of protein loaded on the first-dimension gel of complexes I, V, III and IV in mitochondria isolated from rat kidney. The absorbances are also listed in Table 7.2.1a. The absorbances of complexes I, V, III and IV were 0.61 ± 0.04 , 0.89 ± 0.01 , 0.68 ± 0.02 and 0.61 ± 0.03 , respectively, when normalised per mg of protein. When the data shown in figure 7.2.6 were normalised to IV=1, the I:V:III:IV ratio was found to be 1.00:1.48:1.13:1.00 (Table 7.2.1b).

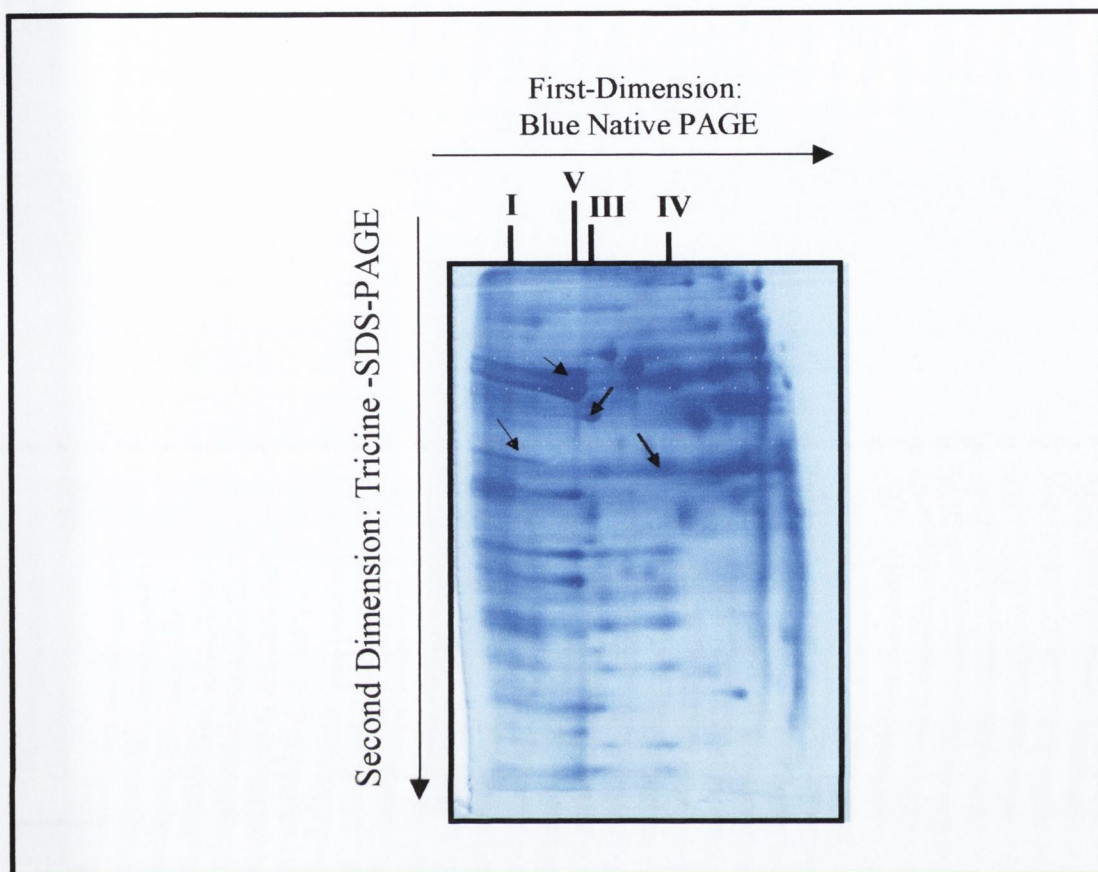


Figure 7.2.5. Two-dimensional resolution of mitochondrial proteins from rat kidney mitochondria using blue native PAGE in the first dimension and denaturing tricine-SDS-PAGE in the second dimension. The membrane protein complexes were separated in the native first-dimension and identified by their characteristic polypeptide patterns in the denaturing second dimension. The gel was fixed, stained using Serva blue G and destained as described in Materials and Methods. The positions of the complexes I, V, III and IV are indicated at the top of the gel. Arrows on the gel mark the protein subunits whose staining intensity or absorbance was measured for the quantification of second-dimension gels. The subunits of the complexes whose staining intensities were measured were as follows: 42kDa subunit of complex I, 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The rat kidney mitochondrial proteins were solubilized using a 1:1 ratio of dodecyl maltoside to protein and resolved using blue native PAGE in the first-dimension (1mm gel, 5.5-13% non-linear gradient gel). A first-dimension lane loaded with 25 μ l (454 μ g protein) of solubilized rat kidney mitochondrial protein was cut from the first-dimension gel and the in-gel native proteins were denatured using 20mM DDT/1%SDS (see Materials and Methods). The mitochondrial proteins were resolved using a uniform 1mm 10% tricine-SDS gel in the second-dimension. The gel shown is representative of three gels that were used for the quantification of rat kidney mitochondria.

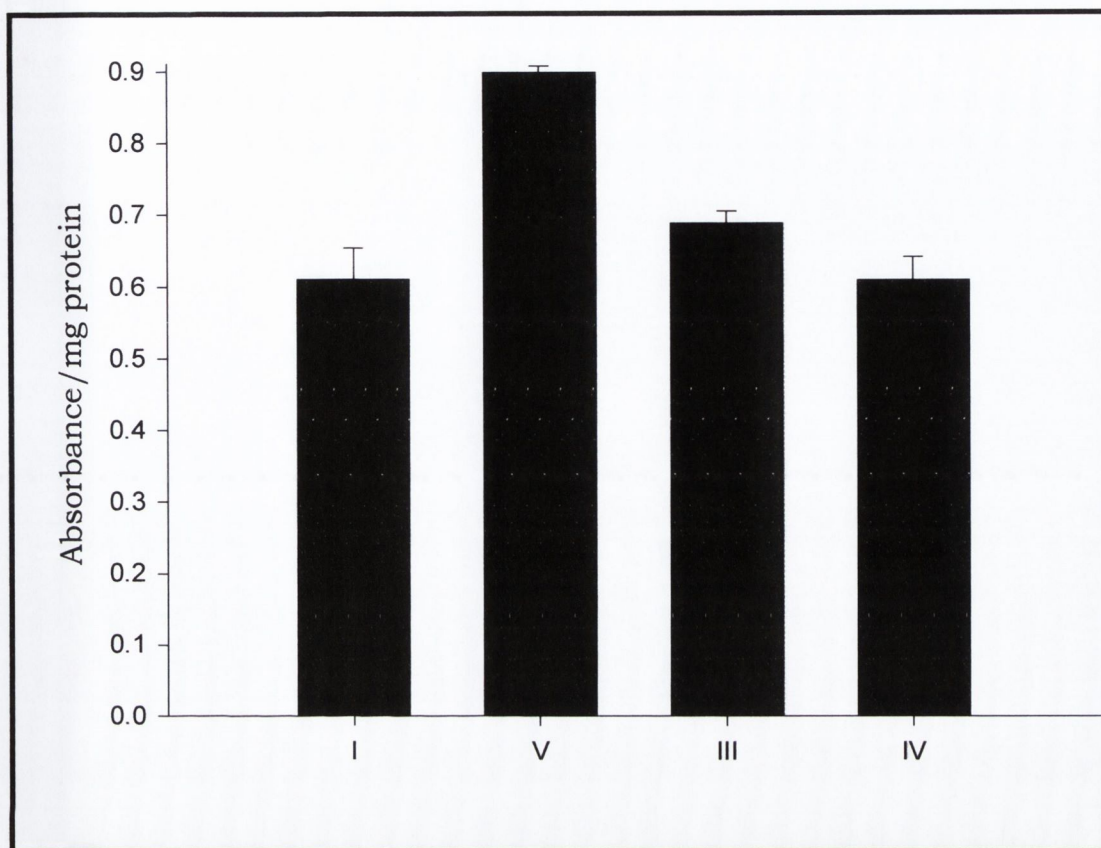


Figure 7.2.6. Comparison of the absorbances of complexes I, V, III and IV normalised per mg protein in mitochondria isolated from rat kidney. All mitochondrial proteins were solubilized using dodecyl maltoside. The membrane-protein complexes I, V, III and IV were separated using blue native PAGE in the first-dimension and identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second-dimension. The second-dimension gel, which was fixed, stained using Serva blue G and destained as described in Materials and Methods, was used for densitometric analysis. The subunits used for densitometric analysis are indicated by arrows on the gel shown in fig. 7.2.5. The subunits whose staining intensities were measured were as follows: 42kDa subunit of complex I, 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The graph labels are as follows: I: complex I, V: complex V, III: complex III, IV: complex IV. 454 μ g rat kidney mitochondria was loaded on the first-dimension gel. All of the data represent the mean $\pm$ SEM of three determinations. The data are also represented in Table 7.2.1a and 7.2.1b.

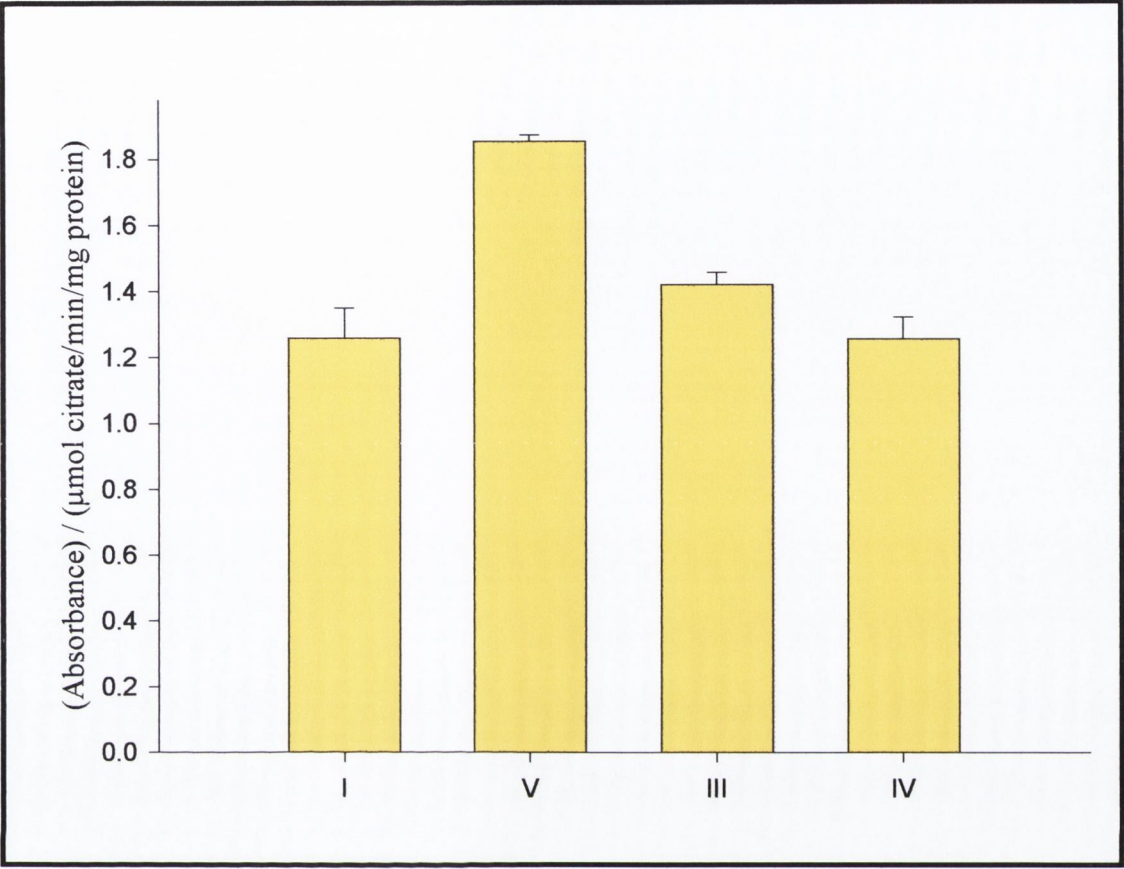


Figure 7.2.7. Comparison of the absorbances of complexes I, V, III and IV per μmol citrate synthase activity in mitochondria isolated from rat kidney. All mitochondrial proteins were solubilized using dodecyl maltoside. The membrane-protein complexes I, V, III and IV were separated using blue native PAGE in the first-dimension and identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second- dimension. The second-dimension gel, which was fixed, stained using Serva blue G and destained as described in Materials and Methods, was used for densitometric analysis. The subunits used for densitometric analysis are indicated by arrows on the gel shown in fig. 7.2.5. The subunits whose staining intensities were measured were as follows: 42kDa subunit of complex I, 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The graph labels are as follows: I: complex I, V: complex V, III: complex III, IV: complex IV. 454 μg rat kidney mitochondria was loaded on the first-dimension gel. All of the data represent the mean $\pm$ SEM of three determinations. The data are also represented in Table 7.2.2a and 7.2.2b.

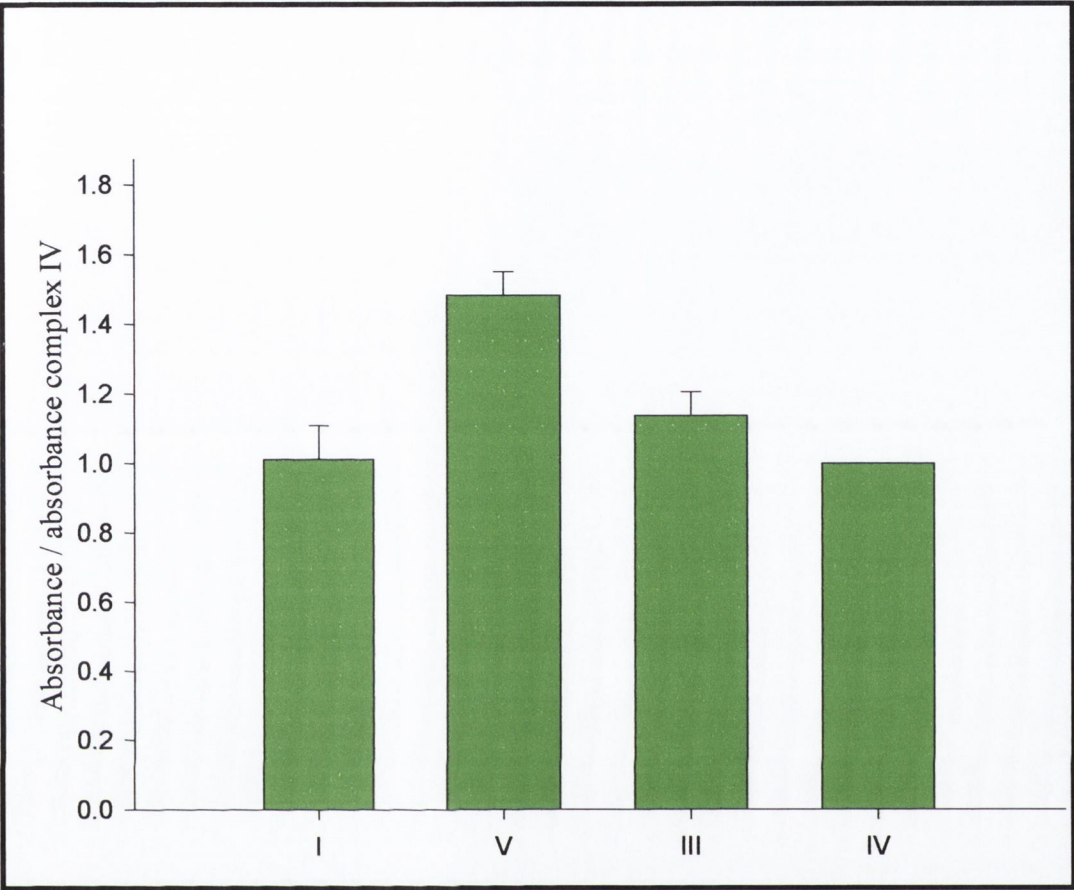


Figure 7.2.8. Comparison of the absorbances of complexes I, V, III and IV normalised to the absorbance of complex IV in mitochondria isolated from rat kidney. All mitochondrial proteins were solubilized using dodecyl maltoside. The membrane- protein complexes I, V, III and IV were separated using blue native PAGE in the first- dimension and identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second-dimension. The second-dimension gel, which was fixed, stained using Serva blue G and destained as described in Materials and Methods, was used for densitometric analysis. The subunits used for densitometric analysis are indicated by arrows on the gel shown in fig. 7.2.5. The subunits of the complexes whose staining intensities were measured were as follows: 42kDa subunit of complex I, 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The graph labels are as follows: I: complex I; V: complex V; III: complex III and IV: complex IV. A 454 μ g amount of rat kidney mitochondria was loaded on the first-dimension gel. All of the data represent the mean $\pm$ SEM of three determinations. The data are also represented in Table 7.2.3.

Figure 7.2.7 shows the absorbances normalised per μmol of citrate synthase activity of complexes I, V, III and IV in mitochondria isolated from rat kidney. The absorbances are also listed in Table 7.2.2a. The absorbances of complexes I, V, III and IV were 1.26 ± 0.09 , 1.85 ± 0.02 , 1.42 ± 0.04 and 1.26 ± 0.07 , respectively, when normalised to citrate synthase activity. When the data shown in figure 7.2.7 were normalised to IV=1, the I:V:III:IV ratio was found to be 1.00:1.47:1.13:1.00 (Table 7.2.2b).

Figure 7.2.8 shows the absorbances of complexes I, V and III normalised to the absorbance of complex IV in mitochondria isolated from rat kidney. The absorbances are also listed in Table 7.2.3. The absorbances of complexes I, V and III were 1.01 ± 0.10 , 1.48 ± 0.07 and 1.14 ± 0.07 , respectively, when normalised to the absorbance of complex IV.

Figure 7.2.9 shows the resolution in the second-dimension of rat liver mitochondrial proteins. The gel was stained using Serva blue G. First-dimension gel lanes loaded with $454\mu\text{g}$ of protein had the in-gel proteins denatured using 20mM DDT/1%SDS and the denatured proteins were resolved on a second-dimension denatured 10%-tricine-SDS gel. Complexes I, V, III and IV were identified on the basis of their characteristic patterns in the second-dimension. Complex I was quite diffuse and complex II could not be identified in rat liver mitochondria because of a large number of unidentified proteins in the same region of the gel. Arrows on the gel mark the protein subunits whose staining intensity was measured for the quantification of second dimension gels. The gel shown in Fig 7.2.9 is representative of three gels that were used for the analysis of rat liver mitochondria.

Figure 7.2.10 shows the absorbances normalised per mg of protein loaded on the first dimension gel of complexes I, V, III and IV in mitochondria isolated from rat liver. The absorbances are also listed in Table 7.2.1a. The absorbances of complexes I, V, III and IV were 0.39 ± 0.06 , 0.66 ± 0.06 , 0.36 ± 0.03 and 0.38 ± 0.05 , respectively, when normalised per mg of protein. When the data shown in figure 7.2.10 was normalised to IV=1, the I:V:III:IV ratio was found to be 1.02:1.73:0.94:1.00 (Table 7.2.1b).

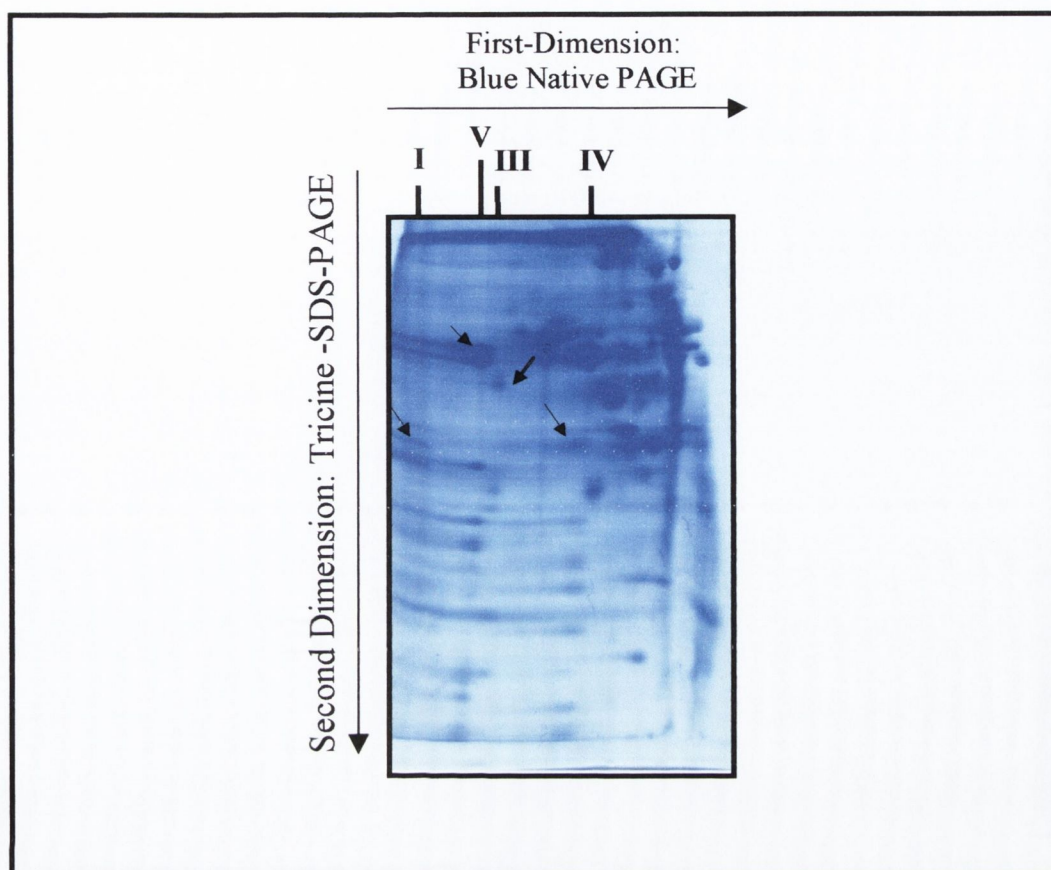


Figure 7.2.9. Two-dimensional resolution of mitochondrial proteins from rat liver mitochondria using blue native PAGE in the first-dimension and denaturing tricine-SDS-PAGE in the second-dimension. The membrane protein complexes were separated in the native first-dimension and identified by their characteristic polypeptide patterns in the denaturing second-dimension. The gel was fixed, stained using Serva blue G and destained as described in Materials and Methods. The positions of the complexes I, V, III and IV are indicated at the top of the gel. Arrows on the gel mark the protein subunits whose staining intensities or absorbances was measured for the quantification of second-dimension gels. The subunits of the complexes whose staining intensities were measured were as follows: 42kDa subunit of complex I, 55kDa α subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The rat liver mitochondrial proteins were solubilized using a 1:1 ratio of dodecyl maltoside to protein and resolved using blue native PAGE in the first-dimension (1mm gel, 5.5-13% non-linear gradient gel). A first-dimension lane loaded with 25 μ l (454 μ g protein) of solubilized rat liver mitochondrial protein was cut from the first-dimension gel and the in-gel native proteins were denatured using 20mM DDT/1%SDS (see Materials and Methods). The mitochondrial proteins were resolved using a uniform 1mm 10% tricine-SDS gel in the second-dimension. The gel shown is representative of three gels that were used for the quantification of liver mitochondria.

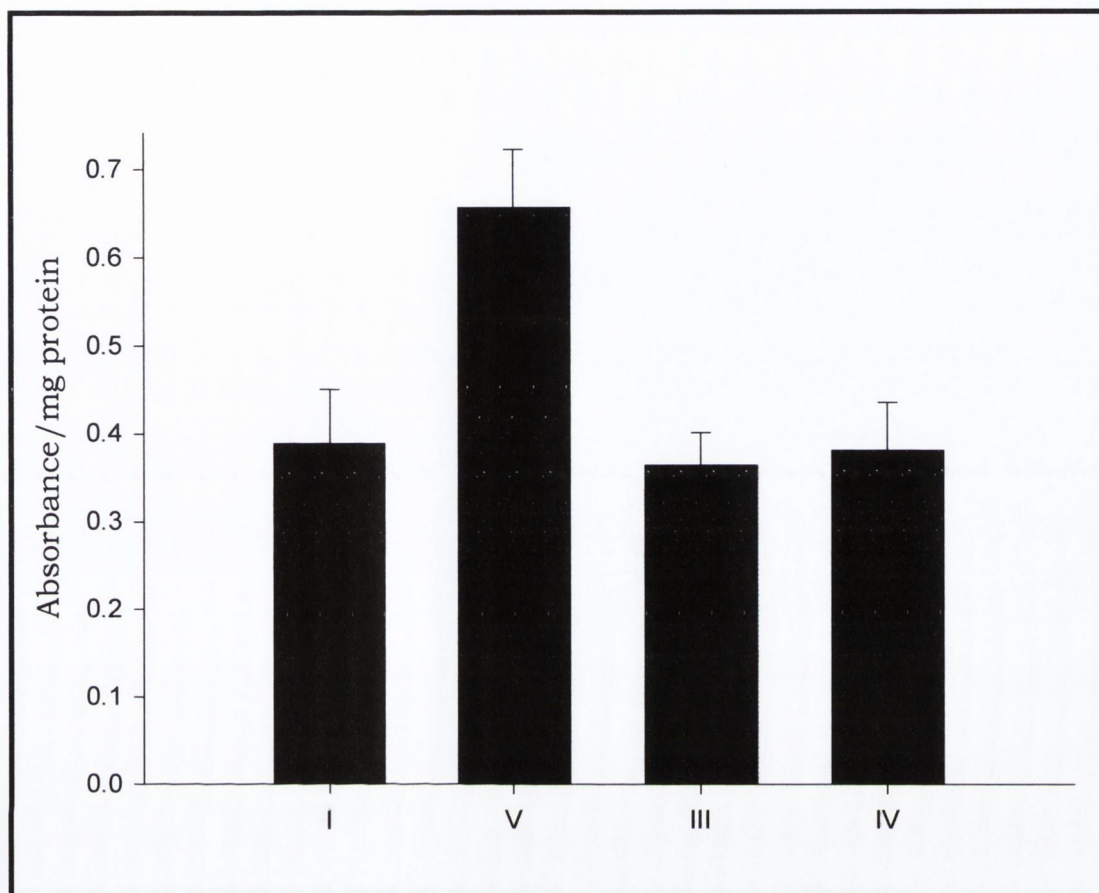


Figure 7.2.10. Comparison of the absorbances of complexes I, V, III and IV normalised per mg protein in mitochondria isolated from rat liver. All mitochondrial proteins were solubilized using dodecyl maltoside. The membrane-protein complexes I, V, III and IV were separated using blue native PAGE in the first-dimension and identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second-dimension. The second-dimension gel, which was fixed, stained using Serva blue G and destained as described in Materials and Methods, was used for densitometric analysis. The subunits used for densitometric analysis are indicated by arrows on the gel shown in Fig. 7.2.9. The subunits whose staining intensities were measured were as follows: 42kDa subunit of complex I, 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The graph labels are as follows: I: complex I; V: complex V; III: complex III and IV: complex IV. 454 μ g rat liver mitochondria was loaded on the first-dimension gel. All of the data represents the mean $\pm$ SEM of three determinations. The data are also represented in Table 7.2.1a and 7.2.1b.

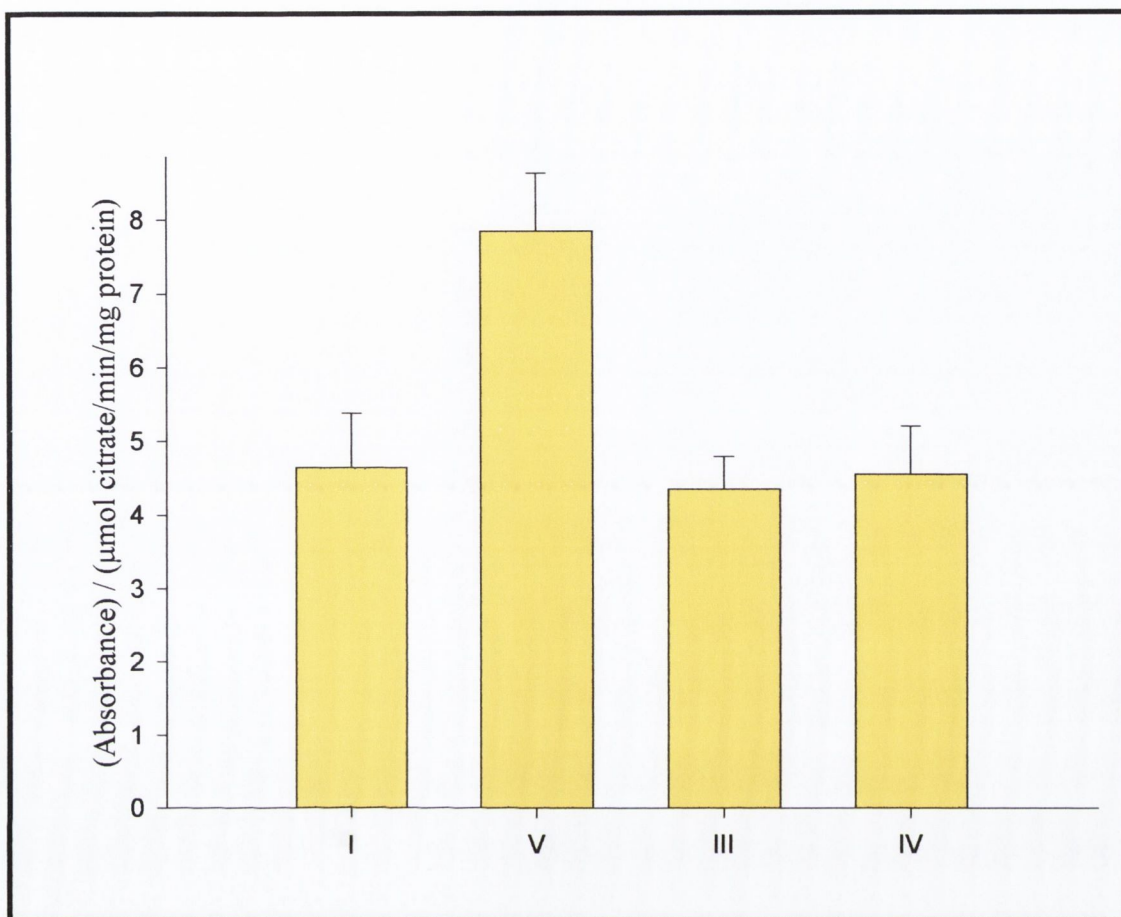


Figure 7.2.11. Comparison of the absorbances of complexes I, V, III and IV per μmol citrate synthase activity in mitochondria isolated from rat liver. All mitochondrial proteins were solubilized using dodecyl maltoside. The membrane-protein complexes I, V, III and IV were separated using blue native PAGE in the first-dimension and identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second-dimension. The second-dimension gel, which was fixed, stained using Serva blue G and destained as described in Materials and Methods, was used for densitometric analysis. The subunits used for densitometric analysis are indicated by arrows on the gel shown in Fig. 7.2.9. The subunits whose staining intensities were measured were as follows: 42kDa subunit of complex I, 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The graph labels are as follows: I: complex I; V: complex V; III: complex III and IV: complex IV. 454 μg rat liver mitochondria was loaded on the first-dimension gel. All of the data represent the mean $\pm$ SEM of three determinations. The data are also represented in Table 7.2.2a and 7.2.2b.

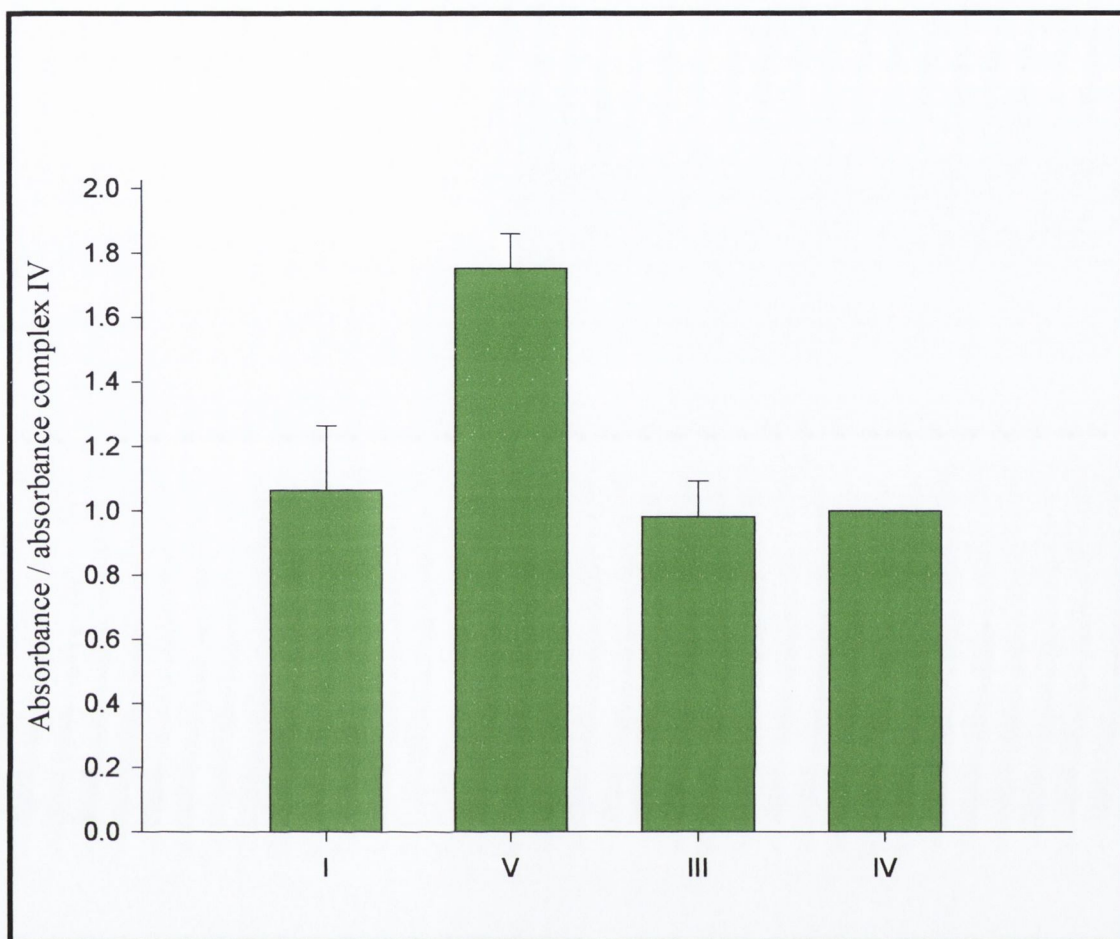


Figure 7.2.12. Comparison of the absorbances of complexes I, V, III and IV normalised to the absorbance of complex IV in mitochondria isolated from rat liver. All mitochondrial proteins were solubilized using dodecyl maltoside. The membrane-protein complexes I, V, III and IV were separated using blue native PAGE in the first-dimension and identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second-dimension. The second-dimension gel, which was fixed, stained using Serva blue G and destained as described in Materials and Methods, was used for densitometric analysis. The subunits used for densitometric analysis are indicated by arrows on the gel shown in Fig. 7.2.9. The subunits of the complexes whose staining intensities were measured were as follows: 42kDa subunit of complex I, 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The graph labels are as follows: I: complex I; V: complex V; III: complex III and IV: complex IV. 454 μ g rat liver mitochondria was loaded on the first-dimension gel. All of the data represent the mean $\pm$ SEM of three determinations. The data are also represented in Table 7.2.3.

Figure 7.2.11 shows the absorbance normalised per μmol of citrate synthase of complexes I, V, III and IV in mitochondria isolated from rat liver. The absorbances are also listed in Table 7.2.2a. The absorbances of complexes I, V, III and IV were 4.65 ± 0.74 , 7.85 ± 0.79 , 4.35 ± 0.44 and 4.55 ± 0.66 , respectively, when normalised to citrate synthase activity. When the data shown in figure 7.2.11 were normalised to IV=1, the I:V:III:IV ratio was found to be 1.02:1.72:0.96:1.00 (table 7.2.2b).

Figure 7.2.12 shows the absorbances of complexes I, V and III normalised to the absorbance of complex IV in mitochondria isolated from rat liver. The absorbances are also listed in Table 7.2.3. The absorbances of complexes I, V and III were 1.06 ± 0.20 , 1.76 ± 0.11 , 0.98 ± 0.11 , respectively, when normalised to the absorbance of complex IV.

Figure 7.2.13 shows the resolution in the second-dimension of rat brown adipose tissue (BAT) mitochondrial proteins. The gel was stained using Serva blue G. First-dimension gel lanes loaded with $227\mu\text{g}$ of protein had the in-gel proteins denatured using 20mM DDT/1%SDS and the denatured proteins were resolved on a second-dimension denatured 10%-tricine-SDS gel. Complexes I, V, III and IV were identified on the basis of their characteristic patterns in the second-dimension. Complex I was quite diffuse and complex II could not be identified in rat BAT mitochondria because of a large number of unidentified proteins in the same region of the gel. Arrows on the gel mark the protein subunits whose staining intensities were measured for the quantification of second-dimension gels. The gel shown in Fig 7.2.13 is representative of three gels that were used for the analysis of rat BAT mitochondria..

Figure 7.2.14 shows the absorbance normalised per mg of protein loaded on the first-dimension gel of complexes I, V, III and IV in mitochondria isolated from rat brown adipose tissue (BAT). The absorbances are also listed in Table 7.2.1a. The absorbances of complexes I, V, III and IV were 0.30 ± 0.06 , 0.41 ± 0.10 , 0.69 ± 0.08 and 0.70 ± 0.09 , respectively, when normalised per mg of protein. When the data shown in Figure 7.2.14 were normalised to IV=1, the I:V:III:IV ratio was found to be 0.42:0.58:0.98:1.00 (Table 7.2.1b).

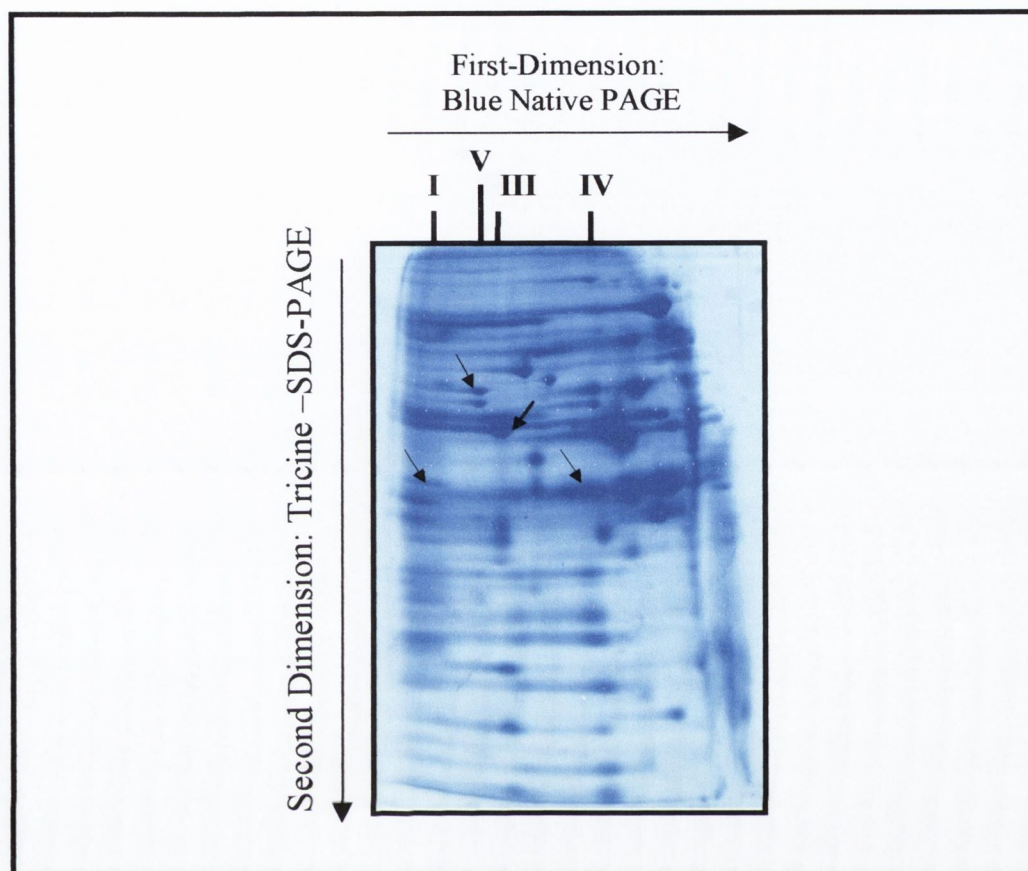


Figure 7.2.13. Two-dimensional resolution of mitochondrial proteins from rat brown adipose tissue (BAT) mitochondria using blue native PAGE in the first-dimension and denaturing tricine-SDS-PAGE in the second-dimension. The membrane-protein complexes were separated in the native first-dimension and identified by their characteristic polypeptide patterns in the denaturing second-dimension. The gel was fixed, stained using Serva blue G and destained as described in Materials and Methods. The positions of the complexes I, V, III and IV are indicated at the top of the gel. Arrows on the gel mark the protein subunits whose staining intensities or absorbances were measured for the quantification of second-dimension gels. The subunits of the complexes whose staining intensity was measured were as follows: 42kDa subunit of complex I, 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The rat BAT mitochondrial proteins were solubilized using a 2:1 ratio of dodecyl maltoside to protein and resolved using blue native PAGE in the first-dimension (1mm gel, 5.5-13% non-linear gradient gel). A first-dimension lane loaded with 25 μ l (227 μ g mitochondrial protein) of solubilized rat liver mitochondrial protein, was cut from the first-dimension gel and the in-gel native proteins were denatured using 20mM DDT/1%SDS (see Materials and Methods). The mitochondrial proteins were resolved using a uniform 1mm 10% tricine-SDS gel in the second-dimension. The gel shown is representative of three gels that were used for the quantification of BAT mitochondria.

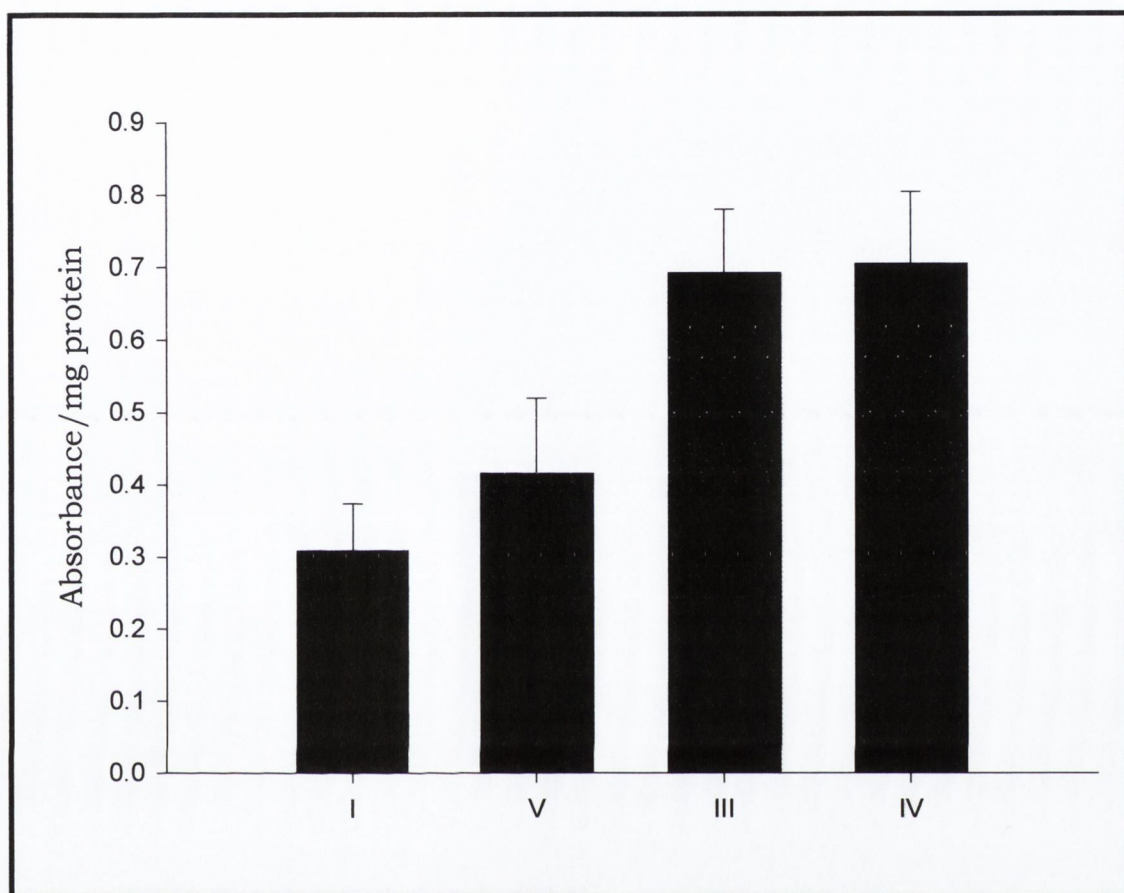


Figure 7.2.14. Comparison of the absorbances of complexes I, V, III and IV normalised per mg protein in mitochondria isolated from rat brown adipose tissue (BAT). All mitochondrial proteins were solubilized using dodecyl maltoside. The membrane-protein complexes I, V, III and IV were separated using blue native PAGE in the first-dimension and identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second-dimension. The second-dimension gel, which was fixed, stained using Serva blue G and destained as described in Materials and Methods, was used for densitometric analysis. The subunits used for densitometric analysis are indicated by arrows on the gel shown in fig. 7.2.13. The subunits whose staining intensities were measured were as follows: 42kDa subunit of complex I, 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The graph labels are as follows: I: complex I; V: complex V; III: complex III and IV: complex IV. 227 μ g rat brown adipose tissue mitochondria was loaded on the first-dimension gel. All of the data represent the mean $\pm$ SEM of three determinations. The data are also represented in Table 7.2.1a and 7.2.1b.

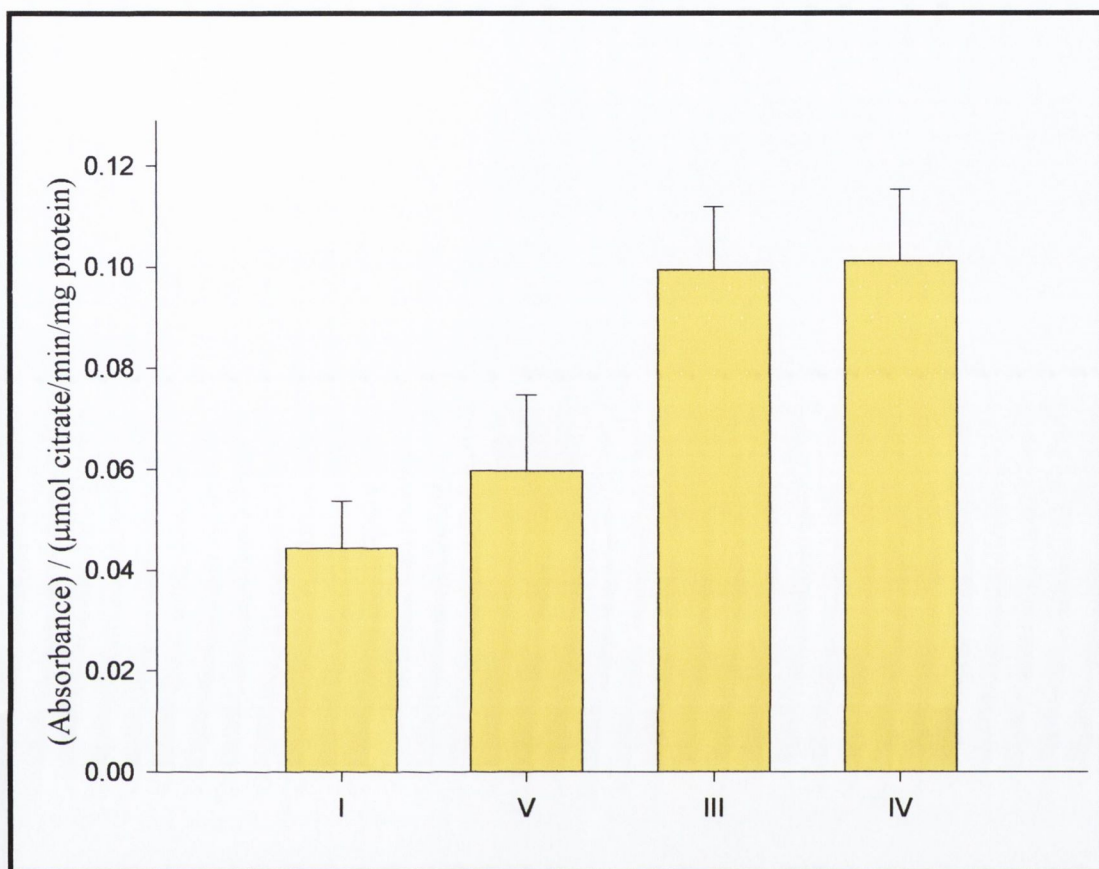


Figure 7.2.15. Comparison of the absorbances of complexes I, V, III and IV per μmol citrate synthase activity in mitochondria isolated from rat brown adipose tissue (BAT). All mitochondrial proteins were solubilized using dodecyl maltoside. The membrane-protein complexes I, V, III and IV were separated using blue native PAGE in the first-dimension and identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second-dimension. The second-dimension gel, which was fixed, stained using Serva blue G and destained as described in Materials and Methods, was used for densitometric analysis. The subunits used for densitometric analysis are indicated by arrows on the gel shown in fig. 7.2.13. The subunits whose staining intensities were measured were as follows: 42kDa subunit of complex I, 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The graph labels are as follows: I: complex I; V: complex V; III: complex III and IV: complex IV. 227 μg rat brown adipose tissue mitochondria was loaded on the first-dimension gel. All of the data represent the mean $\pm$ SEM of three determinations. The data are also represented in Table 7.2.2a and 7.2.2b.

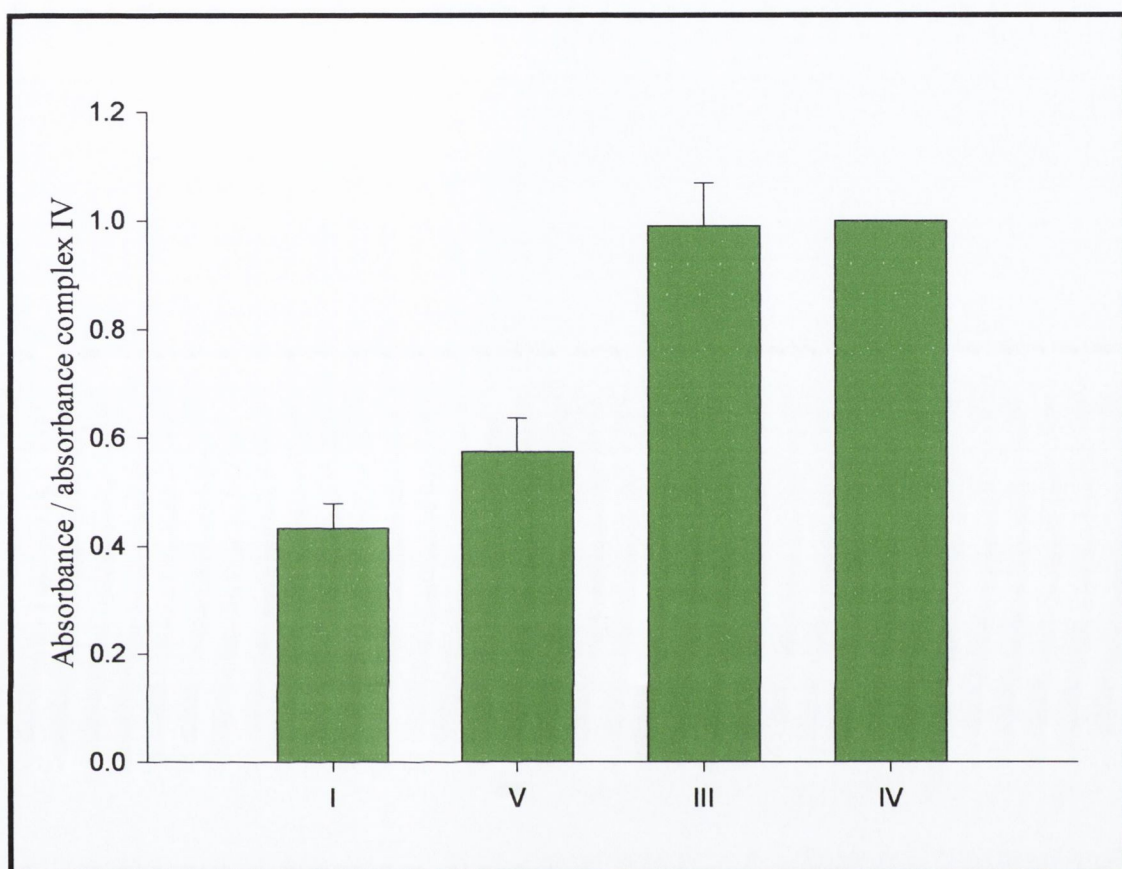


Figure 7.2.16. Comparison of the absorbances of complexes I, V, III and IV normalised to the absorbance of complex IV in mitochondria isolated from rat brown adipose tissue (BAT). All mitochondrial proteins were solubilized using dodecyl maltoside. The membrane-protein complexes I, V, III and IV were separated using blue native PAGE in the first-dimension and identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second-dimension. The second-dimension gel, which was fixed, stained using Serva blue G and destained as described in Materials and Methods, was used for densitometric analysis. The subunits used for densitometric analysis are indicated by arrows on the gel shown in Fig. 7.2.13. The subunits of the complexes whose staining intensities were measured were as follows: 42kDa subunit of complex I, 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The graph labels are as follows: I: complex I; V: complex V; III: complex III and IV: complex IV. 227 μ g rat liver mitochondria was loaded on the first-dimension gel. All of the data represent the mean $\pm$ SEM of three determinations. The data are also represented in Table 7.2.3.

Figure 7.2.15 shows the absorbances normalised per μmol of citrate synthase activity of complexes I, V, III and IV in mitochondria isolated from rat brown adipose tissue (BAT). The absorbances are also listed in Table 7.2.2a. The absorbances of complexes I, V, III and IV were 0.04 ± 0.01 , 0.06 ± 0.02 , 0.10 ± 0.01 and 0.10 ± 0.01 , respectively, when normalised to citrate synthase activity. When the data shown in figure 7.2.15 were normalised to IV=1, the I:V:III:IV ratio was found to be 0.40:0.60:1.00:1.00 (Table 7.2.2b).

Figure 7.2.16 shows the absorbances of complexes I, V and III normalised to the absorbance of complex IV in mitochondria isolated from rat brown adipose tissue (BAT). The absorbances are also listed in Table 7.2.3. The absorbances of complexes I, V and III were 0.43 ± 0.04 , 0.57 ± 0.06 , 0.99 ± 0.08 , respectively, when normalised to the absorbance of complex IV.

Figure 7.2.17 shows the resolution in the second dimension of rat skeletal muscle mitochondrial proteins. The gel was stained using Serva blue G. First-dimension gel lanes loaded with $150\mu\text{g}$ of protein had the in-gel proteins denatured using 20mM DDT/1%SDS and the denatured proteins were resolved on a second-dimension denatured 10%-tricine-SDS gel. Complexes I, V, III and IV were identified on the basis of their characteristic patterns in the second-dimension. Complex I was quite diffuse and complex II could not be identified in rat skeletal muscle mitochondria because of a large number of unidentified proteins in the same region of the gel. Arrows on the gel mark the protein subunits whose staining intensities were measured for the quantification of second-dimension gels. The gel shown in fig 7.2.17 is representative of three gels that were used for the quantification of second-dimension gels.

Figure 7.2.18 shows the absorbances normalised per mg of protein loaded on the first-dimension gel of complexes I, V, III and IV in mitochondria isolated from rat skeletal muscle. The absorbances are also listed in Table 7.2.1a. The absorbances of complexes I, V, III and IV were 0.52 ± 0.34 , 0.78 ± 0.36 , 0.83 ± 0.35 and 0.68 ± 0.30 , respectively, when normalised per mg of protein. When the data shown in figure 7.2.18 were

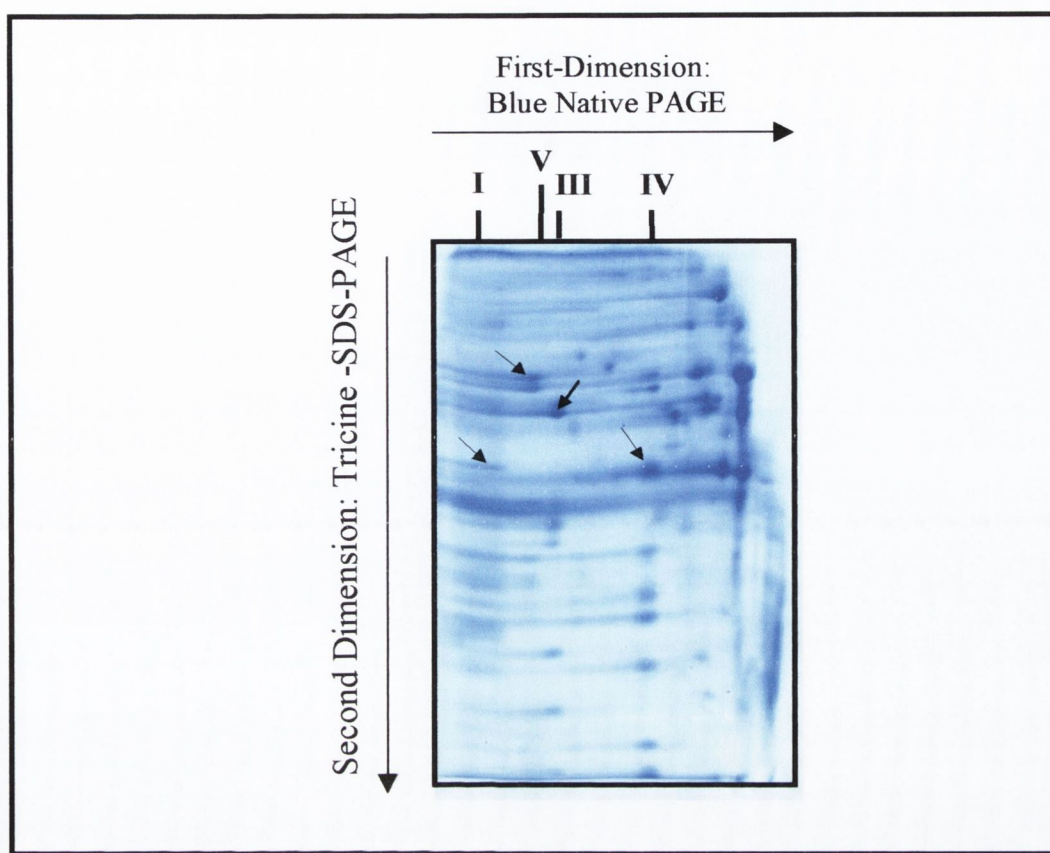


Figure 7.2.17. Two-dimensional resolution of mitochondrial proteins from rat skeletal muscle mitochondria using blue native PAGE in the first-dimension and denaturing tricine-SDS-PAGE in the second-dimension. The membrane-protein complexes were separated in the native first-dimension and identified by their characteristic polypeptide patterns in the denaturing second-dimension. The gel was fixed, stained using Serva blue G and destained as described in Materials and Methods. The positions of the complexes I, V, III and IV are indicated at the top of the gel. Arrows on the gel mark the protein subunits whose staining intensities or absorbances were measured for the quantification of second-dimension gels. The subunits of the complexes whose staining intensities were measured were as follows: 42kDa subunit of complex I, 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The rat skeletal muscle mitochondrial proteins were solubilized using a 3:1 ratio of dodecyl maltoside to protein and resolved using blue native PAGE in the first-dimension (1mm gel, 5.5-13% non-linear gradient gel). A first-dimension lane loaded with 25 μ l (150 μ g protein) of solubilized rat skeletal muscle mitochondrial protein, was cut from the first-dimension gel and the in-gel native proteins were denatured using 20mM DDT/1%SDS (see Materials and Methods). The mitochondrial proteins were resolved using a uniform 1mm 10% tricine-SDS gel in the second-dimension. The gel shown is representative of three gels that were used for the quantification of skeletal muscle mitochondria.

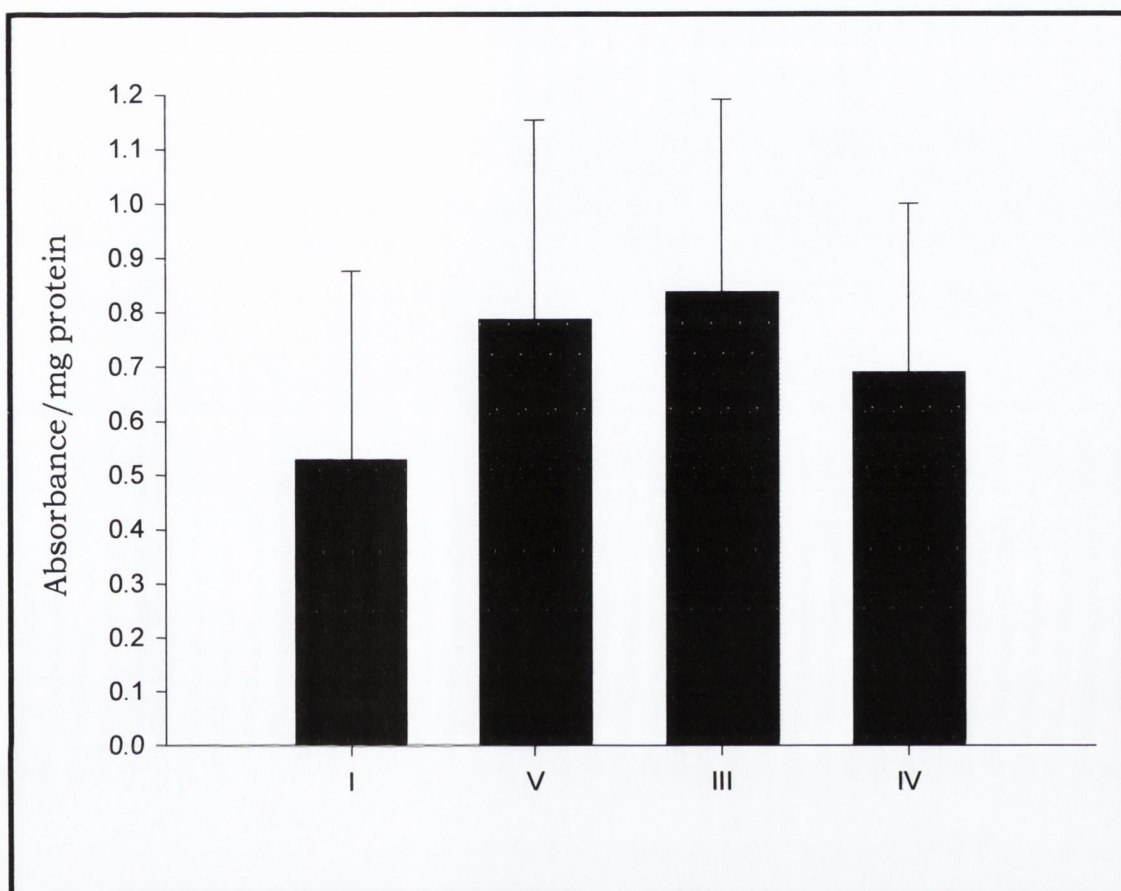


Figure 7.2.18. Comparison of the absorbances of complexes I, V, III and IV normalised per mg protein in mitochondria isolated from rat skeletal muscle. All mitochondrial proteins were solubilized using dodecyl maltoside. The membrane-protein complexes I, V, III and IV were separated using blue native PAGE in the first-dimension and identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second-dimension. The second-dimension gel, which was fixed, stained using Serva blue G and destained as described in Materials and Methods, was used for densitometric analysis. The subunits used for densitometric analysis are indicated by arrows on the gel shown in Fig. 7.2.17. The subunits whose staining intensities were measured were as follows: 42kDa subunit of complex I, 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The graph labels are as follows: I: complex I; V: complex V; III: complex III and IV: complex IV. 150 μ g rat muscle mitochondria was loaded on the first-dimension gel. All of the data represent the mean $\pm$ SEM of three determinations. The data are also represented in Table 7.2.1a and 7.2.1b.

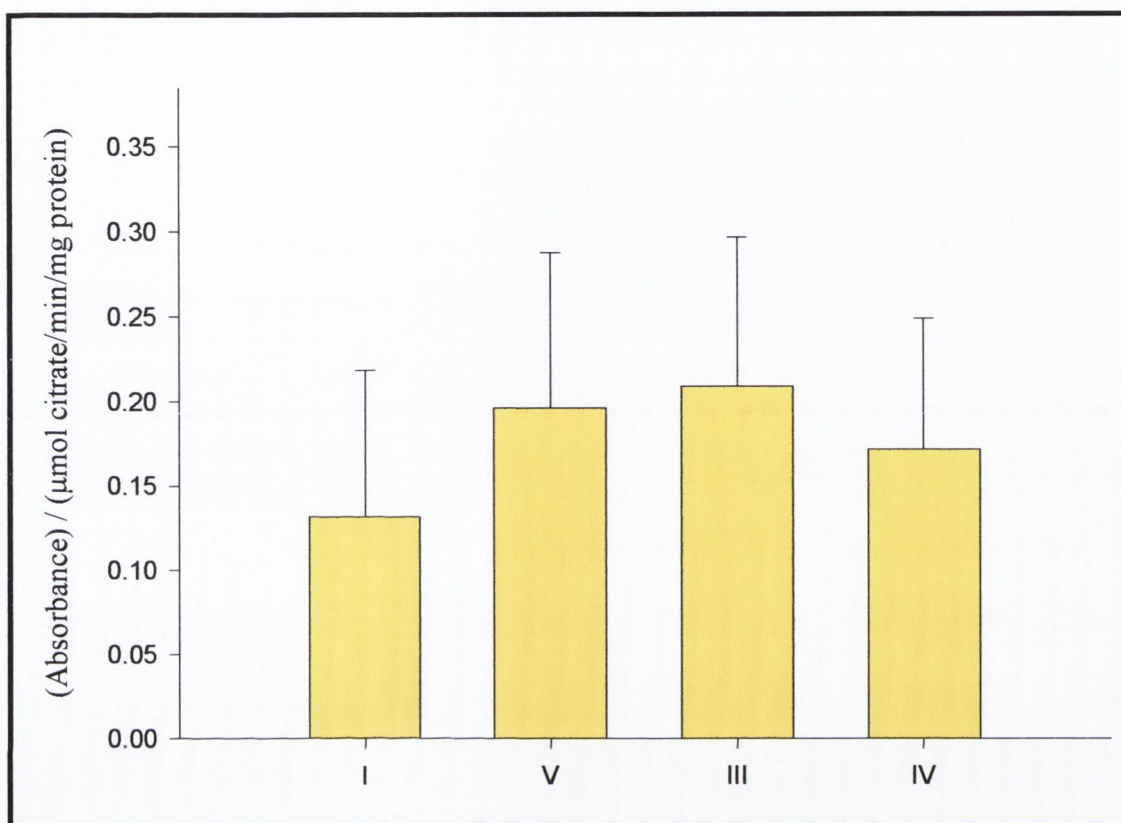


Figure 7.2.19. Comparison of the absorbances of complexes I, V, III and IV per μmol citrate synthase activity in mitochondria isolated from rat skeletal muscle. All mitochondrial proteins were solubilized using dodecyl maltoside. The membrane-protein complexes I, V, III and IV were separated using blue native PAGE in the first-dimension and identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second-dimension. The second-dimension gel, which was fixed, stained using Serva blue G and destained as described in Materials and Methods, was used for densitometric analysis. The subunits used for densitometric analysis are indicated by arrows on the gel shown in Fig. 7.2.17. The subunits whose staining intensities were measured were as follows: 42kDa subunit of complex I, 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The graph labels are as follows: I: complex I; V: complex V; III: complex III and IV: complex IV. 150 μg rat skeletal muscle mitochondria was loaded on the first-dimension gel. All of the data represent the mean $\pm$ SEM of three determinations. The data are also represented in Table 7.2.2a and 7.2.2b.

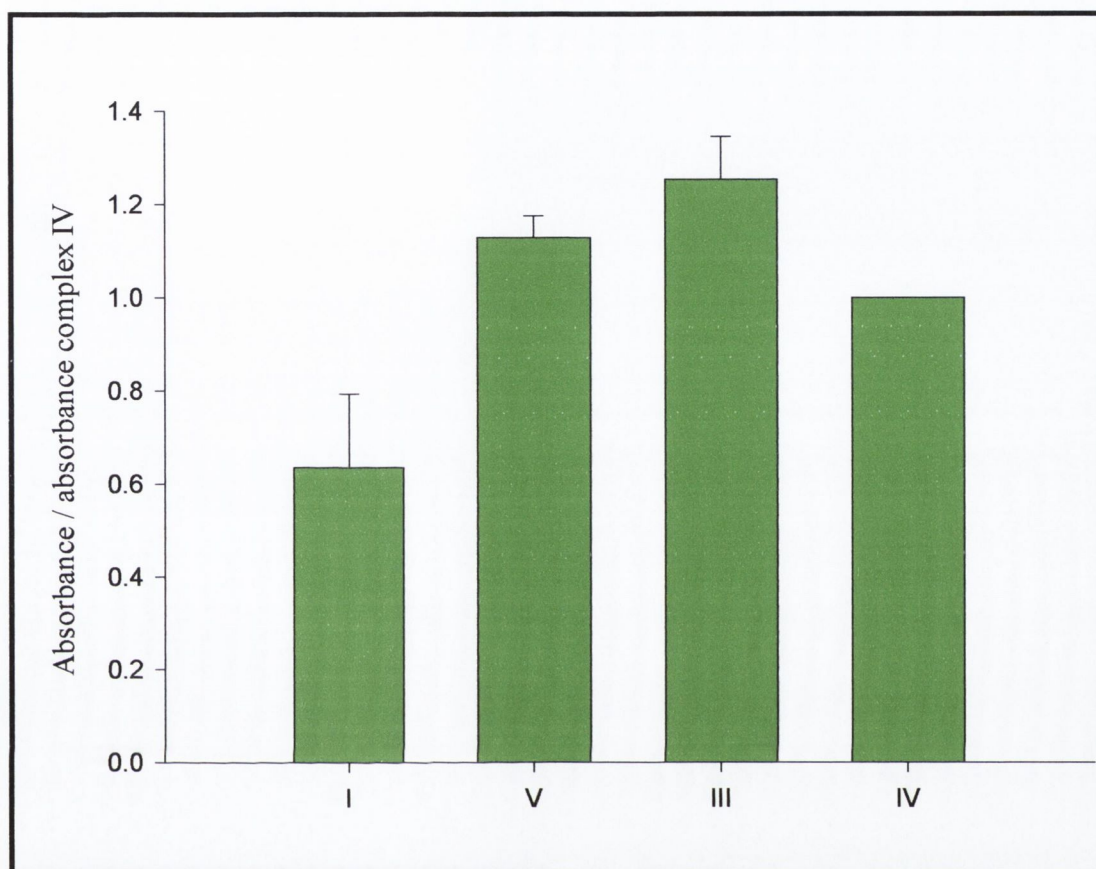


Figure 7.2.20. Comparison of the absorbances of complexes I, V, III and IV normalised to the absorbance of complex IV in mitochondria isolated from rat skeletal muscle. All mitochondrial proteins were solubilized using dodecyl maltoside. The membrane-protein complexes I, V, III and IV were separated using blue native PAGE in the first-dimension and identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second-dimension. The second-dimension gel, which was fixed, stained using Serva blue G and destained as described in Materials and Methods, was used for densitometric analysis. The subunits used for densitometric analysis are indicated by arrows on the gel shown in Fig. 7.2.17. The subunits whose staining intensities were measured were as follows: 42kDa subunit of complex I, 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The graph labels are as follows: I: complex I; V: complex V; III: complex III and IV: complex IV. 150 μ g rat skeletal muscle mitochondria was loaded on the first-dimension gel. All of the data represent the mean $\pm$ SEM of three determinations. The data are also represented in Table 7.2.3.

normalised to IV=1, the I:V:III:IV ratio was found to be 0.76:1.14:1.22:1.00 (Table 7.2.1b).

Figure 7.2.19 shows the absorbances normalised per μmol of citrate synthase activity of complexes I, V, III and IV in mitochondria isolated from rat skeletal muscle. The absorbances are also listed in Table 7.2.2a. The absorbances of complexes I, V, III and IV were 0.13 ± 0.09 , 0.20 ± 0.09 , 0.21 ± 0.09 and 0.17 ± 0.08 , respectively, when normalised to citrate synthase activity. When the data shown in Figure 7.2.19 were normalised to IV=1, the I:V:III:IV ratio was found to be 0.76:1.17:1.23:1.00 (Table 7.2.2b).

Figure 7.2.20 shows the absorbances of complexes I, V and III normalised to the absorbance of complex IV in mitochondria isolated from skeletal muscle. The absorbances are also listed in Table 7.2.3. The absorbances of complexes I, V and III were 0.64 ± 0.16 , 1.13 ± 0.06 , 1.25 ± 0.09 , respectively, when normalised to the absorbance of complex IV.

Figure 7.2.21 shows the resolution in the second-dimension of rat synaptic mitochondrial proteins. The gels were stained using Serva blue G. First-dimension gel lanes loaded with $227\mu\text{g}$ of protein had the in-gel proteins denatured using 20mM DDT/1%SDS and the denatured proteins were resolved on a second-dimension denatured 10%-tricine-SDS gel. Complexes I, V, III and IV were identified on the basis of their characteristic patterns in the second-dimension. Complex I was quite diffuse and complex II could not be identified because of a large number of unidentified proteins in the same region of the gel. Arrows on the gel mark the protein subunits whose staining intensities were measured for the quantification of second-dimension gels. The gel shown in Fig 7.2.21 is representative of three gels that were used for the analysis of rat synaptic mitochondria.

Figure 7.2.22 shows the absorbances normalised per mg of protein loaded on the first-dimension gel of complexes I, V, III and IV in mitochondria isolated from rat brain synaptosomes. The absorbances are also listed in Table 7.2.1a. The absorbances of

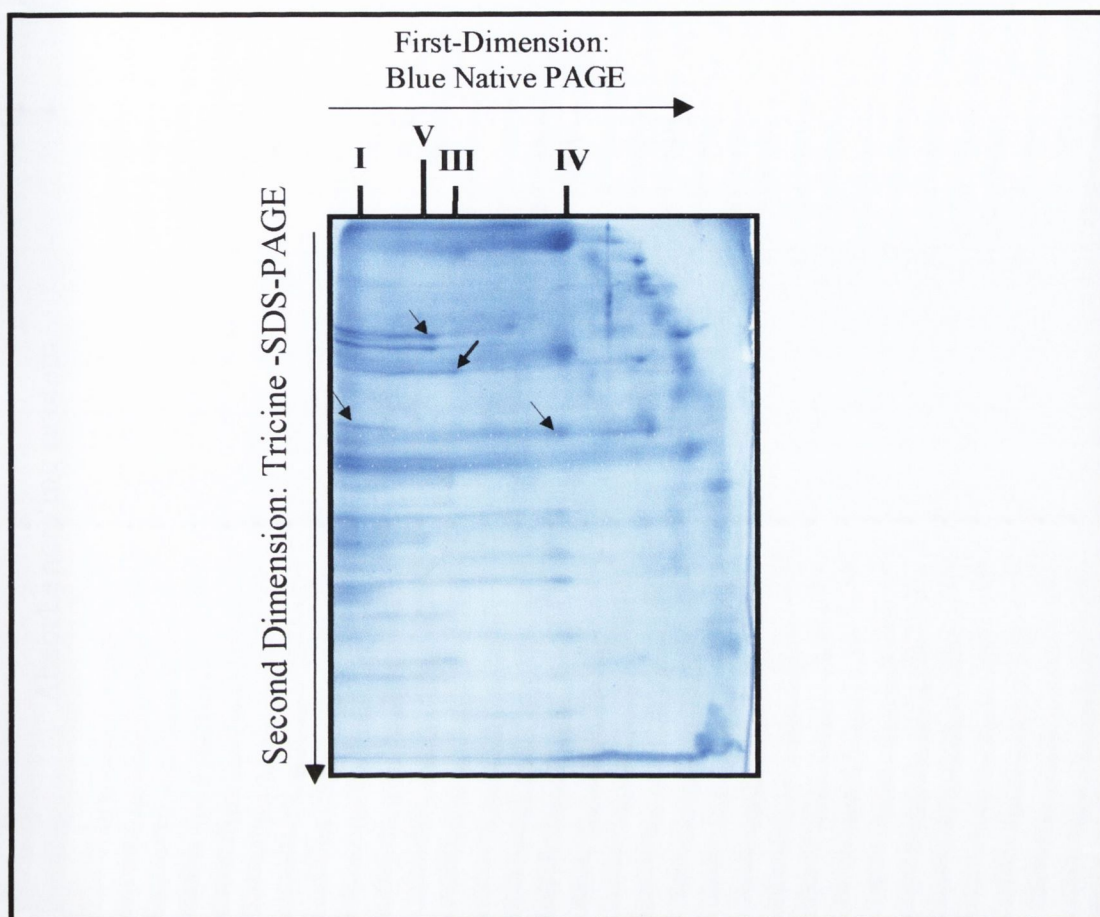


Figure 7.2.21. Two-dimensional resolution of mitochondrial proteins from rat synaptic mitochondria using blue native PAGE in the first-dimension and denaturing tricine-SDS-PAGE in the second-dimension. The membrane-protein complexes were separated in the native first-dimension and identified by their characteristic polypeptide patterns in the denaturing second-dimension. The gel was fixed, stained using Serva blue G and destained as described in Materials and Methods. The positions of complexes I, V, III and IV are indicated at the top of the gel. Arrows on the gel mark the protein subunits whose staining intensities or absorbances were measured for the quantification of second-dimension gels. The subunits of the complexes whose staining intensities were measured were as follows: 42kDa subunit of complex I, 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The rat synaptic mitochondrial proteins were solubilized using a 3:1 ratio of dodecyl maltoside to protein and resolved using blue native PAGE in the first-dimension (1mm gel, 5.5-13% non-linear gradient gel). A first-dimension lane loaded with 25 μ l (227 μ g protein) of solubilized rat synaptic mitochondrial protein, was cut from the first-dimension gel and the in-gel native proteins were denatured using 20mM DDT/1%SDS (see Materials and Methods). The mitochondrial proteins were resolved using a uniform 1mm 10% tricine-SDS gel in the second-dimension. The gel shown is representative of three gels that were used for the quantification of rat synaptic mitochondria.

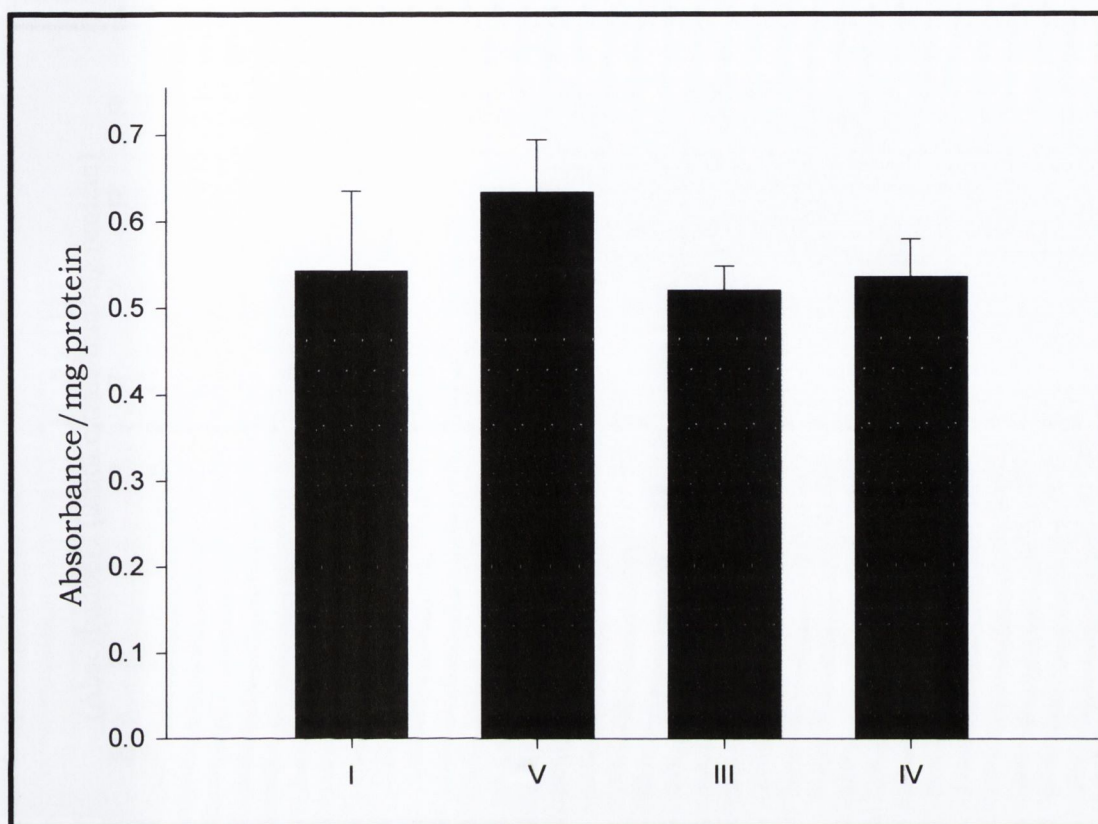


Figure 7.2.22. Comparison of the absorbances of complexes I, V, III and IV normalised per mg protein in mitochondria isolated from rat synaptosomes. All mitochondrial proteins were solubilized using dodecyl maltoside. The membrane-protein complexes I, V, III and IV were separated using blue native PAGE in the first-dimension and identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second-dimension. The second-dimension gel, which was fixed, stained using Serva blue G and destained as described in Materials and Methods, was used for densitometric analysis. The subunits used for densitometric analysis are indicated by arrows on the gel shown in Fig. 7.2.21. The subunits whose staining intensities were measured were as follows: 42kDa subunit of complex I, 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The graph labels are as follows: I: complex I; V: complex V; III: complex III and IV: complex IV. A 227 μ g amount of rat synaptic mitochondria was loaded on the first-dimension gel. All of the data represent the mean $\pm$ SEM of three determinations. The data are also represented in Table 7.2.1a and 7.2.1b.

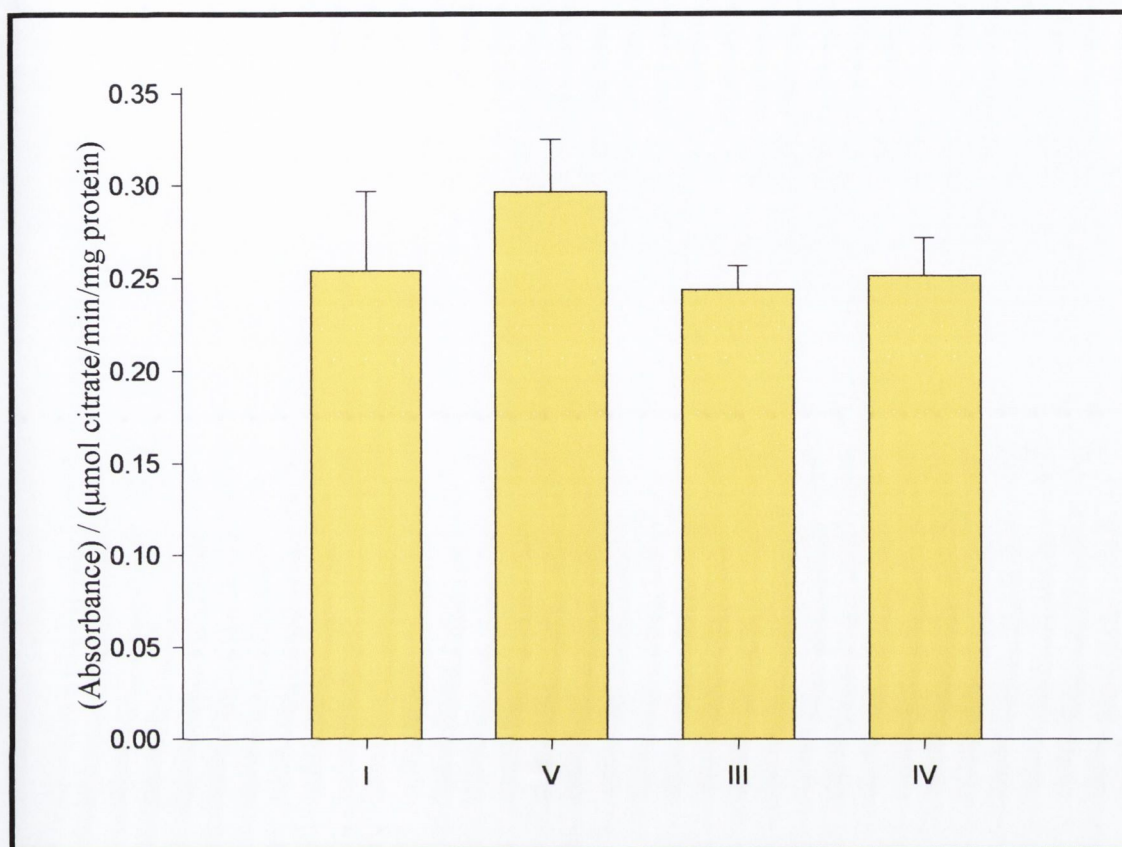


Figure 7.2.23. Comparison of the absorbances of complexes I, V, III and IV per μmol citrate synthase activity in mitochondria isolated from rat synaptosomes. All mitochondrial proteins were solubilized using dodecyl maltoside. The membrane-protein complexes I, V, III and IV were separated using blue native PAGE in the first-dimension and identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second-dimension. The second-dimension gel, which was fixed, stained using Serva blue G and destained as described in Materials and Methods, was used for densitometric analysis. The subunits used for densitometric analysis are indicated by arrows on the gel shown in Fig. 7.2.21. The subunits whose staining intensities were measured were as follows: 42kDa subunit of complex I, 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The graph labels are as follows: I: complex I; V: complex V; III: complex III and IV: complex IV. A 227 μg amount of rat synaptic mitochondria was loaded on the first dimension gel. All of the data represent the mean $\pm$ SEM of three determinations. The data are also represented in Table 7.2.2a and 7.2.2b.

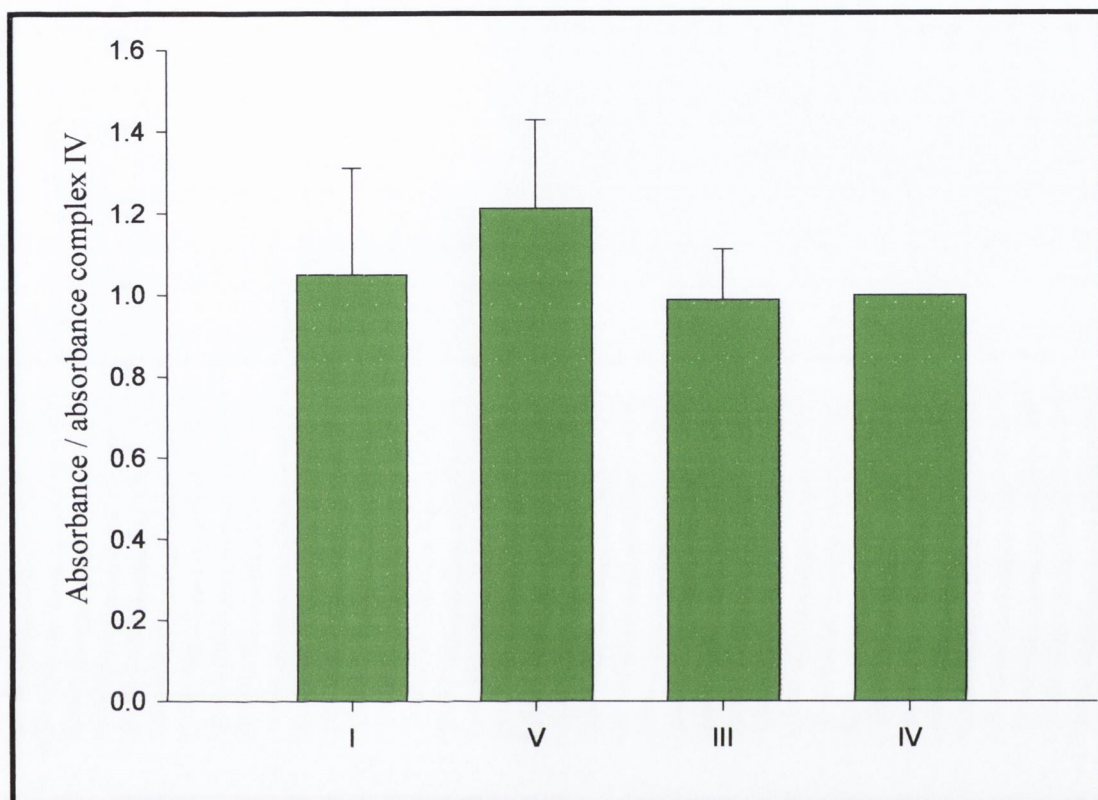


Figure 7.2.24. Comparison of the absorbances of complexes I, V, III and IV normalised to the absorbance of complex IV in mitochondria isolated from rat synaptosomes. All mitochondrial proteins were solubilized using dodecyl maltoside. The membrane-protein complexes I, V, III and IV were separated using blue native PAGE in the first-dimension and identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second-dimension. The second-dimension gel, which was fixed, stained using Serva blue G and destained as described in Materials and Methods, was used for densitometric analysis. The subunits used for densitometric analysis are indicated by arrows on the gel shown in Fig. 7.2.21. The subunits whose staining intensities were measured were as follows: 42kDa subunit of complex I, 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The graph labels are as follows: I: complex I; V: complex V; III: complex III and IV: complex IV. A 227 μ g amount of rat synaptic mitochondria was loaded on the first dimension gel. All of the data represent the mean $\pm$ SEM of three determinations. The data are also represented in Table 7.2.3.

complexes I, V, III and IV were 0.54 ± 0.09 , 0.63 ± 0.06 , 0.52 ± 0.03 and 0.54 ± 0.04 , respectively, when normalised per mg of protein. When the data shown in Figure 7.2.22 were normalised to IV=1, the I:V:III:IV ratio was found to be 1.00:1.16:0.96:1.00 (Table 7.2.1b).

Figure 7.2.23 shows the absorbances normalised per μmol of citrate synthase activity of complexes I, V, III and IV in mitochondria isolated from rat brain synaptosomes. The absorbances are also listed in Table 7.2.2a. The absorbances of complexes I, V, III and IV were 0.25 ± 0.04 , 0.30 ± 0.03 , 0.24 ± 0.01 and 0.25 ± 0.02 , respectively, when normalised to citrate synthase activity. When the data shown in Figure 7.2.23 were normalised to IV=1, the I:V:III:IV ratio was found to be 1.00:1.20:0.96:1.00 (Table 7.2.2b).

Figure 7.2.24 shows the absorbances of complexes I, V and III normalised to the absorbance of complex IV in mitochondria isolated from rat brain synaptosomes. The absorbances are also listed in Table 7.2.3. The absorbances of complexes I, V and III were 1.04 ± 0.26 , 1.21 ± 0.21 and 0.99 ± 0.12 , respectively, when normalised to the absorbance of complex IV.

Figure 7.2.25 shows the resolution in the second-dimension of beef heart mitochondrial proteins. The gels were stained using Serva blue G. First-dimension gel lanes loaded with $150\mu\text{g}$ of protein had the in-gel proteins denatured using 20mM DDT/1%SDS and the denatured proteins were resolved on a second-dimension denatured 10%-tricine-SDS gel. Complexes I, V, III and IV were identified on the basis of their characteristic patterns in the second-dimension. Complex I was quite diffuse and complex II could not be identified because of a large number of unidentified proteins in the same region of the gel. Arrows on the gel mark the protein subunits whose staining intensities were measured for the quantification of second-dimension gels. The gel shown in Fig 7.2.25 is representative of three gels that were used for the analysis of beef heart mitochondria.

Figure 7.2.26 shows the absorbances normalised per mg of protein loaded on the first-dimension gel of complexes I, V, III and IV in mitochondria isolated from beef heart. The absorbances are also listed in Table 7.2.1a. The absorbances of complexes I, V, III and IV were 1.17 ± 0.17 , 2.27 ± 0.15 , 1.65 ± 0.11 and 1.61 ± 0.18 , respectively, when normalised per mg of protein. When the data shown in Figure 7.2.26 were normalised to IV=1, the I:V:III:IV ratio was found to be 0.72:1.40:1.02:1.00 (Table 7.2.1b).

Figure 7.2.27 shows the absorbances normalised per μmol of citrate synthase activity of complexes I, V, III and IV in mitochondria isolated from beef heart. The absorbances are also listed in Table 7.2.2a. The absorbances of complexes I, V, III and IV were 0.43 ± 0.06 , 0.83 ± 0.06 , 0.60 ± 0.04 and 0.58 ± 0.06 , respectively, when normalised to citrate synthase activity. When the data shown in figure 7.2.27 were normalised to IV=1, the I:V:III:IV ratio was found to be 0.74:1.43:1.03:1.00 (Table 7.2.2b).

Figure 7.2.28 shows the absorbances of complexes I, V and III normalised to the absorbance of complex IV in mitochondria isolated from beef heart. The absorbances are also listed in Table 7.2.3. The absorbances of complexes I, V and III were 0.72 ± 0.02 , 1.42 ± 0.08 and 1.04 ± 0.07 , respectively, when normalised to the absorbance of complex IV.

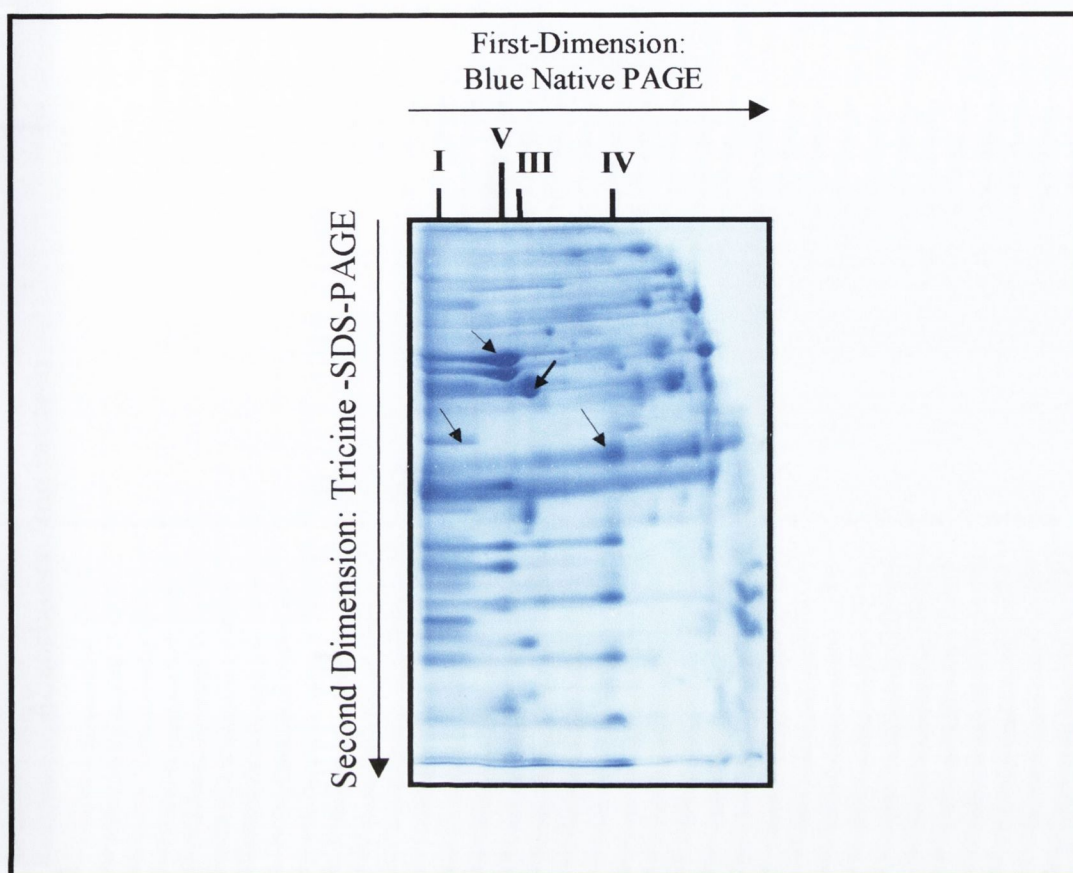


Figure 7.2.25. Two-dimensional resolution of mitochondrial proteins from beef heart mitochondria using blue native PAGE in the first-dimension and denaturing tricine-SDS-PAGE in the second-dimension. The membrane-protein complexes were separated in the native first-dimension and identified by their characteristic polypeptide patterns in the denaturing second-dimension. The gel was fixed, stained using Serva blue G and destained as described in Materials and Methods. The positions of the complexes I, V, III and IV are indicated at the top of the gel. Arrows on the gel mark the protein subunits whose staining intensities or absorbances were measured for the quantification of second-dimension gels. The subunits of the complexes whose staining intensities were measured were as follows: 42kDa subunit of complex I, 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The beef heart mitochondrial proteins were solubilized using a 3:1 ratio of dodecyl maltoside to protein and were resolved using blue native PAGE in the first-dimension (1mm gel, 5.5-13% non-linear gradient gel). A first-dimension lane loaded with 25 μ l (150 μ g protein) of solubilized beef heart mitochondrial protein was cut from the first-dimension gel and the in-gel native proteins were denatured using 20mM DDT/1%SDS (see Materials and Methods). The mitochondrial proteins were resolved using an uniform 1mm 10% tricine-SDS gel in the second-dimension. The gel shown is representative of three gels that were used for the quantification of beef heart mitochondria.

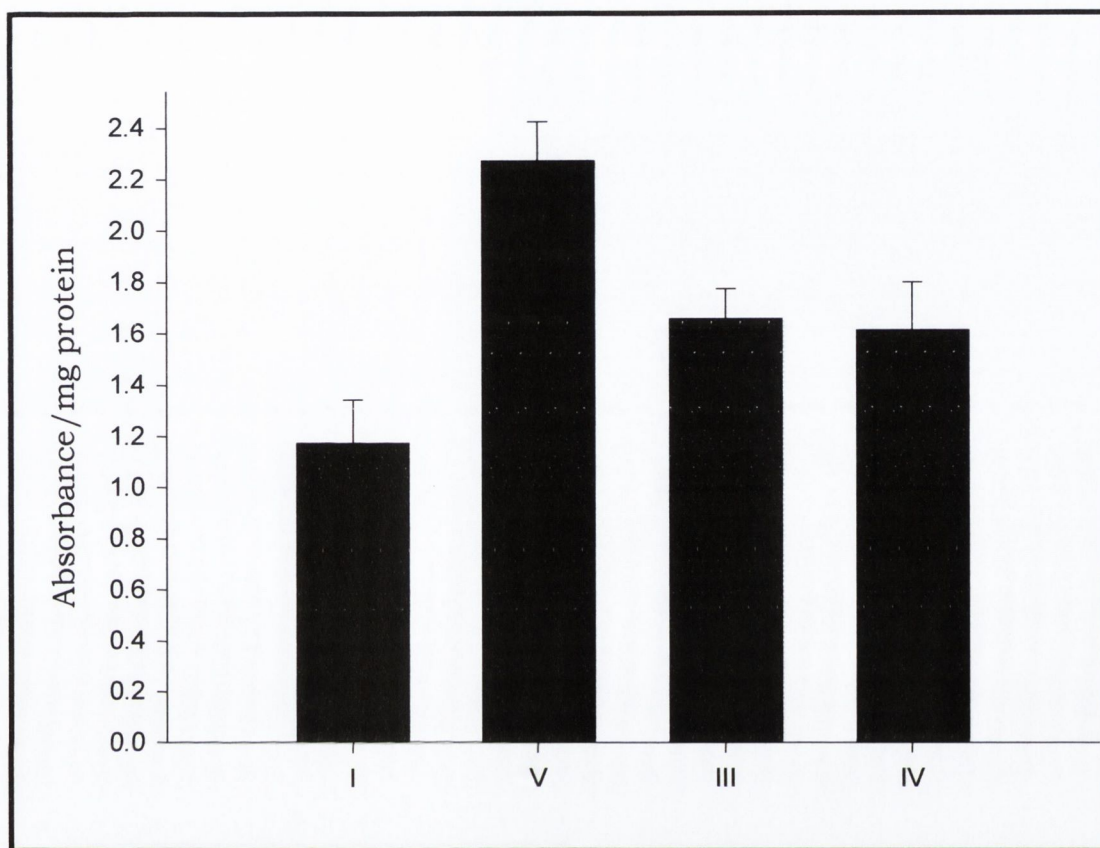


Figure 7.2.26. Comparison of the absorbances of complexes I, V, III and IV normalised per mg protein in mitochondria isolated from beef heart. All mitochondrial proteins were solubilized using dodecyl maltoside. The membrane-protein complexes I, V, III and IV were separated using blue native PAGE in the first-dimension and identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second-dimension. The second-dimension gel, which was fixed, stained using Serva blue G and destained as described in Materials and Methods, was used for densitometric analysis. The subunits used for densitometric analysis are indicated by arrows on the gel shown in Fig. 7.2.25. The subunits whose staining intensities were measured were as follows: 42kDa subunit of complex I, 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The graph labels are as follows: I: complex I; V: complex V; III: complex III and IV: complex IV. A 150 μ g amount of beef heart mitochondria was loaded on the first-dimension gel. All of the data represent the mean $\pm$ SEM of three determinations. The data are also represented in Table 7.2.1a and 7.2.1b.

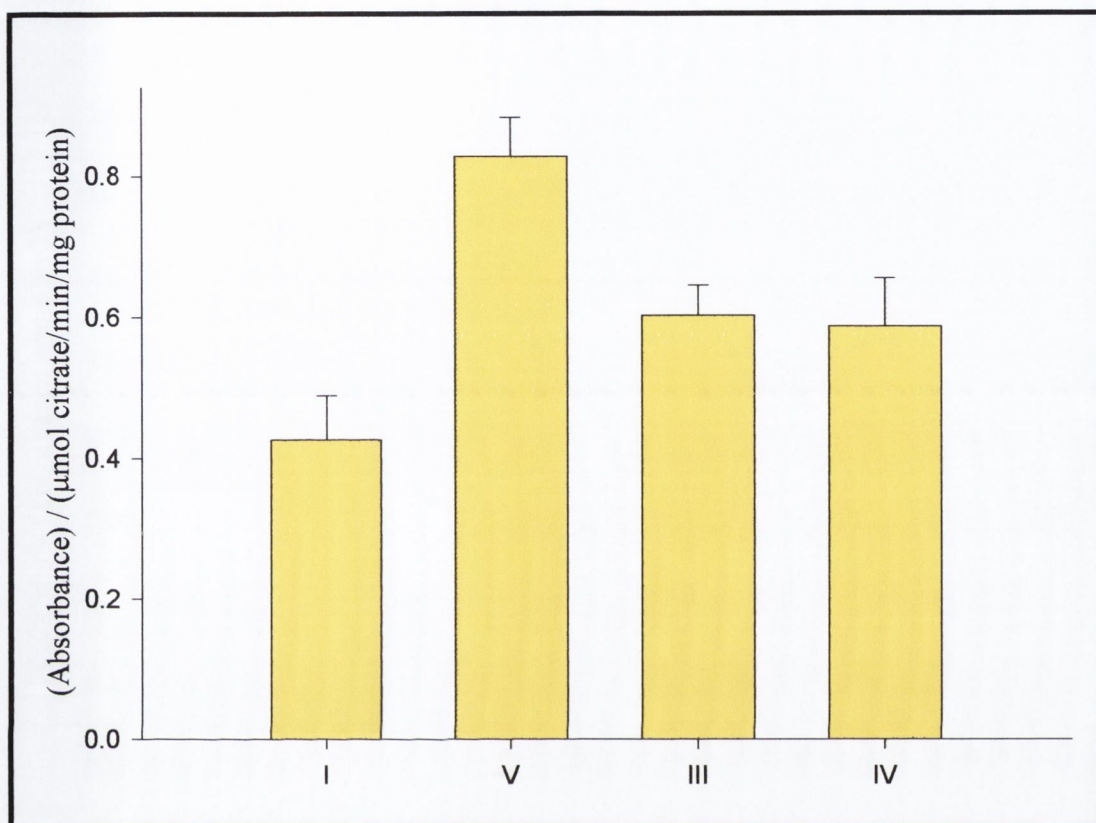


Figure 7.2.27. Comparison of the absorbances of complexes I, V, III and IV per μmol citrate synthase activity in mitochondria isolated from beef heart. All mitochondrial proteins were solubilized using dodecyl maltoside. The membrane-protein complexes I, V, III and IV were separated using blue native PAGE in the first-dimension and identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second-dimension. The second-dimension gel, which was fixed, stained using Serva blue G and destained as described in Materials and Methods, was used for densitometric analysis. The subunits used for densitometric analysis are indicated by arrows on the gel shown in Fig. 7.2.25. The subunits whose staining intensities were measured were as follows: 42kDa subunit of complex I, 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The graph labels are as follows: I: complex I; V: complex V; III: complex III and IV: complex IV. A 150 μg amount of beef heart mitochondria was loaded on the first-dimension gel. All of the data represent the mean $\pm$ SEM of three determinations. The data are also represented in Table 7.2.2a and 7.2.2b.

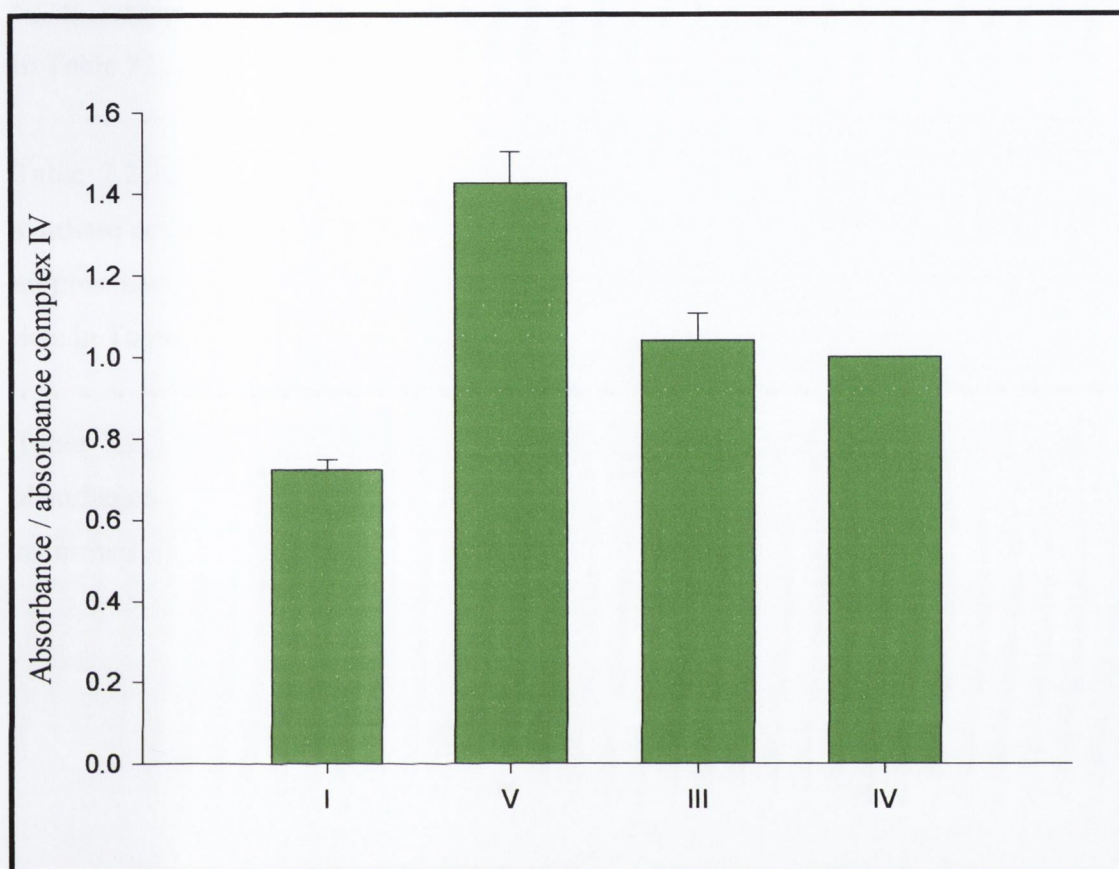


Figure 7.2.28. Comparison of the absorbances of complexes I, V, III and IV normalised to the absorbance of complex IV in mitochondria isolated from beef heart. All mitochondrial proteins were solubilized using dodecyl maltoside. The membrane-protein complexes I, V, III and IV were separated using blue native PAGE in the first-dimension and identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second-dimension. The second-dimension gel, which was fixed, stained using Serva blue G and destained as described in Materials and Methods, was used for densitometric analysis. The subunits used for densitometric analysis are indicated by arrows on the gel shown in Fig. 7.2.25. The subunits whose staining intensities were measured were as follows: 42kDa subunit of complex I, 55kDa α -subunit of complex V, the 47kDa core protein II of complex III and the 26kDa subunit 2 of complex IV. The graph labels are as follows: I: complex I; V: complex V; III: complex III and IV: complex IV. A 150 μ g amount of beef heart mitochondria was loaded on the first-dimension gel. All of the data represent the mean $\pm$ SEM of three determinations. The data are also represented in Table 7.2.3.

Table 7.2.1a lists the absorbances of complexes I, V, III and IV normalised per mg protein in mitochondria isolated from rat heart, rat kidney, rat liver, rat brown adipose tissue, rat skeletal muscle, rat synaptosomes and beef heart. Table 7.2.1b shows the data in Table 7.2.1a normalised to IV=1.

Table 7.2.2a lists the absorbances of complexes I, V, III and IV per μmol of citrate synthase activity in mitochondria isolated from rat heart, rat kidney, rat liver, rat brown adipose tissue, rat skeletal muscle, rat synaptosomes and beef. Table 7.2.2b shows the data in Table 7.2.2a normalised to IV=1

Table 7.2.3 lists the absorbances of complexes I, V, III and IV normalised to the absorbance of complex IV in mitochondria isolated from rat heart, rat kidney, rat liver, rat brown adipose tissue, rat skeletal muscle, rat synaptosomes and beef heart.

Table 7.2.1a

	I	V	III	IV
Rat heart mitochondria	0.52 ± 0.06	0.81 ± 0.03	0.68 ± 0.03	0.64 ± 0.02
Rat kidney mitochondria	0.61 ± 0.04	0.89 ± 0.01	0.68 ± 0.02	0.61 ± 0.03
Rat liver Mitochondria	0.39 ± 0.06	0.66 ± 0.06	0.36 ± 0.03	0.38 ± 0.05
Rat brown adipose tissue mitochondria	0.30 ± 0.06	0.41 ± 0.10	0.69 ± 0.08	0.70 ± 0.09
Rat skeletal muscle mitochondria	0.52 ± 0.34	0.78 ± 0.36	0.83 ± 0.35	0.68 ± 0.30
Rat synaptic mitochondria	0.54 ± 0.09	0.63 ± 0.06	0.52 ± 0.03	0.54± 0.04
Beef heart Mitochondria	1.17 ± 0.17	2.27 ± 0.15	1.65 ± 0.11	1.61 ± 0.18

Table 7.2.1a. Comparison of the absorbances of complexes I, V, III and IV normalised per mg protein in mitochondria isolated from rat heart, rat kidney, rat liver, rat brown adipose tissue, rat skeletal muscle, rat synaptosomes and beef heart.

All mitochondrial proteins were solubilized using dodecyl maltoside. The membrane-protein complexes I, V, III and IV were separated using blue native PAGE in the first-dimension and identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second-dimension. The second-dimension gel, which was fixed, stained using Serva blue G and destained as described in Materials and Methods, was used for densitometric analysis. The subunits used for densitometric analysis are indicated by arrows on the relevant gels (Figs. 7.2.1, 7.2.5, 7.2.9, 7.2.13, 7.2.17, 7.2.21, 7.2.24). The amounts of mitochondrial protein loaded per gel were: rat heart mitochondria (227µg), rat kidney mitochondria (454µg), rat liver mitochondria (454µg), rat brown adipose tissue mitochondria (227µg), rat skeletal muscle mitochondria (150µg), rat synaptic mitochondria (227µg) and beef heart mitochondria (150µg). All of the data represent the mean ± SEM of three determinations.

Table 7.2.1b

	I	V	III	IV
Rat heart mitochondria	0.81	1.26	1.06	1.00
Rat kidney mitochondria	1.00	1.48	1.13	1.00
Rat liver Mitochondria	1.02	1.73	0.94	1.00
Rat brown adipose tissue mitochondria	0.42	0.58	0.98	1.00
Rat skeletal muscle mitochondria	0.76	1.14	1.22	1.00
Rat synaptic mitochondria	1.00	1.16	0.96	1.00
Beef heart Mitochondria	0.72	1.40	1.02	1.00

Table 7.2.1b. Comparison of the data in Table 7.2.1a normalised to the absorbance of complex IV in mitochondria isolated from rat heart, rat kidney, rat liver, rat brown adipose tissue, rat skeletal muscle, rat synaptosomes and beef heart. The data shown in Table 7.2.1b are the data from Table 7.2.1a normalised to the absorbance of complex IV.

Table 7.2.2a

	I	V	III	IV
Rat heart mitochondria	0.12 ± 0.01	0.18 ± 0.01	0.16 ± 0.01	0.14 ± 0.01
Rat kidney mitochondria	1.26 ± 0.09	1.85 ± 0.02	1.42 ± 0.04	1.26 ± 0.07
Rat liver Mitochondria	4.65 ± 0.74	7.85 ± 0.79	4.35 ± 0.44	4.55 ± 0.66
Rat brown adipose tissue mitochondria	0.04 ± 0.01	0.06 ± 0.02	0.10 ± 0.01	0.10 ± 0.01
Rat skeletal muscle mitochondria	0.13 ± 0.09	0.20 ± 0.09	0.21 ± 0.09	0.17 ± 0.08
Rat synaptic mitochondria	0.25 ± 0.04	0.30 ± 0.03	0.24 ± 0.01	0.25 ± 0.02
Beef heart mitochondria	0.43 ± 0.06	0.83 ± 0.06	0.60 ± 0.04	0.58 ± 0.06

Table 7.2.2.a Comparison of the absorbances of complexes I, V, III and IV per μ mol citrate synthase activity in mitochondria isolated from rat heart, rat kidney, rat liver, rat brown adipose tissue, rat skeletal muscle, rat synaptosomes and beef heart. All mitochondrial proteins were solubilized using dodecyl maltoside. The membrane-protein complexes I, V, III and IV were separated using blue native PAGE in the first-dimension and identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second-dimension. The second-dimension gel, which was fixed, stained using Serva blue G and destained as described in Materials and Methods, was used for densitometric analysis. The subunits used for the densitometric analysis are indicated by arrows on the relevant gels (Figs. 7.2.1, 7.2.5, 7.2.9, 7.2.13, 7.2.17, 7.2.21, 7.2.24). All of the data represent the mean $\pm$ SEM of three determinations.

Table 7.2.2b

	I	V	III	IV
Rat heart mitochondria	0.86	1.29	1.14	1.00
Rat kidney mitochondria	1.00	1.47	1.13	1.00
Rat liver Mitochondria	1.02	1.72	0.96	1.00
Rat brown adipose tissue mitochondria	0.40	0.60	1.00	1.00
Rat skeletal muscle mitochondria	0.76	1.17	1.23	1.00
Rat synaptic mitochondria	1.00	1.20	0.96	1.00
Beef heart mitochondria	0.74	1.43	1.03	1.00

Table 7.2.2b. Comparison of the data in Table 7.2.2a normalised to the absorbance of complex IV in mitochondria isolated from rat heart, rat kidney, rat liver, rat brown adipose tissue, rat skeletal muscle, rat synaptosomes and beef heart. The data shown in table 7.2.2b are the data from Table 7.2.2a normalised to the absorbance of complex IV.

Table 7.2.3

	I	V	III	IV
Rat heart mitochondria	0.82 ± 0.12 <i>p</i> =0.039 a	1.27 ± 0.09 <i>p</i> =0.003 b	1.05 ± 0.08	1.00
Rat kidney mitochondria	1.01 ± 0.10 <i>p</i> =0.005 a	1.48 ± 0.07 <i>p</i> =0.001 b	1.14 ± 0.07	1.00
Rat liver mitochondria	1.06 ± 0.20 <i>p</i> =0.037 a	1.76 ± 0.11 <i>p</i> =0.001 b	0.98 ± 0.11	1.00
Rat brown adipose tissue mitochondria	0.43 ± 0.04 a	0.57 ± 0.06 b	0.99 ± 0.08	1.00
Rat skeletal muscle mitochondria	0.64 ± 0.16	1.13 ± 0.06 <i>p</i> =0.002 b	1.25 ± 0.09	1.00
Rat synaptic mitochondria	1.04 ± 0.26	1.21 ± 0.21 <i>p</i> =0.046 b	0.99 ± 0.12	1.00
Beef heart mitochondria	0.72 ± 0.02 <i>p</i> =0.004 a	1.42 ± 0.08 <i>p</i> =0.001 b	1.04 ± 0.07	1.00

Table 7.2.3. Comparison of the absorbances of complexes I, V, III and IV normalised to the absorbance of complex IV in mitochondria isolated from rat heart, rat kidney, rat liver, rat brown adipose tissue, rat skeletal muscle, rat synaptosomes and beef heart. All mitochondrial proteins were solubilized using dodecyl maltoside. The membrane-protein complexes I, V, III and IV were separated using blue native PAGE in the first-dimension and identified by their characteristic polypeptide patterns in the denaturing tricine-SDS-PAGE second-dimension. The second-dimension gel, which was fixed, stained using Serva blue G and destained as described in Materials and Methods, was used for densitometric analysis. The subunits used for the densitometric analysis are indicated by arrows on the relevant gels (Figs. 7.2.1, 7.2.5, 7.2.9, 7.2.13, 7.2.17, 7.2.21, 7.2.24). All of the data represent the mean ± SEM of three determinations. The labels a and b highlight the fact that an unpaired *Student's t*-test, two-tailed *p* value indicated a significant difference between brown adipose tissue complex I and the other labeled mitochondria (a) and between brown adipose tissue complex V and the other labeled mitochondria (b). *p* < 0.05 was considered significant.

7.3 Discussion

7.3.1 Rat heart mitochondria

Mitochondrial proteins from rat heart mitochondria were resolved in two dimensions using blue native PAGE in the first-dimension followed by tricine-SDS-PAGE in the second-dimension. Four complexes could be identified on the second-dimension gel after staining using Serva blue G: namely complexes I, V, III and IV (fig. 7.2.1). Complex II could not be detected using a Coomassie stain. The second-dimension tricine gel was used for densitometric analysis.

The absorbances of selected subunits of the complexes were determined in the rat heart mitochondria. When the absorbances were normalised to protein loaded on the gel (Fig 7.2.2 and Table 7.2.1a), the values for complexes I, V, III and IV were 0.52 ± 0.06 , 0.81 ± 0.03 , 0.68 ± 0.03 and 0.64 ± 0.027 , respectively. When these values were normalised to IV=1, the I:V:III:IV ratio was found to be 0.81:1.26:1.06:1.00 (Table 7.2.1b). When the absorbances of the selected subunits of the complexes were normalised to mitochondrial citrate synthase activity (Fig 7.2.3 and Table 7.2.2a), the values for complexes I, V, III and IV were 0.12 ± 0.01 , 0.18 ± 0.01 , 0.16 ± 0.01 and 0.14 ± 0.01 , respectively. When these values were normalised to IV=1, the I:V:III:IV ratio was found to be 0.86:1.29:1.14:1.00 (table 7.2.2b). When the absorbances of the selected subunits of the complexes were normalised to the in-gel absorbance of complex IV (Fig 7.2.4 and Table 7.2.3), the values for complexes I, V, III and IV were 0.82 ± 0.12 , 1.27 ± 0.09 , 1.05 ± 0.08 and 1.00, respectively.

When the data from the three different methods were normalised to IV=1, it was clear that the results were similar and provided essentially the same results. Complex V was present in greatest abundance in rat heart mitochondria, followed by approximately equal amounts of complexes III and IV. Complex I was present in the least amount relative to the other complexes in rat heart mitochondria.

7.3.2 Rat kidney mitochondria

Mitochondrial proteins from rat kidneys were resolved in two dimensions using blue native PAGE in the first-dimension followed by tricine-SDS-PAGE in the second-dimension. Four complexes could be identified on the second-dimension gel after staining using Serva blue G: namely complexes I, V, III and IV (fig. 7.2.5). Complex II could not be detected using a Coomassie stain. The second-dimension tricine gel was used for densitometric analysis.

The absorbances of selected subunits of the complexes were determined in the rat kidney mitochondria. When the absorbances were normalised to protein loaded on the gel (Fig 7.2.6 and Table 7.2.1a), the values for complexes I, V, III and IV were 0.61 ± 0.04 , 0.89 ± 0.01 , 0.68 ± 0.02 and 0.61 ± 0.03 , respectively. When these values were normalised to IV=1, the I:V:III:IV ratio was found to be 1.00:1.48:1.13:1.00 (Table 7.2.1b). When the absorbances of the selected subunits of the complexes were normalised to mitochondrial citrate synthase activity (Fig 7.2.7 and Table 7.2.2a), the values for complexes I, V, III and IV were 1.26 ± 0.09 , 1.85 ± 0.02 , 1.42 ± 0.04 and 1.26 ± 0.07 , respectively. When these values were normalised to IV=1, the I:V:III:IV ratio was found to be 1.00:1.47:1.13:1.00 (Table 7.2.2b). When the absorbances of the selected subunits of the complexes were normalised to the in-gel absorbance of complex IV (Fig 7.2.8 and Table 7.2.3), the values for complexes I, V, III and IV were 1.01 ± 0.10 , 1.48 ± 0.07 , 1.14 ± 0.07 and 1.00 respectively.

When the data from the three different methods were normalised to IV=1, it was clear that the results were similar and provided essentially the same results. Complex V was present in greatest abundance in rat kidney mitochondria, followed by complex III. Complexes I and IV were present in the same amount relative to the other complexes in rat kidney mitochondria.

7.3.3 Rat liver mitochondria

Mitochondrial proteins from rat liver were resolved in two dimensions using blue native PAGE in the first-dimension followed by tricine-SDS-PAGE in the second-dimension.

Four complexes could be identified on the second-dimension gel after staining using Serva blue G: namely complexes I, V, III and IV (fig. 7.2.9). Complex II could not be detected using a coomassie stain. The second dimension tricine gel was used for densitometric analysis.

The absorbances of selected subunits of the complexes were determined in the rat liver mitochondria. When the absorbances were normalised to protein loaded on the gel (Fig 7.2.10 and Table 7.2.1a), the values for complexes I, V, III and IV were 0.39 ± 0.06 , 0.66 ± 0.06 , 0.36 ± 0.03 and 0.38 ± 0.05 , respectively. When these values were normalised to IV=1, the I:V:III:IV ratio was found to be 1.02:1.73:0.94:1.00 (Table 7.2.1b). When the absorbances of the selected subunits of the complexes were normalised to mitochondrial citrate synthase activity (Fig 7.2.11 and Table 7.2.2a), the values for complexes I, V, III and IV were 4.65 ± 0.74 , 7.85 ± 0.79 , 4.35 ± 0.44 and 4.55 ± 0.66 , respectively. When these values were normalised to IV=1, the I:V:III:IV ratio was found to be 1.02:1.72:0.96:1.00 (Table 7.2.2b). When the absorbances of the selected subunits of the complexes were normalised to the in-gel absorbance of complex IV (Fig 7.2.12 and Table 7.2.3), the values for complexes I, V, III and IV were 1.06 ± 0.20 , 1.76 ± 0.11 , 0.98 ± 0.11 and 1.00, respectively.

When the data from the three different methods were normalised to IV=1, it was clear that the results were similar and provided essentially the same results. Complex V was present in greatest abundance in rat liver mitochondria. Complexes I, III and IV were present in approximately the same amount relative to the other complexes in rat liver mitochondria.

7.3.4 Rat brown adipose tissue (BAT) mitochondria

Mitochondrial proteins from rat BAT were resolved in two dimensions using blue native PAGE in the first-dimension followed by tricine-SDS-PAGE in the second-dimension. Four complexes could be identified on the second-dimension gel after staining using Serva blue G: namely complexes I, V, III and IV (fig. 7.2.13). Complex II could not be

detected using a Coomassie stain. The second-dimension tricine gel was used for densitometric analysis.

The absorbances of selected subunits of the complexes were determined in the rat BAT mitochondria. When the absorbances were normalised to protein loaded on the gel (Fig 7.2.14 and Table 7.2.1a), the values for complexes I, V, III and IV were 0.30 ± 0.06 , 0.41 ± 0.10 , 0.69 ± 0.08 and 0.70 ± 0.09 , respectively. When these values were normalised to IV=1, the I:V:III:IV ratio was found to be 0.42:0.58:0.98:1.00 (Table 7.2.1b). When the absorbances of the selected subunits of the complexes were normalised to mitochondrial citrate synthase activity (Fig 7.2.15 and Table 7.2.2a), the values for complexes I, V, III and IV were 0.04 ± 0.01 , 0.06 ± 0.02 , 0.10 ± 0.01 and 0.10 ± 0.01 , respectively. When these values were normalised to IV=1, the I:V:III:IV ratio was found to be 0.40:0.60:1.00:1.00 (Table 7.2.2b). When the absorbances of the selected subunits of the complexes were normalised to the in-gel absorbance of complex IV (Fig 7.2.16 and Table 7.2.3), the values for complexes I, V, III and IV were 0.43 ± 0.04 , 0.57 ± 0.06 , 0.99 ± 0.08 and 1.00, respectively.

When the data from the three different methods were normalised to IV=1, it was clear that the results were similar and provided essentially the same results. Complexes III and IV were present in approximately the same amount in rat brown adipose tissue mitochondria, with complexes I and V present in lesser amounts. The situation was different in BAT mitochondria compared with the mitochondria isolated from other tissues in the rat. The relative levels of complexes I and V were very much reduced in relation to the content of complex IV. The relative level of complex I in rat brown adipose tissue was compared to the relative levels of complex I in the other rat tissues and in beef heart mitochondria. It was found that relative to complex IV, the level of complex I in rat brown adipose tissue (0.43 ± 0.04 , table 7.2.3) was significantly less than the level of complex I found in rat heart mitochondria ($p=0.039$, table 7.2.3), rat kidney mitochondria ($p=0.005$, table 7.2.3), rat liver mitochondria ($p=0.037$, table 7.2.3) and beef heart mitochondria ($p=0.004$, table 7.2.3). However, the reduced quantity of

complex I was unexpected, as there have been no reports of reduced amounts of complex I in BAT mitochondria.

Also, the relative level of complex V in rat brown adipose tissue was compared to the relative levels of complex V in the other rat tissues and in beef heart mitochondria. It was found that relative to complex IV, the level of complex V in rat brown adipose tissue (0.57 ± 0.06 , table 7.2.3) was significantly less than the level of complex V found in rat heart mitochondria ($p=0.003$, table 7.2.3), rat kidney mitochondria ($p=0.005$, table 7.2.3), rat liver mitochondria ($p=0.001$, table 7.2.3), rat skeletal muscle mitochondria ($p=0.002$, table 7.2.3), rat synaptic mitochondria ($p=0.046$, table 7.2.3), and beef heart mitochondria ($p=0.001$, table 7.2.3). The results for BAT presented here are in good agreement with other reports which show that the content of mitochondrial ATP synthase is exceptionally low in mouse brown adipose tissue (Cannon and Vogel, 1977; Houstek *et al.*, 1978) and that complex V content was reduced in hamster brown adipose tissue (Houstek and Drahota, 1977). Indeed, Houstek *et al.*, (1991) determined the content of complex IV and complex V in various mouse tissues using an antibody to the COX IV subunit of complex IV and an antibody to the β -subunit of complex V, the results of which showed a lesser level of complex V in mouse brown adipose tissue. The complex V/IV ratio was 0.17 in mouse brown adipose tissue, 0.97 in mouse liver, 1.00 in mouse heart, 1.88 in mouse skeletal muscle and 0.85 in brain mitochondria.

7.3.5 Rat skeletal muscle mitochondria

Mitochondrial proteins from rat skeletal muscle mitochondria were resolved in two dimensions using blue native PAGE in the first-dimension followed by tricine-SDS-PAGE in the second-dimension. Four complexes could be identified on the second-dimension gel after staining using Serva blue G: namely complexes I, V, III and IV (Fig. 7.2.17). Complex II could not be detected using a Coomassie stain. The second-dimension tricine gel was used for densitometric analysis.

The absorbances of selected subunits of the complexes were determined in the rat skeletal muscle mitochondria. When the absorbances were normalised to protein loaded on the

gel (Fig 7.2.18 and Table 7.2.1a), the values for complexes I, V, III and IV were 0.52 ± 0.34 , 0.78 ± 0.36 , 0.83 ± 0.35 and 0.68 ± 0.30 , respectively. When these values were normalised to IV=1, the I:V:III:IV ratio was found to be 0.76:1.14:1.22:1.00 (Table 7.2.1b). When the absorbances of the selected subunits of the complexes were normalised to mitochondrial citrate synthase activity (Fig 7.2.19 and Table 7.2.2a), the values for complexes I, V, III and IV were 0.13 ± 0.09 , 0.20 ± 0.09 , 0.21 ± 0.09 and 0.17 ± 0.08 , respectively. When these values were normalised to IV=1, the I:V:III:IV ratio was found to be 0.76:1.17:1.23:1.00 (Table 7.2.2b). When the absorbances of the selected subunits of the complexes were normalised to the in-gel absorbance of complex IV (Fig 7.2.20 and Table 7.2.3), the values for complexes I, V, III and IV were 0.64 ± 0.16 , 1.13 ± 0.06 , 1.25 ± 0.09 and 1.00, respectively.

When the data from the three different methods were normalised to IV=1, it was clear that the results were similar and provided essentially the same results. Complex III was present in the greatest amount in rat skeletal muscle mitochondria, with complexes I, IV and V present in lesser amounts in the order $V > IV > I$.

7.3.6. Rat synaptic mitochondria

Mitochondrial proteins from rat brain synaptosomal mitochondria were resolved in two dimensions using blue native PAGE in the first-dimension followed by tricine-SDS-PAGE in the second-dimension. Four complexes could be identified on the second-dimension gel after staining using Serva blue G: namely complexes I, V, III and IV (Fig. 7.2.21). Complex II could not be detected using a Coomassie stain. The second-dimension tricine gel was used for densitometric analysis. The synaptic mitochondria analyzed in the study were isolated from synaptosomes using hypotonic lysis. As the mitochondria isolated from the synaptosomes were not further purified using a Percoll gradient, there were probably some synaptic-membrane proteins associated with the synaptic mitochondria.

The absorbances of selected subunits of the complexes were determined in the rat brain synaptosomal mitochondria. When the absorbances were normalised to protein loaded on

the gel (Fig 7.2.22 and Table 7.2.1a), the values for complexes I, V, III and IV were 0.54 ± 0.09 , 0.63 ± 0.06 , 0.52 ± 0.03 , respectively. When these values were normalised to IV=1, the I:V:III:IV ratio was found to be 1.00:1.16:0.96:1.00 (Table 7.2.1b). When the absorbances of the selected subunits of the complexes were normalised to mitochondrial citrate synthase activity (Fig 7.2.23 and Table 7.2.2a), the values for complexes I, V, III and IV were 0.25 ± 0.04 , 0.30 ± 0.03 , 0.24 ± 0.01 and 0.25 ± 0.02 , respectively. When these values were normalised to IV=1, the I:V:III:IV ratio was found to be 1.00:1.20:0.96:1.00 (Table 7.2.2b). When the absorbances of the selected subunits of the complexes were normalised to the in-gel absorbance of complex IV (Fig 7.2.24 and Table 7.2.3), the values for complexes I, V, III and IV were 1.04 ± 0.26 , 1.21 ± 0.21 , 0.99 ± 0.12 and 1.00, respectively.

When the data from the three different methods were normalised to IV=1, it was clear that the results were similar and provided essentially the same results. Complex V was present in the greatest amount, with complexes, I, III and IV being present in approximately equal amounts in rat synaptic mitochondria.

7.3.7. Beef heart mitochondria

Mitochondrial proteins from beef heart mitochondria were resolved in two dimensions using blue native PAGE in the first-dimension followed by tricine-SDS-PAGE in the second-dimension. Four complexes could be identified on the second-dimension gel after staining using Serva blue G: namely complexes I, V, III and IV (fig. 7.2.24). The second-dimension tricine gel was used for densitometric analysis.

The absorbances of selected protein subunits of the complexes were determined in the beef heart mitochondria. When the absorbances were normalised to protein loaded on the gel (Fig 7.2.25 and Table 7.2.1a), the values for complexes I, V, III and IV were 1.17 ± 0.17 , 2.27 ± 0.15 , 1.65 ± 0.11 and 1.61 ± 0.18 , respectively. When these values were normalised to IV=1, the I:V:III:IV ratio was found to be 0.72:1.40:1.02:1.00 (Table 7.2.1b). When the absorbances of the selected subunits of the complexes were normalised to mitochondrial citrate synthase activity (Fig 7.2.26 and Table 7.2.2a), the

values for complexes I, V, III and IV were 0.43 ± 0.06 , 0.83 ± 0.06 , 0.60 ± 0.04 and 0.58 ± 0.06 , respectively. When these values were normalised to IV=1, the I:V:III:IV ratio was found to be 0.74:1.43:1.03:1.00 (Table 7.2.2b). When the absorbances of the selected subunits of the complexes were normalised to the in-gel absorbance of complex IV (Fig 7.2.24 and Table 7.2.3), the values for complexes I, V, III and IV were 0.72 ± 0.02 , 1.42 ± 0.08 , 1.04 ± 0.07 and 1.00, respectively.

When the data from the three different methods were normalised to IV=1, it was clear that the results were similar and provided essentially the same results. Complex V was present in the greatest amount in beef heart mitochondria. Complexes III and IV were present in lesser amounts than complex V but were present in equal amounts to each other. Complex I is present in the least amount in beef heart mitochondria.

7.4. Conclusions

The results shown in Chapter 7 are novel because this is the first time mitochondria isolated from rat heart, rat kidney, rat brown adipose tissue, rat skeletal muscle mitochondria and rat brain synaptic mitochondria were resolved using blue native/tricine-SDS-PAGE two-dimensional electrophoresis. The polypeptide patterns of the mitochondrial proteins were different in all of the mitochondria examined, however the significance of these differences isn't known because the identity of many of the proteins isn't known. The polypeptide pattern of complexes I, III, IV and V was identical when resolved using blue native/tricine-SDS-PAGE two-dimensional electrophoresis in all of the mitochondria examined.

In BAT mitochondria, complex I and complex V were greatly reduced relative to the level of complex IV. The relative level of complex I in rat brown adipose tissue was compared to the relative levels of complex I in the other rat tissues and in beef heart mitochondria. Relative to complex IV, the level of complex I in rat brown adipose tissue was significantly less than the level of complex I found in rat heart mitochondria, rat kidney mitochondria, rat liver mitochondria and beef heart mitochondria. The finding that brown adipose tissue mitochondria have a reduced content of complex I, as shown by

blue native/tricine-SDS-PAGE two-dimensional electrophoresis is novel and unexpected as there have been no reports of reduced amounts of complex I in BAT mitochondria.

The relative level of complex V in rat brown adipose tissue was compared to the relative levels of complex IV in the other rat tissues and in beef heart mitochondria. It was found that relative to complex IV, the level of complex V in rat brown adipose tissue was significantly less than the level of complex V found in rat heart mitochondria, rat kidney mitochondria, rat liver mitochondria, rat skeletal muscle mitochondria, rat synaptic mitochondria, and beef heart mitochondria. Our results showing a decreased level of complex V in rat brown adipose tissue mitochondria confirm previous reports that showed the content of complex V was exceptionally low in brown adipose tissue (Cannon and Vogel, 1977, Houstek *et al.*, 1978). Here is the first time that the low content of complex V in BAT was demonstrated using two dimensional blue native/tricine-SDS-PAGE two dimensional electrophoresis. Similar to the results discussed here, Houstek *et al.*, (1991) determined the content of complex IV and complex V in various mouse tissues using an antibody to the cytochrome oxidase IV subunit of complex IV and an antibody to the β -subunit of complex V. The results of which showed, relative to the level of the in gel complex IV, a lesser level of complex V in mouse brown adipose tissue than in mouse liver, mouse heart, mouse skeletal muscle and in mouse brain mitochondria.

The finding that different amounts of dodecyl maltoside were required to solubilize mitochondrial proteins from different mitochondria was novel. The fact that different amounts of dodecyl maltoside were required to solubilize the various mitochondria could be due to different lipid compositions of the mitochondrial inner membranes. Rolfe *et al.* (1994) examined the lipid:protein ratio (mg/mg) of mitochondria isolated from rat liver, kidney, brain and skeletal muscle. The lipid:protein ratios of liver and kidney mitochondria was quite similar being, 0.19 and 0.21, respectively. A dodecyl maltoside:protein ratio of 1:1 was required to solubilize both rat liver and kidney mitochondrial proteins. The lipid:protein (mg/mg) ratio of brain mitochondria was 0.35 and a dodecyl maltoside:protein ratio of 2:1 was required to solubilize rat synaptic

mitochondrial proteins. The lipid:protein (mg/mg) ratio of skeletal muscle mitochondria was 0.46 and a dodecyl maltoside:protein (mg/mg) ratio of 3:1 was required to solubilize rat skeletal muscle mitochondrial proteins. The results listed here seem to suggest that the higher the lipid:protein ratio of mitochondria, the more dodecyl maltoside required to solubilize mitochondrial membrane proteins.

Chapter 8

General Discussion

Experiments were designed to investigate for the first time *in situ* synaptosomal mitochondrial bioenergetic function using synaptosomes prepared from the whole brain of rats and to compare bioenergetic functions in synaptosomes isolated from young (3 month) and old (24 month) rats. The investigation describes for the first time the pattern of control of mitochondrial energy metabolism based on the kinetic responses of the substrate oxidation, proton leak and phosphorylation systems to changes in the mitochondrial membrane potential ($\Delta\Psi_m$) in synaptosomes isolated from the whole brain of rat. In addition, a comparison was made of the aforementioned kinetic responses, in synaptosomes isolated from old and young animals.

Mitochondria in synaptosomes, *in situ*, from whole rat brain do not work very hard under resting conditions. The majority of the energy requirements for maintaining resting synaptosomal function are met from other metabolic sources. It was demonstrated that 85% of the resting oxygen consumption of synaptosomes isolated from both young and old rats, was a result of mitochondrial proton leak (and possibly other ion cycling) across the mitochondrial inner membrane. There was a significantly lower rate of oxygen consumption in the presence of uncoupler by synaptosomes isolated from the old rats, when compared with synaptosomes isolated from young rats. These results demonstrate that the essential difference between synaptosomes isolated from old rats, when compared with synaptosomes isolated from young rats, is an impaired flux through the synaptosomal substrate oxidation system. There were no significant age differences in the kinetic responses of the substrate oxidation system, the mitochondrial proton leak and the phosphorylation system to changes in the proton electrochemical gradient. Thus the age-dependent decreases in the amounts of certain respiratory chain complexes, which have been reported to occur, do not significantly affect mitochondrial energy producing functions within the synaptosome. These results have implications for the theory that an age-dependent decline in mitochondrial function may underlie some neurodegenerative diseases. However, as synaptosomes from whole rat brain were used in these studies, it is possible that greater levels of impairment may occur in specific brain regions.

Metabolic control analysis was applied to synaptosomal *in situ* mitochondria isolated from young and old rats to determine if the flux control coefficients change with age. This was the first time metabolic control analysis was applied to synaptosomal *in situ* mitochondria. The pattern of control was similar in synaptosomes from young and old animals. The flux control coefficients show that the control of flux through the substrate oxidation system, the proton leak and control of the mitochondrial membrane potential was mainly by the substrate oxidation system at maximal ATP-producing capacity. Proton leak, as expected, had little control at maximal ATP-producing capacity. Control of the flux through the phosphorylation system was mainly by the substrate oxidation system in young rat brain synaptosomes, but mainly by the phosphorylating system itself in the old rat brain synaptosomes, however, the substrate oxidation system has a similar degree of control of flux through the phosphorylating system. The control over the mitochondrial membrane potential lies with the substrate oxidation system in synaptosomal *in situ* mitochondria isolated from young and old rat brains. The P/O ratio was controlled mainly by the phosphorylating system in young and old rat brain synaptosomes.

Future work in this area would involve investigation of *in situ* synaptosomal mitochondrial bioenergetic function using synaptosomes prepared from specific brain regions such as the hippocampus. Due to the quantity of material required to perform these experiments, it would not be possible to carry out a comparison of specific brain regions using rat brain. For this reason, future work to investigate the *in situ* synaptosomal mitochondrial bioenergetic function using synaptosomes prepared from specific brain regions would have to be carried out using rabbits or pigs.

Chapter 5 showed that the use of nagarse in the isolation of muscle mitochondria, namely beef heart, altered the polypeptide pattern of mitochondrial membrane proteins and in particular complexes I, II, III, IV and V when resolved using blue native/tricine-SDS-PAGE two-dimensional electrophoresis. Any work on muscle tissue mitochondria isolated using nagarse will have to consider that mitochondrial proteins are digested so

that the characteristic protein pattern of mitochondrial proteins resolved in two dimensions is absent when compared with mitochondria isolated without using nargarse.

The purpose of chapter 6 and 7 was to compare levels of complexes I, III, IV and V as resolved using blue native/tricine-SDS-PAGE two-dimensional electrophoresis in different types mitochondria. The approach used in chapters 6 and 7 to compare the mitochondria isolated from young and old rats and to compare mitochondria from various rat tissues and beef heart mitochondria is similar to a technique used by Schägger and Ohm (1995) and Bentlage *et al.* (1995). Schägger and Ohm (1995) used blue native/tricine-SDS-PAGE two-dimensional electrophoresis to compare hippocampal mitochondrial proteins of control and Alzheimer's brain. The levels of complexes IV and V were determined relative to the in-gel complex III and a complex V deficiency was detected. Bentlage *et al.* (1995) used blue native/tricine-SDS-PAGE two-dimensional electrophoresis to compare skeletal muscle mitochondrial proteins of control and encephalomyopathy patients. Bentlage *et al.* (1995) determined the levels of complexes I, III and IV relative to the in-gel complex V and found where there was a decrease in the activity of a complex, the level of that complex decreased relative to complex V.

The results in Chapter 6 are novel in that this is the first time blue native/tricine-SDS-PAGE two-dimensional electrophoresis was used in an aging comparison or for a comparison of mitochondria isolated from euthyroid and hyperthyroid animals. In the age comparison, significant differences were found in a comparison of mitochondria isolated from young rat liver with mitochondria isolated from old rat liver. The levels of complexes I, V, III and IV, when normalised per mg protein and when normalised to citrate synthase activity, were shown to be significantly less in liver mitochondria isolated from old rats compared with liver mitochondria isolated from young rats. However, only complex IV showed a significant decrease when normalised to the in gel complex V in liver mitochondria isolated from old rats compared with liver mitochondria isolated from young rats. The results found in the rat liver mitochondria comparison were surprising given that liver is a mitotic tissue and aging effects are hypothesized to be manifest greater in post mitotic tissues. The levels of complexes I, III, IV and V were

shown to have increased in synaptic mitochondria isolated from old rats compared with synaptic mitochondria isolated from young rats when the absorbances were normalised to citrate synthase activity. When the levels of complexes I, III and V were normalised to the in gel complex IV, there were no significant differences in mitochondria isolated from young and old rat brain synaptosomes. The results obtained for synaptic mitochondria were novel in that this was the first time proteins from synaptic mitochondria isolated from whole brain were resolved using blue native/tricine-SDS-PAGE two-dimensional electrophoresis. Previously, Schägger and Ohm (1995) used blue native/tricine-SDS-PAGE two-dimensional electrophoresis to compare hippocampal mitochondrial proteins of control and Alzheimer's brain, that is to say, there was no distinction between synaptic and non-synaptic mitochondria. No significant difference was found in the levels of complexes I, III, IV and V in a comparison of young and old rat heart mitochondria or in a comparison of euthyroid and hyperthyroid rat liver mitochondria.

Future work in an aging comparison would involve comparing mitochondria isolated from discrete regions of rat brain or specific regions of human brain (*e.g.* hippocampus) to determine if there are any age-related changes.

The results shown in Chapter 7 are novel because this is the first time mitochondria isolated from rat heart, rat kidney, rat brown adipose tissue, rat skeletal muscle mitochondria and rat brain synaptic mitochondria were resolved using blue native/tricine-SDS-PAGE two-dimensional electrophoresis. Rat brown adipose tissue mitochondria were different when compared to mitochondria from the other rat tissues examined and mitochondria from beef heart. In rat brown adipose tissue mitochondria, complex I and complex V were greatly reduced relative to the level of complex IV. The relative level of complex I in rat brown adipose tissue was compared to the relative levels of complex I in the other rat tissues and in beef heart mitochondria. It was found that relative to complex IV, the level of complex I in rat brown adipose tissue was significantly less than the level of complex I found in rat heart mitochondria, rat kidney mitochondria, rat liver mitochondria and beef heart mitochondria. The finding that rat brown adipose tissue mitochondria have a reduced content of complex I, as shown by blue native/tricine-SDS-

PAGE two-dimensional electrophoresis is novel and unexpected as there have been no reports of reduced amounts of complex I in BAT mitochondria.

The relative level of complex V in rat brown adipose tissue was compared to the relative levels of complex IV in the other rat tissues and in beef heart mitochondria. It was found that relative to complex IV, the level of complex V in rat brown adipose tissue was significantly less than the level of complex V found in rat heart mitochondria, rat kidney mitochondria, rat liver mitochondria, rat skeletal muscle mitochondria, rat synaptic mitochondria, and beef heart mitochondria. Our results showing a decreased level of complex V in rat brown adipose tissue mitochondria confirm previous reports that showed the content of complex V was exceptionally low in brown adipose tissue (Cannon and Vogel, 1977, Houstek et al., 1978). Here is the first time that the low content of complex V in BAT was demonstrated using blue native/tricine-SDS-PAGE two dimensional electrophoresis.

The finding that different amounts of maltoside were required to solubilize mitochondrial proteins from different mitochondria was novel. Based on a comparison of the work of Rolfe *et al.* (1994) who determined the lipid:protein ratio (mg/mg) of mitochondria isolated from rat liver, kidney, brain and skeletal muscle, it would seem that the higher the lipid:protein ratio of mitochondria, the more maltoside required to solubilize mitochondrial membrane proteins.

In general the polypeptide patterns of the mitochondrial membrane proteins were different in all of the mitochondria examined, however the significance of these differences isn't known because the identity of many of the proteins isn't known. Specifically, the polypeptide pattern of complexes I, III, IV and V was identical when resolved using blue native/tricine-SDS-PAGE two-dimensional electrophoresis in all of the mitochondria examined.

Future work would involve identifying the unknown proteins on the second dimension tricine gel as has been done for *Arabidopsis* (Kruft *et al.*, 2001). Also, as mentioned

previously, synaptic mitochondria would need to be further purified using percoll or ficoll gradients as there may have been non-mitochondrial protein present on the second dimension tricine gels. A comparison of synaptic with non-synaptic mitochondria would be a future aim as would the comparison of synaptic and non-synaptic mitochondria from specific brain regions of rat and human brain.

Chapter 9

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