Biochemical Analyses of Deficiencies in the Oxidative Phosphorylation System in Human Muscle

BY

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This dissertation is dedicated to my parents,

Marthinus & Sarah du Toit

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Abstract

Mitochondrial disorders are caused by biochemical abnormalities of the mitochondrial respiratory chain (RC), a key component of oxidative phosphorilation (OXPHOS). The diagnosis of a RC deficiency can be invasive, expensive, time consuming and labour intensive.

The corner stone for diagnosis of mitochondrial disorders and the only two definitive tests are DNA analysis and respiratory chain enzyme analysis (Naviaux, 2004).

The aim of this study was to use biochemical analysis (polarographic analysis and enzyme assay) to identify patients with an OXPHOS defect by setting up reference values from healthy control data. The most commonly used approach in many centres is the interpretation of enzyme activity data based on retrospectively compiled reference values obtained from the data from diseased children, because of the unavailability of muscle tissue from healthy children (Thorburn, 2004). Ethics approval (protocol 91/98) was given to obtain muscle biopsies from selected healthy children.

Biochemical tests were done on the muscle biopsies of the healthy children (control group) and those of the selected possible patients, based on their clinical phenotypes. The methods used in the literature differ significantly, so the first aim was to evaluate and partly standardise the method. Reference values were determined from the control group, based on four different approaches used in the literature. The most accurate approach was chosen, the patient data were compared to the reference range and the patients with OXPHOS deficiencies were diagnosed.

During this study 18 controls were collected and 26 possible patients identified. In 69% of the patients an OXPHOS defect could be diagnosed based on their enzymatic assay data. Only 11% of the enzymatic assay data were comparable with the respiratory analysis data. It is thus recommended that in future respiratory analysis is no longer utilised.

Opsomming

Mitochondriale afwykings word veroorsaak deur biochemiese abnormaliteite van die mitochondriale respiratoriese ketting (RK), 'n sleutelkomponent van oksidatiewe fosforilase ("OXPHOS"). Die diagnose vir 'n RK-defek kan ingrypend, duur, tydrowend en arbeidsintensief wees.

Die hoeksteen vir die diagnose van 'n mitochondriale defek en die enigste twee definitiewe toetse is DNA-analise en respiratorieseketting-ensiemanalise (Naviaux, 2004).

Die doel van hierdie studie was om deur middel van biochemiese analise (polarografiese analise en ensiemanalises) pasiente met 'n OXPHOS-defek te identifiseer deur verwysingswaardes op grond van gesonde kontroledata op te stel. Die mees algemeen gebruikte benadering in baie sentrums is die vertolking van ensiemaktiwiteitdata gebaseer op retrospektief saamgestelde verwysingswaardes. Hierdie waardes is afkomstig van data van siek kinders, omdat spierweefsel van gesonde kinders nie beskikbaar is nie (Thornburn, 2004). Etiekgoedkeuring (protokol 91/98) is toegestaan om spierbiopsies van gesonde kinders te verkry.

Biochemiese toetse is op die spierbiopsies van gesonde kinders (die kontrolegroep) en dié van geselekteerde moontlike pasiënte op grond van hulle kliniese fenotipes gedoen. Die metodes wat in die literatuur gebruik word, verskil baie van mekaar, dus was die eerste doel om die metode te evalueer en gedeeltelik te standaardiseer. Verwysingswaardes is vanaf die kontrolegroepdata bepaal deur van vier verskillende benaderings gebruik te maak. Die akkuraatste benadering is gekies en die pasiëntdata is met die verwysingswaardes vergelyk om pasiënte met 'n mitochondriale defek te identifiseer.

Gedurende die studie is 18 kontroles versamel en 26 moontlike pasiënte geïdentifiseer. In 69% van die pasiënte kon n OXPHOS-defek op grond van hulle ensiembepalingsdata gediagnoseer word. Slegs 11% van die data van die ensiembepalings was vergelykbaar met die respirasie-analises. Dit word dus aanbeveel dat respirasie-analises nie meer in die toekoms gebruik word nie.

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CI	respiratory chain complex I
CII	respiratory chain complex II
CIII	respiratory chain complex III
CIV	respiratory chain complex IV
CV	respiratory chain complex V
α	alpha
β	beta
μ	micro: 10^{-6}
ε	epsilon

List of Abbreviations

ADP	adenosine diphophate
Ag^+	silver ion
AgCl	silverchloride
ATP	adenosine triphosphate
BN-PAGE	blue-native polyacrylamide gel electrophoresis
BSA	bovine serum albumin
BCA	bicinchoninic acid
CO_2	carbon dioxide
CoA	coenzyme A
CoASH	acyl-coA thioesterase
CoQ	coenzyme Q
$CoQH_2$	reduced coenzyme Q
COX	cytochrome c oxidase
CPEO	chronic progressive external ophthalmoplegia
Cu	copper
cytochrome C_{ox}	oxidized cytochrome c
cytochrome C_{red}	reduced cytochrome c
$CuSO_4.5H_2O$	copper (II) sulphate pentahydrate
C	control
CP	control and patient
Da	Daltons

DNA	deoxyribonucleic acid
e^-	electron
e.g	*exempli gratia*: latin abbreviation for "for example"
Eq	equation
ETC	electron transport chain
EDTA	ethylenediamine tetraacetate
F_0	transmembrane proton channel of complex V
F_1	hydrophobic component of complex V
FAD+	flavin adenine dinucleotide
FADH	reduced flavin adenine dinucleotide
Fe^{2+}	iron - ferrous oxidation state
Fe^{3+}	iron – ferric oxidation state
FeS	iron-sulphur proteins
FMN	flavin mononucleotide
G+M	glutamate and malate
ΔH^+	electrochemical gradient
H^+	hydrogen
H_2O	water
HPLC	high-performance liquid chromatography
IMM	inner mitochondrial membrane
K^+	potassium ion
KCl	potassium chloride
kDa	kilo daltons
KCN	potassium cyanide

KH_2PO_4	potassium dihydrogen orthophosphate
K_2HPO_4	di-potassium hydrogen phosphate
LHON	Leber's hereditary optic neuropathy
MCAD	medium chain acyl-CoA dehydrogenase
MELAS	mitochondrial encephalomyopathy, lactic acidosis and stroke like episodes
MERRF	myoclonic epilepsy and ragged red muscle fibres
Mg	milligram
mtDNA	mitochondrial DNA
MEGS	mitochondrial energy generating system
MRC	Medical Research Council
µmol	micromole
min	minute
M	molar: moles per litre
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NARP	neurogenic ataxia and retinitis pigmentosa
NBT	nitrotetrazolium
nDNA	nuclear DNA
NWU	North West University
Na_2SO_4	sodium sulfate
Na_2SO_3	sodium sulfite anhydrous
O_2	oxygen
OMM	outer mitochondrial membrane

OXPHOS	oxidative phosphorylation
OAA	oxaloacetate
PDH	pyruvate dehydrogenase
PDH_C	pyruvate dehydrogenase complex
Pi	inorganic phosphate
P	patient
P+M	pyruvate and malate
QH_2	ubiquinol
RC	respiratory chain
RCC	respiratory chain complexes
RCR	respiratory control ratio
RNA	ribonucleic acid
SCAD	small chain acyl-CoA dehydrogenase
SOP	standard operating procedure
SMH	standard mitochondria homogenate
S+R	succinate and rotenone
SD	standard deviation
t	time
TCA	tricarboxylic acid
TKDEP	Transformation Kernel Density Estimation Program
TPP	thiamine pyrophosphate
tris.HCl	tris-hydrochloric acid (2-amino-2-hydroxymethyl)-1,3-proganediol hydrochloride: $C_4H_{11}NO_3H_3O$
Triton X-100®	Triton X-100®[1]: Octylphenolpoly(ethylene-glycoether)n: $C_{24}H_{62}O_{11}$; for n=10

UP	University of Pretoria
UQ	ubiquinone
UQH_2	dihydro-ubiquinone
VLCAD	very long chain acyl-CoA dehydrogenase
v/v	volume per total volume
w/v	weight per volume

Chapter 1
Introduction

Each of our cells contains, on average, 500 to 2000 "factories" called mitochondria (singular: mitochondrion), which are responsible for supplying our energy needs. Energy sources such as glucose are metabolised in the cytoplasm, products are imported into mitochondria and the catabolism of the products continues, using metabolic pathways. The end products of these pathways include two energy-rich electron donors, NADH and $FADH_2$. Electrons from these donors are passed through an electron transport chain (respiratory chain) to oxygen, which is reduced to water.

The respiratory chain in the inner membrane of the mitochondrion consists of a number of integral protein complexes, namely complex I (NADH-UQ oxidoreductase), complex III (UQ-cytochrome c oxidoreductase) and complex IV (cytochrome c oxidase); complex II (succinate dehydrogenase) catalyzes a reaction that is also part of the Krebs cycle; and two freely diffusible electron-transporting molecules, coenzyme Q and cytochrome c. The main function of the respiratory chain is to produce a transmembrane electrochemical potential gradient through a series of redox reactions (Schapira, 1996). This potential gradient is then used by a fifth membrane enzyme complex, complex V (ATP synthase), which phosphorylates ADP to form ATP, the main cellular source of chemical energy. The combined series of five enzyme complexes is called "oxidative phosphorylation" (OXPHOS), which in humans has a daily turnover (formation of ATP from ADP) of approximately a person's own body weight (Gareth and Grisham, 1999). It is therefore understandable that deficiencies in this important anabolic process, which is often referred to as "mitochondrial disorders", have a major effect on normal developmental and physiological processes.

The phenotype is the perceptible characteristics of the defect. Clinical phenotype is highly variable with a disorder of the respiratory chain. Although tissues with a high demand for OXPHOS such as brain and skeletal muscle are frequently affected, virtually any tissue can be involved, as listed in table 2.3. The same biochemical defect may cause diverse clinical phenotypes and, conversely, symptoms (table 2.3) may be similar in patients with different biochemical defects. Patients may become symptomatic at any age and may show variable symptoms and outcomes.

Diagnosis of dysfunction of the OXPHOS system relies upon a combination of clinical, biochemical, histological, functional and genetic features (van den Heuvel et al, 2004). Among these features/evaluations, DNA analysis and respiratory chain enzyme analysis are still the corner stone of the diagnosis of "mitochondrial disorders" and thus the only two definitive tests when such a disorder is suspected in a patient (Naviaux, 2004).

Over the past decade the identification of mitochondrial disorders in South African paediatric patients who present with associated clinical features has been enhanced by the addition of biochemical (enzyme) assays in mitochondria isolated from muscle tissue. However, the interpretation of the enzyme activity data was based on retrospectively compiled reference values obtained from data from diseased children. This was done by retrospectively assigning values as "normal" or not. Although this is a commonly used approach in many centres because of the unavailability of muscle tissue from healthy children (Thorburn et al, 2004), it is obviously not the best approach, or possibly not an accurate approach either.

The aim of this study therefore was to further enhance and evaluate key biochemical analyses to identify deficiencies of the respiratory chain and pyruvate dehydrogenase complex in South African paediatric patients. The first objective was to set up procedures for enzyme assays and respiration analysis in mitochondrial preparations and evaluate their responsiveness to protein content in reactions. The second objective was to obtain reference material (muscle biopsies) from healthy children and determine the (presumably) normal distribution of enzyme and respiration activities in these biopsies, which could then be used as reference values for the identification of mitochondrial disorders.

Reference values for the identification of mitochondrial disorders in the South African population do not exist, because of the difficulty of obtaining ethical approval and the lack of research on this topic. Our laboratory has the equipment to do the biochemical analysis and to identify patients with mitochondrial disorders. For every child in whom a firm diagnosis can be obtained there is the possibility of effective treatment. It also relieves the parents of such children of the uncertainty of not knowing what is wrong with their children.

Chapter 2
Literature Review

2.1 THE MITOCHONDRION

The organelles called mitochondria are found in the cytoplasm of nearly all eukaryotic cells. It is postulated that mitochondria are derived from an ancient aerobic bacterium that invaded a primitive cell during the early stages of evolution and is an example of endosymbiosis (Borst, 1977). The presence of the mitochondrial DNA (mtDNA) is the only primary remaining evidence because the mtDNA's structural characteristics are very similar to the deoxyribonucleic acid (DNA) of primitive bacteria (Leblanc et al., 1997). The mitochondrion is known as the power source of the cell because of the central role in the provision of energy for cellular metabolism (Duchen, 2004).

2.2 STRUCTURE OF THE MITOCHONDRION

A typical mitochondrion is approximately the size of a cell of an *E. coli* bacterium. The molecular weight of a mitochondrion is 10^7 Daltons (Da) and they range from 0.5 μm to 1 μm in length (Duchen, 2004). Mitochondria vary considerably in shape and size but can be rod-shaped or round, although they have the same basic architecture (Figure 2.1).

A mitochondrion is made up of two concentric membranes, a smooth outer mitochondrial membrane (OMM) and a folded inner mitochondrial membrane (IMM), each ~5-7 nm, thick as illustrated in Figure 2.1. The membranes of the mitochondria contain integral membrane proteins but both have specific functions according to their location. The OMM is a smooth and somewhat elastic phospholipid bilayer and consists of 60-70% proteins and 30-40% lipids. The proteins are mainly porin proteins, which ensure permeability of the membrane to molecules smaller than 10 kDa by forming large channels across the membrane. It is suggested that the function of the OMM is to maintain the mitochondrion's shape (LaNoue and Schoolwerth, 1984; Garrett and Grisham, 1999).

The IMM structure is highly complex and includes the electron transport system, the ATP synthase complex and transport proteins. The IMM has inward folds or invaginations called *cristae* that increase the surface area of the membrane (Scheffler, 2001) and act as a permeability barrier for a variety of compounds. This membrane is only freely permeable to

oxygen, carbon dioxide and water (Duchen, 2004). Substances like ions, substrates and fatty acid for oxidation that must cross the IMM are carried by specific transport proteins in the membrane (Garrett and Grisham, 1999).

The IMM divides the mitochondria into two compartments, namely the inter membrane space, located between the OMM and the IMM, and the matrix, enclosed by the IMM (Passarella et al., 2003). The inter membrane space has an important role in the primary function of the mitochondrion, which is oxidative phosphorylation (Duchen, 2004). Most of the enzymes of the tricarboxylic acid (TCA) cycle and the fatty acid oxidation pathway are located in the matrix, along with the mtDNA molecules, ribosomes and enzymes required for mtDNA replication and protein synthesis (Garrett and Grisham, 1999).

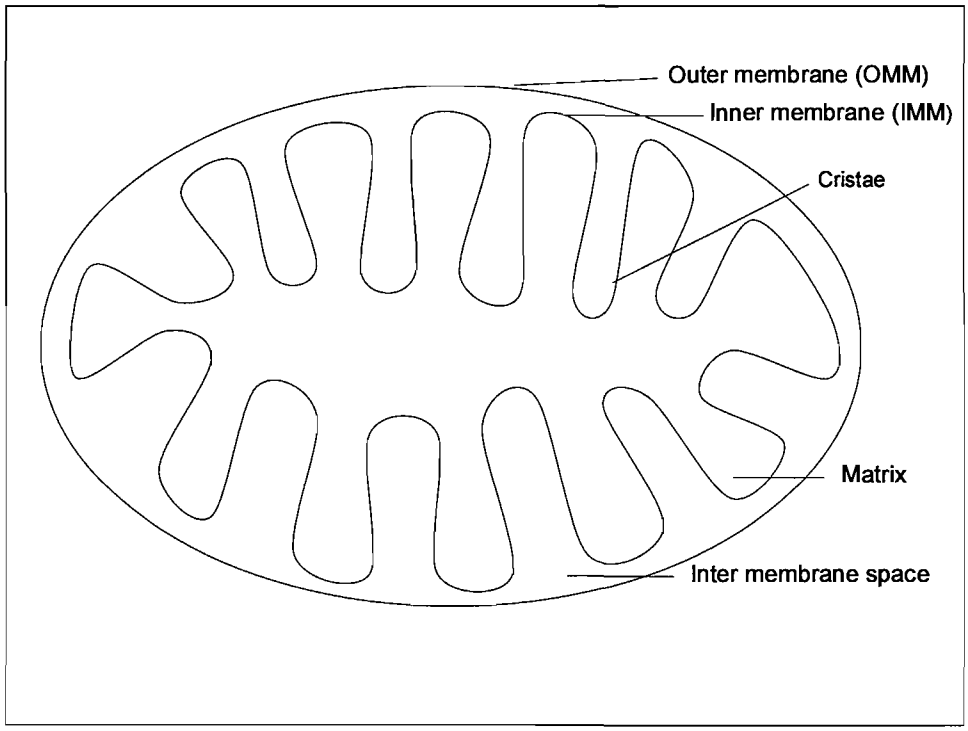


Figure 2.1 Simplified structure of a typical mitochondrion.

It shows the different compartments; the OMM, IMM, cristae, matrix and intermembrane space, within the mitochondrion. Adapted from Garrett and Grisham (1999).

2.3 FUNCTION OF THE MITOCHONDRION

The mitochondrion is the site of numerous metabolic pathways, but one of the most important functions is the production of energy in the form of adenosine triphosphate (ATP) by means of the process of oxidative phosphorylation (OXPHOS). This energy is required for cell survival and function (Schon et al., 1993).

Mitochondria are involved in the building, breaking down and recycling of products needed for proper cell functioning. Mitochondria are also involved in the synthesising of blood and hormones, such as estrogens and testosterone. They are involved in cholesterol metabolism, neurotransmitter metabolism and detoxification of ammonia in the urea cycle. Thus, if mitochondria do not function properly, energy production as well as cell-specific products needed for normal cell functioning will be affected (Schon et al., 1993).

2.4 THE PRODUCTION OF ENERGY IN THE MITOCHONDRION

Respiration can be divided into three main pathways: glycolysis, the mitochondrial TCA cycle and OXPHOS (Fernie et al., 2004).

The food ingested is first converted to basic chemicals, which the cell may use to produce energy as illustrated in Figure 2.2. Some of the best energy-supplying foods contain sugars or carbohydrates (Fernie et al., 2004). Figure 2.2 illustrates all the processes that take place in the mitochondria and how the food is broken down through these pathways to produce energy.

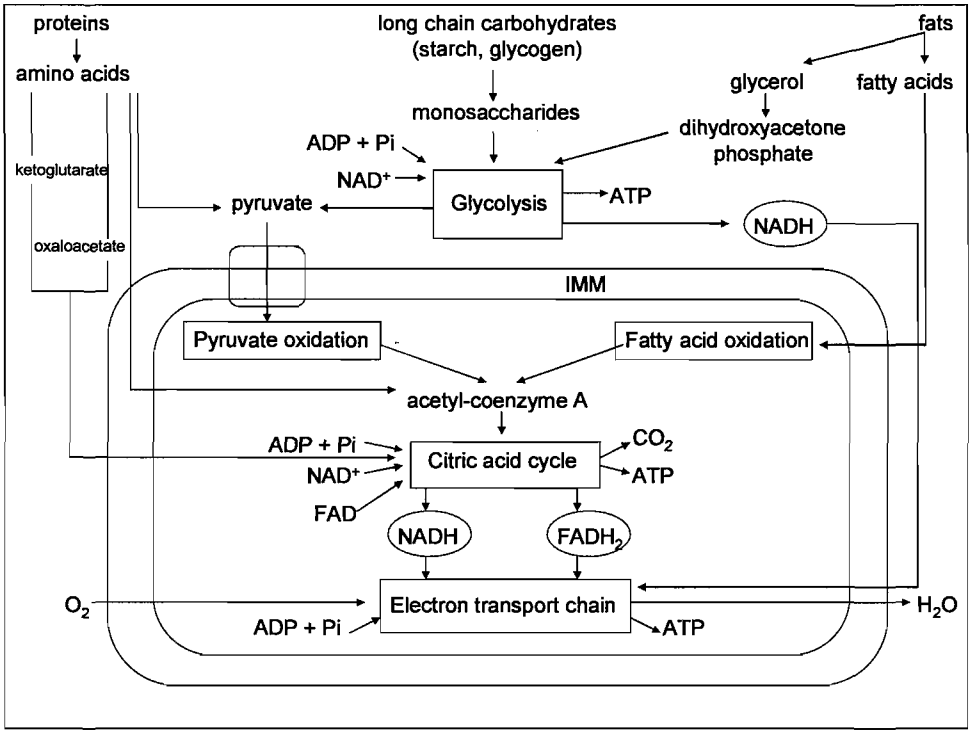


Figure 2.2: Metabolic pathways within the mitochondria.

The integration of all the pathways in the mitochondria. Adapted from Blau et al. (2003).

2.4.1 Glycolysis

Glucose enters the cell via "glucose transporters" (Fernie et al., 2004). Once inside the cell, glucose is broken down in two distinct pathways to produce ATP containing high-energy phosphate bonds. The first pathway requires no oxygen and is called glycolysis (Figure 2.3); it forms part of anaerobic metabolism. Glycolysis occurs in the cytoplasm outside the mitochondria and is responsible for the oxidization of glucose to pyruvate, as illustrated below (Fernie et al., 2004).

Equation 2.1: Oxidization of glucose to pyruvate.

$$\text{Glucose} + 2\,\text{ADP} + 2\,\text{NAD}^+ + 2\,\text{P}_i \rightarrow 2\,\text{Pyruvic acid} + 2\,\text{ATP} + 2\,\text{NADH} + 2\,\text{H}^+$$

ADP = adenosine diphosphate; NAD^+ = nicotinamide adenine dinucleotide; Pi = inorganic phosphate

During glycolysis, glucose is broken down into two molecules of pyruvic acid[1], two reduced nicotinamide adenine dinucleotide (NADH) molecules and a net gain of two ATP molecules. The two ATP molecules are used as a source of energy and the remaining pyruvic acid is drawn into the mitochondrial matrix (Mader, 2002; Fernie et al., 2004).

[1] Free acid form of *pyruvate*, which is the commonly used term for salt-conjugated form, e.g. K^+-pyruvic acid)

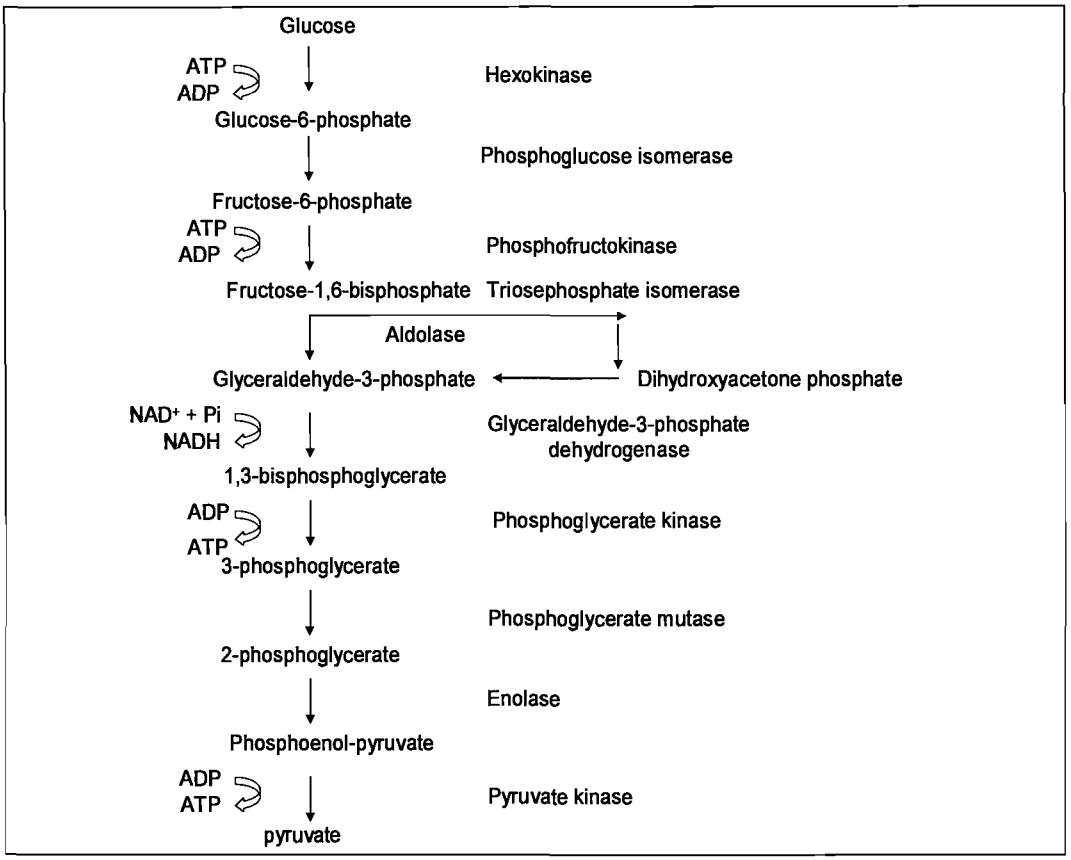


Figure 2.3: Schematic illustration of the glycolysis pathway in mitochondria.

On the right-hand side all the enzymes involved and on the left hand side the reducing equivalents or ATP molecules formed in the process are shown. Adapted from Garrett & Grisham (1999).

2.4.2 Pyruvate dehydrogenase complex (PDH_C)

In eukaryotic cells, glycolysis occurs in the cytoplasm, whereas the TCA cycle reactions and all subsequent steps of aerobic metabolism take place in the mitochondria; pyruvate first has to enter the mitochondria in order to enter the TCA cycle. The oxidative decarboxylation of pyruvate to acetyl CoA (Equation 2.2) is the connecting link between glycolysis and the TCA cycle.

The reaction is catalysed by pyruvate dehydrogenase, a multienzyme complex (PDHc) that consists of 60 polypeptides. Once pyruvate has been converted to acetyl-CoA, this substrate can enter the TCA cycle. For this reason the PDH_C is considered to be one of the most important enzyme complexes in controlling aerobic energy metabolism (Maragos, 1989). The complex consists of multiple copies of enzymes E1 ($\alpha_2\beta_2$ heterotetramer), E2 and E3, as illustrated in Table 2.1. These three enzymes and the five coenzymes (coenzyme A (CoA), nicotinamide adenine dinucleotide (NAD^+), flavin adenine dinucleotide

(FAD^+), lipoic adic and thiamine pyrophosphate (TPP)), which assist these enzymes, are collectively known as the pyruvate dehydrogenase complex (PDH_C) (Garrett and Grisham, 1999).

Table 2.1: The three PDHc enzymes

Enzyme number	EC number	Enzyme name
E1	EC 1.2.4.1	pyruvate dehydrogenase
E2	EC 2.3.1.12	dihydrolipoamide acetyltransferase
E3	EC 1.6.4.3	dihydrolipoamide dehydrogenase

EC = Enzyme Commission number

Equation 2.2: oxidative decarboxylation of pyruvate to acetyl CoA

$$\text{Pyruvate} + \text{CoA} + NAD^+ \rightarrow \text{acetyl}-\text{CoA} + CO_2 + \text{NADH} + H^+$$

2.4.3 Tricarboxylic acid (TCA) or Krebs cycle

The second pathway requires oxygen and is called aerobic metabolism. The main function of the TCA cycle is to derive electrons from the two-carbon sources that enter the mitochondrion. The acetyl-CoA is the connecting link between glycolysis and the TCA cycle and enters the cycle as shown in Figure 2.4. The acetyl CoA enters the TCA cycle by joining with a four-carbon molecule called oxaloacetate, a reaction catalysed by citrate synthase.

The resulting six-carbon molecule, citric acid, is then processed further in a stepwise fashion by a series of seven enzyme-catalysed reactions, including four dehydrogenases, as illustrated in Figure 2.4. The result of the cycle is a net formation of 6 NADH, 2 $FADH_2$ and 2 ATP molecules and a number of intermediates, of which oxaloacetate re-enters the next cycle. (Garrett & Grisham, 1999, Mader, 2002; Fernie et al., 2004). The reducing equivalents, NADH and $FADH_2$ formed in the TCA cycle, are then utilised in the respiratory chain (ETC).

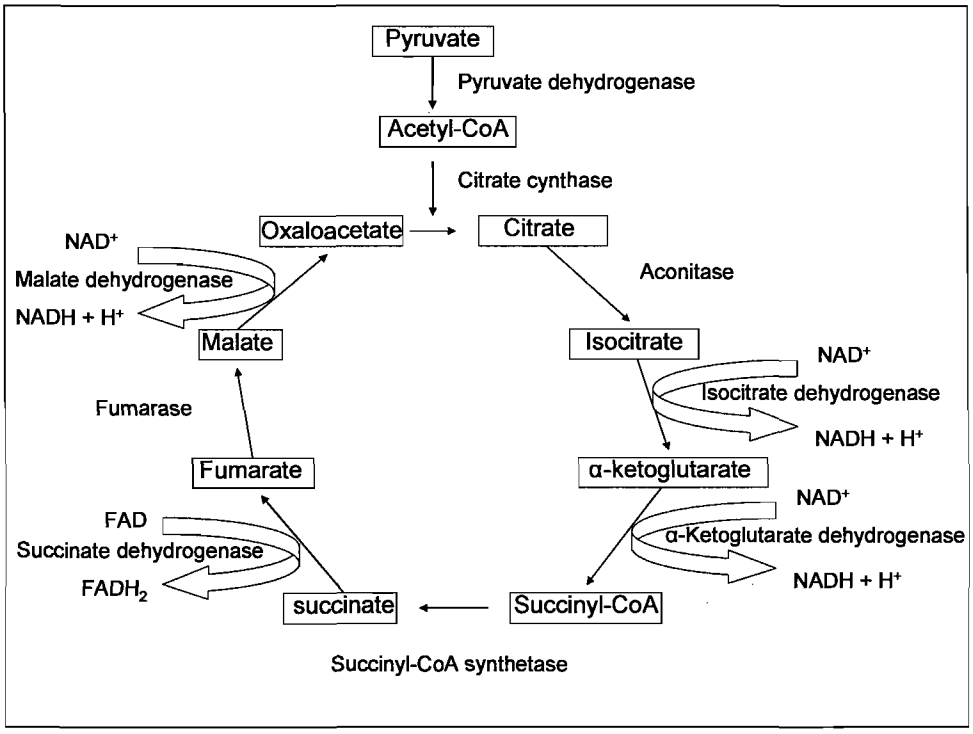


Figure 2.4: Illustration of the TCA (Krebs) cycle in the mitochondrion.

This cycle consists of the stepwise breakdown of the pyruvate through the TCA cycle to produce electrons to enter the electron transport chain. Adapted from Garrett & Grisham (1999).

2.4.4 Oxidative Phosphorylation

The ETC or respiratory chain (RC), as illustrated in Figure 2.5, is localised in the IMM and utilises electrons available from NADH and $FADH_2$ (prosthetic group of complex II) to generate electron flow through this chain. This ultimately results in an electrochemical gradient (ΔH^+) over the IMM. The respiratory chain consists of four enzyme complexes, as illustrated in Table 2.2, complex I, complex II, complex III, complex IV and two freely-diffusible molecules, coenzyme Q (CoQ), or also called ubiquinone, and cytochrome c (Munnich et al., 1995). A fifth complex, ATP synthase, also forms part of (the) OXPHOS (system).

Table 2.2: The names of the OXPHOS enzymes

Complex number	Complex name	EC number
Complex I	NADH:ubiquinone oxidoreductase	EC 1.6.5.3
Complex II	Succinate coenzyme Q dehydrogenase	EC 1.3.5.1
Complex III	Cytochrome c reductase	EC 1.10.2.2
Complex IV	Cytochrome c oxidase	EC 1.9.3.1
Complex V	ATP synthase	EC 3.6.1.34

EC = Enzyme commission number

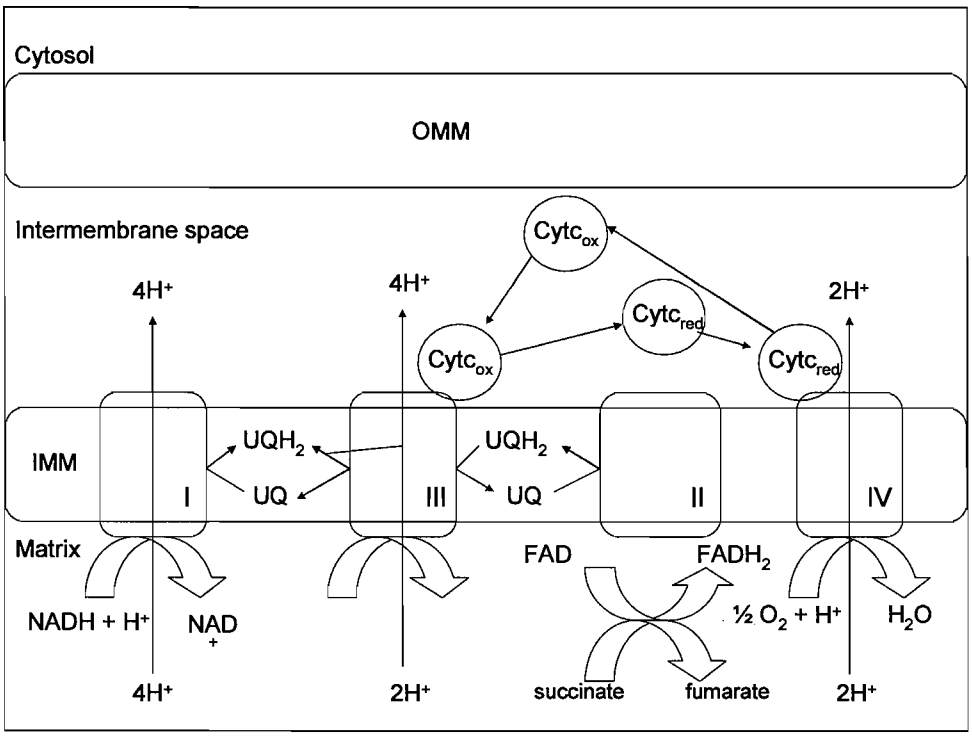


Figure 2.5: The electron transport chain.

The electron transport chain in the IMM with all the protein complexes. Adapted from Garrett and Grisham (1999).

2.4.4.1 Complex I

Complex I is a very large (750 KDa) L-shaped complex and contains 46 subunits. CI is composed of proteins that contain iron-sulphur clusters, a flavoprotein that oxidizes NADH, and other protein subunits. As illustrated in Equation 2.3, the reactions occurring in complex I occur in several steps.

Equation 2.3: CI catalyses the oxidation of NADH

a. $NADH + [FMN] + H^+ \rightarrow [FMNH_2] + NAD^+$

b. $[FMNH_2] + Fe\text{-}S_{oxidised} \rightarrow [FMN] + Fe\text{-}S_{reduced} + 2H^+$

c. $Fe\text{-}S_{reduced} + CoQ + 2H^+ \rightarrow Fe\text{-}S_{oxidised} + CoQH_2$

FMN = flavin mononucleotide (oxidized); $FMNH_2$ = flavin mononucleotide (reduced); CoQ = coenzyme Q (ubiquinone); $CoQH_2$ = reduced coenzyme Q (ubiquinol); Fe-S = iron – sulphur; H^+ = hydrogen ion; NAD^+ = oxidised nicotinamide adenine dinucleotide.

The initial process is the transfer of electrons, as indicated in Equation 2.3 (a), NADH binds complex I and passes two electrons to a flavin mononucleotide-oxidised (FMN) prosthetic group, which is reduced to flavin mononucleotide ($FMNH_2$). Each electron is

transferred with a proton. The second, as illustrated in Equation 2.3 (b), involves the re-oxidation of the reduced flavoprotein, followed by the transfer of electrons to the iron-sulphur clusters and subsequent reduction of CoQ to reduced CoQ ($CoQH_2$), as depicted in Equation 2.3 (c) (Adams and Turnbull, 1996; Nichols and Ferguson, 2001). CoQ can pass electrons to the third complex for further transport to oxygen. Its isoprenoid tail makes it highly hydrophobic, and it diffuses freely in the hydrophobic core of the inner mitochondrial membrane. As a result, it shuttles electrons from complex I and complex II to complex III (Garrett and Grisham, 1999).

2.4.4.2 Complex II

Complex II is the only complex not embedded in the IMM. It contains the enzyme succinate dehyrogenase, has a molecular mass of 140 KDa and is encoded exclusively by the nDNA. Complex II also transfers electrons to the ubiquinone pool, as illustrated in Equation 2.4. Equation 2.4 (a) illustrates the oxidation of succinate to fumarate and is followed by Equation 2.4 (b), where the oxidation of the flavin group occurs, and the reduction of CoQ to $CoQH_2$ (Nicholls and Ferguson, 2001).

Equation 2.4: Electron transfer reaction in CII

a.	$Succinate + [FAD] \rightarrow Fumarate + [FADH_2]$
b.	$[FADH_2] + CoQ \rightarrow [FAD] + CoQH_2$

FAD = Flavin adenine dinucleotide (oxidised); FADH2 = flavin adenine dinucleotide (reduced); CoQ = coenzyme Q (ubiquinone); $CoQH_2$ = reduced coenzyme Q (ubiquinol).

2.4.4.3 Coenzyme Q (ubiquinone)

Coenzyme Q (CoQ) is a mobile electron carrier. It is the smallest electron carrier (non-protein) and is freely soluble in the membrane layer. Its isoprenoid tail makes it highly hydrophobic, and it diffuses freely in the hydrophobic core of the inner mitochondrial membrane. As a result, it shuttles electrons from complex I and complex II to complex III. Figure 2.6 illustrates the redox cycle of UQ. It is a three oxidation step of CoQ, CoQ, oxidized form (Q, ubiquinone); semiquinone intermediates ($QH\cdot$); CoQ, reduced form (QH_2 ubiquinol) (Nicholls and Ferguson, 2001). The UQ redox cycle is initiated when a molecule of UQH_2 diffuses to a site called Q_p on complex III near the cytosolic face of the membrane.

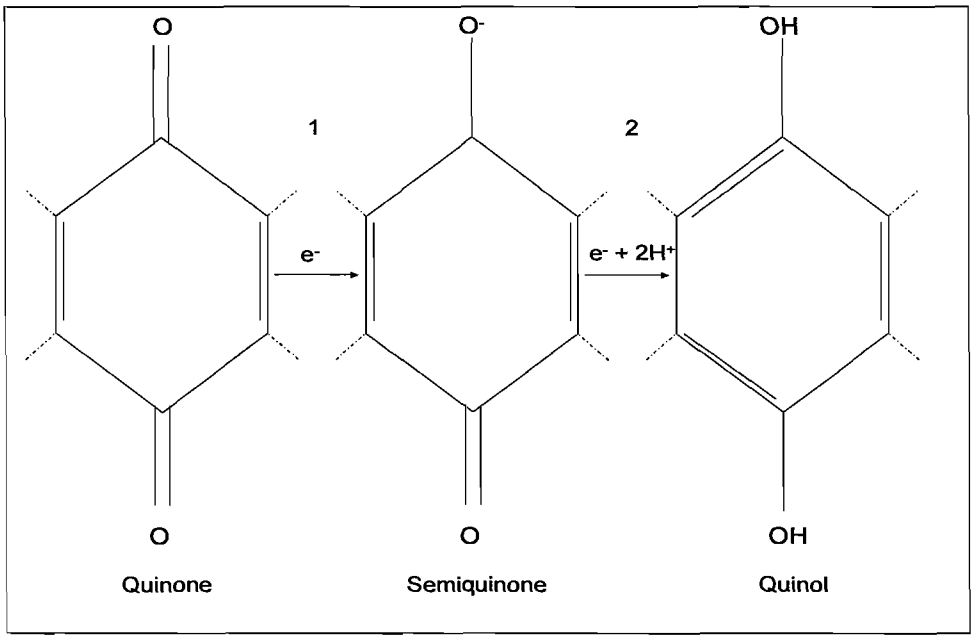


Figure 2.6: The two oxidation states of coenzyme Q.

The oxidation of UQH_2 to UQ takes place in two stages: 1 – The first electron is transferred from UQH_2 to the Rieske protein (a Fe-S protein named after its discoverer), releasing two protons to the cytoplasm and leaving the free radical semiquinone anion species UQ^- at the Q_p site; and 2 – The second electron is transferred to the bL haem, which is also close to the P-face. Adapted from Nicholls and Ferguson (2001).

2.4.4.4 Complex III

Complex III is a dimer in which the two monomeric units do not function independently. Each monomer consists of as many as 11 subunits. Complex III accepts electrons from coenzyme QH_2 that is generated by electron transfer in complex I and complex II. Complex III contains cytochrome b, cytochrome c1 and FeS proteins (Nicholls and Ferguson, 2001).

Cytochrome c1 is a prosthetic group within complex III which reduces cytochrome c, the electron donor, to complex IV. The iron atoms alternate between +3 and +2 oxidation states, as they pass on the electrons. $CoQH_2$ passes two electrons to cytochrome b, causing the Fe^{3+} to be reduced to Fe^{2+} (Nicholls and Ferguson, 2001).

2.4.4.5 Cytochrome c

Cytochrome c is a small mobile protein that is loosely bound to the outer surface of the inner membrane facing the intermembrane space. It accepts electrons from complex III

(Fe^{3+} is reduced to Fe^{2+}) and shuttles them to the last electron transport protein in the chain (complex IV) (Nicholls and Ferguson, 2001).

2.4.4.6 Complex IV

Complex IV contains cytochrome and cytochrome a3 (both use Fe and Cu atoms to transport the electrons). Four cytochrome c molecules pass on four electrons to complex IV. These are eventually transferred with four H^+ atoms to O_2 to form two water molecules (Nicholls and Ferguson, 2001)

Meanwhile, oxygen molecules diffuse into the cell and are captured by the iron-copper core at heme a3. Oxygen is electrophilic, but adding less than four electrons at a time makes oxygen unstable. Therefore heme a3 holds them apart until four electrons accumulate (Nicholls and Ferguson, 2001).

When four electrons have accumulated at heme a3, they are simultaneously fed to oxygen, resulting in the production of H_2O, as illustrated in Equation 2.5. Meanwhile, protons have been drawn out of the watery medium of the matrix and are trapped in the outer mitochondria chamber by their attraction to high-energy electrons (Nicholls and Ferguson, 2001).

Equation 2.5: Reaction that occurs in complex IV

$$4 \text{ cytochrome c } (Fe^{+2}) + 4H^+ + O_2 \rightarrow 4 \text{ cytochrome c } (Fe^{+3}) + 2H_2O$$

Fe^{+2} = iron – ferrous oxidation state; Fe^{+3} = iron – ferric oxidation state.

2.4.4.7 Complex V

Complex V is also known as ATP synthase, and the entire complex has a molecular weight of 400 kDa. Complex V is embedded in the cristae of the IMM and includes two major subunits. F_1, the catalytic subunit that is made up of five polypeptides and F_0, a complex of integral membrane proteins that mediates proton transport. The F_1F_0 complex couples ATP synthesis to H^+ transport into the mitochondrial matrix (Nicholls and Ferguson, 2001). The coupling of ATP to electron transport is termed the phosphate/oxygen (P/O) ratio. This ratio gives the number of moles Pi required in the reaction with ADP to yield ATP for each mole of oxygen atoms consumed, as illustrated in Equation 2.6. The pH and electrical gradients created by respiration together are the driving force for H^+ uptake. The

return of protons to the matrix via F_0 used up the pH and electrical gradients (Koolman and Röhm, 1996).

Equation 2.6: Reaction that occurs in complex V

$$ADP_{free} + Pi_{free} \leftrightarrow ATP_{free}$$

Electrons are transferred from protein to protein in the electron transport chain. Each successive protein in the transport chain can accept a lower-energy electron. As electrons travel from a high-energy state to a low-energy state, energy is released. This energy is used to pump protons across the membrane to set up a proton gradient. The final electron acceptor is oxygen. Oxygen has high electro-negativity and its high affinity for electrons makes it an ideal acceptor for low-energy electrons. With the electrons, hydrogen is added to oxygen, forming water as the final product (Mader, 2002).

This series of hydrogen pumping steps creates a gradient, the proton motive force. The potential energy in this gradient is used by ATP synthase during oxidative phosphorilation (OXPHOS) to form ATP from ADP and inorganic phosphate. The electron transport cycles occur simultaneously, helping to ensure that the proton gradient is always maintained. In total, 36 ATP molecules are generated from the complete breakdown of one molecule of glucose (Nicholls and Ferguson, 2001).

2.5 MITOCHONDRIAL DISORDERS[2]

Mitochondrial disorders are characterised by biochemical abnormalities (deficiencies) of the mitochondrial respiratory chain, a key component of oxidative phosphorylation (McFarland et al., 2002: DiMauro and Schon, 2003). Clinically, mitochondrial disorders are a heterogeneous group of disorders that can affect various systems; it affects the function of single organs or can result in a multisystem disease. Although tissues with a high demand for oxidative phosphorylation such as brain and skeletal muscle are frequently affected, virtually any tissue can be involved, as listed in Table 2.3. The same biochemical defect may cause diverse clinical phenotypes and, conversely, symptoms (Table 2.3) may be similar in patients with different biochemical defects. Patients may become symptomatic at any age and may show variable symptoms and outcome.

[2]In this dissertation, the term "disorder" will be used when referring to disturbance of function, structure or both. This includes an interaption or disorder of body functions,systems or organs. The term "deficiency" will be used when referring to anything resulting from lack of calories, protein, essential amino acids, fatty acids, vitamins or trace minerals. The term "disease" refers to an illness, sickness to an interruption or disorder of body functions, systems or organs.

Table 2.3: Tissue affected by mitochondrial disorder and most frequently associated symptoms. Adapted from Blau et al. (2003).

Tissue affected	Symptoms
CNS	seizures, tremors, developmental delays, deafness, dementia, problems with peripheral nerves, ataxia, blindness, hypotonia
Gastrointestinal	gastro-esophageal reflux, vomiting, chronic diarrhea, intestinal obstruction
Skeletal muscles	muscle weakness, exercise intolerance, cramps, easy fatiguability
kidneys	fanconi syndrome (loss of essential metabolites in urine)
Heart	cardiomyopathy (heart failure), conduction abnomalities
Liver	liver failure
Eyes	drooping eyelids (ptosis), inability to move eyes from side to side (extemal ophtalmopiegia), blindness (retinitis pigmentosa), strabismus, cataract
Endocrine	diabetes insipidus, delayed puberty, hypothroidism, diabetes mellitus, primary ovarian dysfunction

Genetically, mitochondrial disorders can be due to mutations of either the nuclear or the mitochondrial genome. As discussed in Section 2.4.4 the inner mitochondrial membrane contains the respiratory chain and comprises > 80 individual polypeptides, the vast majority of which are encoded by nuclear DNA. Many of the proteins responsible for the maintenance, replication and transcription of the mitochondrial genome are nuclear-encoded (DiMauro and Schon, 2001; Shoubridge, 2001). The only non-chromosomal DNA in human cells is the mitochondrial genome (DiMauro and Schon, 2001; Shoubridge, 2001). The human mitochondrial DNA (mtDNA), maternally transmitted, encodes 13 subunits of the respiratory chain enzyme complex I, complex III, complex IV and complex V. Mitochondrial DNA also codes for 22 transfer RNAs (tRNA) and two ribosomal RNAs (rRNA). Each individual mitochondrion contains two to ten copies of the mtDNA genome, the number depending on the energy demands of the cell in which it is located. Homoplasmy refers to the presence of identical copies of mtDNA, be it normal or affected. In contrast, heteroplasmy is the occurrence of two populations of mtDNA, thus a mixture of normal and mutant mtDNA within the cell (Wallace, 1997)

This makes the oxidative phosphorylation system, which includes the respiratory chain and complex V, unique as the different components are encoded by nuclear DNA (Mendelian inheritance) and mtDNA (maternal inheritance), with the exception of complex II, which is entirely nuclear-encoded (Blau et al., 2003).

The mutation rate of mtDNA is much higher than that of nDNA, evolving 10 to 20 times faster than nDNA. The reason is that the mitochondrial genome is an open circular molecule and lacks the protective histones surrounding the nuclear chromosomes. The clinical phenotype and the severity of the mitochondrial disorder result from the type of mtDNA mutation, the proportion of mutant DNA and the tissue distribution (Wallace, 1992).

Mutations of the mtDNA occur in the mitochondrial genome. Mutations in mtDNA generally cause functional deficiencies in the respiratory chain, and these mutations manifest in tissues with high-energy demands. Nuclear DNA mutations occur in the nDNA genes coding for mitochondrial components and result in defective oxidative phosphorylation (Shoubridge, 1998). In Table 2.4 examples of different disorders due to mutations within the mtDNA and nDNA are listed. Primary OXPHOS defects are thus defined as those caused by mutations of mtDNA or nuclear genes encoding subunits of complexes I-V. Secondary OXPHOS deficiencies encompass genetic and environmental factors (Schapira 1999)

Table 2.4: Mitochondrial disorders due to mtDNA and nDNA mutations.

List of known mitochondrial disorders that are due to a mutation in either the mtDNA or the nDNA.

Disorders associated with mtDNA mutations	Disorders assosiated with nDNA mutations
MELAS	Luft disease
MERRF	Leigh Syndrome (CI, COX, PDH)
NARP	Alpers disease
LHON	MCAD, SCAD,VLCAD
Pearson Marrow syndrome	Glutaric aciduria II
Kearns-Sayre-CPEO	Lethal infantile cardiomyopathy
Diabetes with deafness	Friedreich ataxia
Leigh Syndrome	Maturity onset diabetes
	Malignant hyperthermia
	Disorders of ketone utilisation
	mtDNA depletion syndrome
	Pyruvate dehydrogenase deficiency
	Fumarase deficiency
	Pyruvate carboxylase deficiency

MELAS = mitochondrial encephalomyopathy, lactic acidosis and stroke like episodes; MERRF = myoclonic epilepsy and ragged red muscle fibres; NARP = neurogenic ataxia and retinitis pigmentosa; CPEO = chronic progressive external ophthalmoplegia; LHON = Leber's hereditary optic neuropathy; COX = cytochrome c oxidase; PDH = pyruvate dehydrogenase; MCAD = medium chain acyl-CoA dehydrogenase; SCAD = small chain acyl-CoA dehydrogenase; VLCAD = very long chain acyl-CoA dehydrogenase. Adapted from Naviaux (1997).

2.6 DIAGNOSIS OF MITOCHONDRIAL DISORDERS

The diagnosis of mitochondrial disorders[3] is invasive, expensive, time-consuming and labour-intensive. Due to the ubiquitous nature of the oxidative phosphorylation, when a patient presents an unexplained association of neuromuscular and/or non-neuromuscular symptoms, with a rapidly progressive course, involving seemingly unrelated organs or tissues, a defect in the mitochondrial respiratory chain is considered (Munnich et al., 1995). Diagnosis of mitochondrial dysfunction relies upon a combination of clinical evaluations, measurement of metabolites, histological staining of tissues (biopsies), analysis of respiratory chain function or enzyme activities and DNA studies (van den Heuvel et al., 2004). Table 2.5 summarises tests that are used to assist in the diagnosis of mitochondrial disorders.

Table 2.5: Tests performed to assist in the diagnosis of mitochondrial disorders.

Types	Test	What it shows
Blood test	1. Lactate and pyruvate levels	1. If elevated, may indicate deficiency in RC; abnormal ratios of the two may help identify part of RC that is blocked.
	2. Serum creatine kinase	2. May be slightly elevated in mitochondrial disease but is usually only high in cases of mitochondrial DNA depletion.
Muscle biopsy	1. Histochemistry	1. Detects abnormal proliferation of mitochondria and deficiencies in cytochrome C oxidase (COX) activity.
	2. Immuno-histochemistry	2. Detects presence or absence of specific proteins.
	3. Electron microscopy	3. May confirm abnormal appearance of mitochondria.
	4. Biochemistry	4. Measures activities of OXPHOS enzymes. Polarography measures oxygen consumption in mitochondria.
Molecular tests	1. Known mutations	1. Blood or muscle sample to screen for known mutations, looking for common mutations first.
	2. Rare or unknown mutations	2. Look for rare or unknown mutations. Require samples from family members; more expensive and time-consuming.
Family History	Clinical exam or oral history of family members	Can sometimes indicate inheritance pattern.

[3]Mitochondrial diseases result from failures of the mitochondrion. Mitochondrial dysfunction is manifested by a decreased activity of the mitochondrial energy generating system, the MEGS (Janssen, 2007)

However, DNA analysis and respiratory chain enzyme analyses are still the cornerstone of the diagnosis of mitochondrial disorders and the only two definitive tests (Naviaux, 2004). Successful approaches for the diagnosis of mitochondrial disease always use a combination of clinical and biochemical criteria (Naviaux, 2004). The criteria used in this study are the Nijmegen Criteria for Mitochondrial Disease (Wolf and Smeitink, 2002). These criteria are given in Appendix A. Other criteria that also received attention is the Modified Walker Criteria for Mitochondrial Disease (Bernier et al., 2002), the Nonaka Criteria for Mitochondrial Encephalomyopathy (Nonaka, 2002), Wolfson Criteria for Mitochondrial Disease (Nissenkorn et al., 1999).

The Nijmegen Criteria for Mitochondrial Disease (Appendix A) consist of two subsets of criteria – general (clinical, metabolic, imaging and pathologic) and biochemical. The general criteria are used to identify the patients who must undergo a muscle biopsy for further biochemical tests (Naviaux, 2004). In the paediatric population a muscle biopsy and biochemical investigation are performed only if there is a reasonable suspicion of a respiratory chain disorder (Wolfand and Smeitink, 2002). The Nijmegen Criteria format as given in the Appendix differs somewhat from the criteria used in the article by Naviaux refered to. The criteria in the Appendix in particular does not contain the biochemical criteria. Suggest using the most recent criteria as well as the reference in the Appendix. The patients who score as probable or definite using the general criteria of Nijmegen Criteria must undergo a muscle (or liver) biopsy. When the biopsy has been done, a biochemical test follows on either frozen or freshly prepared mitochondrial enriched preparations. The results are evaluated and a diagnosis made by a clinician (Naviaux, 2004). The tests that are done include one or more of the following:

- Functional tests (intact freshly prepared mitochondria preparations)
    - Polarographic measurment (RC) (Section 2.6.2.1)
    - ATP synthesis (OXPHOS) (Section 2.6.2.2)
    - Pyruvate oxidation (NAD/NADH) (Section 2.6.2.3)
- Respiratory chain enzyme (single) activities

These functional tests are labour-intensive, dependent on the quality of the biopsy, quality of mitochondrial preparations and limited time after preparations. Specialised apparatus, experience and a permit to use radioactive material (oxidation analysis) are additional requirements. In the case of enzyme tests only (except complex V, which requires coupled intact mitochondria), frozen material can be used; in fact samples can be frozen for several

years without loss of activities. Thus the tests that are selected for diagnosis at a specific institution depend on the equipment of the laboratory, experience of personnel and financial and practical considerations, such as where and when biopsies are taken and processed.

2.6.1 Tissue biopsy

When a patient with a suspected RC defect is investigated, the choice of the specific tissue used is important. Tussue that could be obtained in a minimal invasive manner, like blood or skin fibroblasts, but in the literature Faivre, (2000); Niers, (2003) and Thorburn et al., (2004) found that half of the children with a RC enzyme defect in skeletal muscle have normal activities in cultured skin fibroblasts, white blood cells or platelets and the latter have an additional problem of limited sample volume (Thorburn et al., 2004). Skeletal muscle is the tissue most widely used for RC enzyme studies.

There are two methods of obtaining the muscle. The method most commonly used, especially in the case of paediatric patients, also applied to this study, namely via an open muscle biopsy where an incision is made through the skin and the sample of muscle is obtained by means of a surgical incision through the muscle fascia. The muscle used in this study is the *vastus lateralis.* The advantage of the open biopsy method is that the muscle is directly exposed and the required size of the biopsy (20-500 mg) can be taken, although there are certain limitations, e.g. the age of the patient (Bourgeois and Tarnopolsky, 2004). The disadvantage is that a full surgical procedure is required, leaving a scar. The second method is a "needle" muscle biopsy, where a needle device (modified Bergström needle) is passed through a small incision in the skin and fascia, through which a sample of muscle tissue is obtained (Magistris et al., 1998). Approximately 200 mg of tissue can be obtained with a single pass of the needle. The advantage is that only local anaesthesia is required, which simplifies the procedure. The important disadvantage is that local anaesthetics (lidocaine) used during the needle biopsy procedure may inhibit complex I activity (Chazotte and Vanderkooi, 1981).

2.6.2 Functional tests

The integrated functional activity of the RC coupled to ATP synthesis, membrane transport, dehydrogenase activities and the structural integrity of the mitochondria (Puchowicz et al., 2004) can be assessed by measuring oxygen consumption. A variety of functional tests can be performed, including polarographic analysis (RC), ATP synthesis

(OXPHOS) and pyruvate oxidation (NAD/NADH) (van den Heuvel et al., 2004). Studies of RC function require fresh, intact and well-coupled mitochondria.

2.6.2.1 Polarographic measurements (RC)

In polarographic measurements changes in the dissolved oxygen concentration are measured. These occur during respiration of intact mitochondria. The polarographic oxygen sensor is referred to as a Clark cell (Clark, 1822-1898). The Clark cell was invented in 1873 by Josiah Latimer Clark. It is a wet-chemical cell producing a highly stable voltage that is useful as a laboratory standard (Nicholls and Ferguson, 2001). When a polarographic system is used the measuring device is a Clark oxygen electrode, the processing/coupling device an oxygen monitor and the recording device a computer-assisted data acquisition system. The principle of the Clark oxygen electrode is illustrated in Figure 2.7.

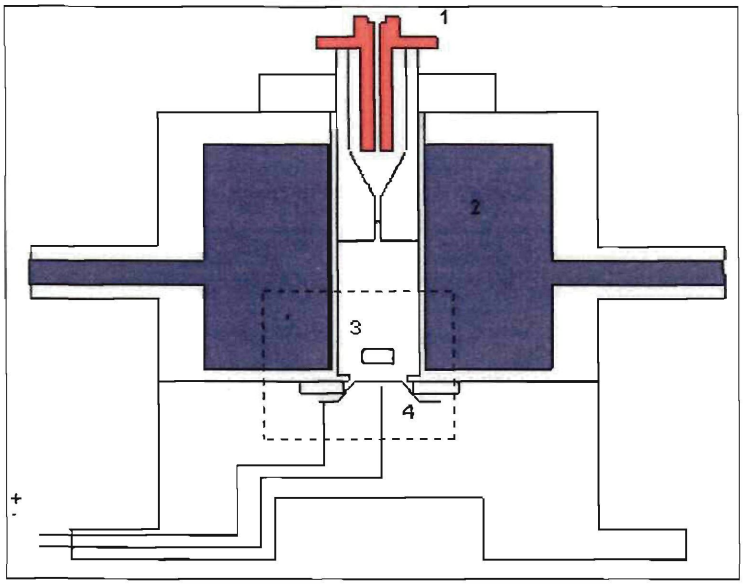


Figure 2.7: A cross-section through a typical polarography apparatus.

1 = stopper, 2 = water jacket, 3 = sample and magnetic stirrer, 4 = electrodes (silver anode (+) and platinum cathode (-)). Adapted from University of Leeds (2006).

The upper section contains a transparent, temperature-controlled sample chamber filled up with the solution or suspension to be assayed. It is then secured to the lower electrode assembly with a screwed ring. The stopper is used to seal the incubation chamber and prevent ambient air from entering. The small hole in the centre of the stopper permits the expulsion of air bubbles and allows small volumes of reagents to be added with a microlitre syringe. The content of the chamber is stirred continuously with a magnetic flea to ensure homogeneity and to ensure that oxygen can freely diffuse into the electrode. A thin

membrane separates the incubation medium from the strong KCl electrolyte in the electrode compartment (Nicholls and Ferguson, 2001). The reaction occurring in the electrode is shown in Figure 2.8.

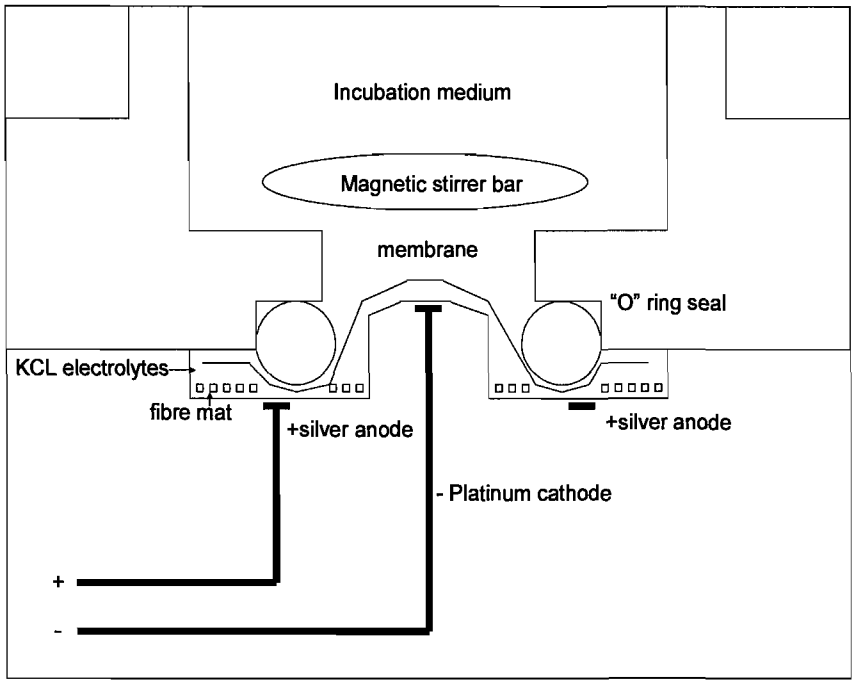


Figure 2.8: Principles of the Clark oxygen electrode. Adapted from University of Leeds (2006).

The Clark oxygen electrode is composed of two half cells separated by a salt bridge. The platinum cathode is separated from a solid silver anode by means of insulating material. A membrane holds concentrated KCl in place across the surface of the electrode and is attached by an O-ring that surrounds the electrodes. The coupling device, an oxygen monitor, maintains a constant voltage difference across the two electrodes so that the platinum electrode is negatively charged with respect to the positively charged silver electrode. Platinum is a strong catalyst for the covalent dissociation or reassociation of water. Electrons "boil" off the platinum electrode and combines with dissolved molecular oxygen that diffuses through the membrane and hydrogen ions to produce water. The rate at which electrons boil off is proportional to the concentration of oxygen that is available to "grab" them. The movement of electrons is induced by an electrical current, which is converted into a voltage by the oxygen monitor (Nicholls and Ferguson, 2001).

The following occurs at the anode (Equation 2.7) and cathode (Equation 2.8) respectively:

Equation 2.7: Reaction at the anode of the Clark oxygen electrode

$$4Ag^{+} + 4Cl^{-} \rightarrow 4AgCl + 4e^{-}$$

Equation 2.8: Reaction at the cathode of the Clark oxygen electrode

$$4H^{+} + 4e^{-} + O_2 \rightarrow 2H_2O$$

Current flows from the silver electrode to the platinum electrode as electrons boil off into solution from the latter. Removal of electrons from solid silver produces silver ions. The silver ions combine with chloride ions in solution to precipitate silver chloride on the silver electrode surface, leaving behind potassium ions. However, since hydrogen ions are taken out of solution by the consumption of oxygen, the charge remains balanced. The recording device is a computer-assisted data acquisition system, the result appearing exactly as indicated in Figure 2.9 (Nicholls and Ferguson, 2001).

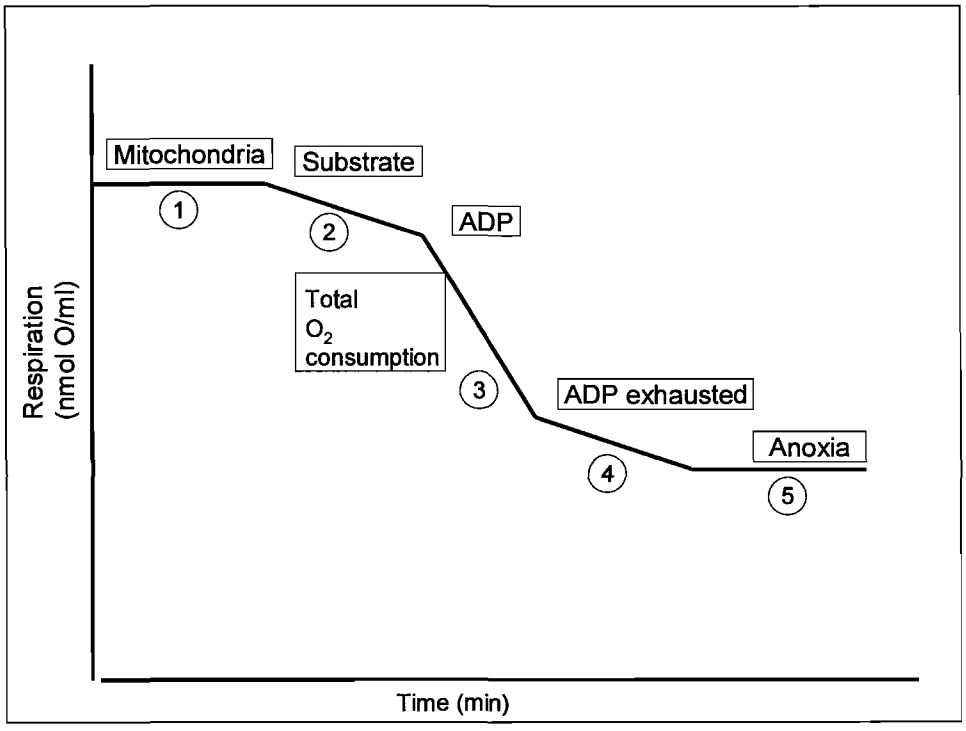


Figure 2.9: Respiration of intact mitochondria.

The figure illustrates respiration (nmol O/ml) against time (min) and the various states of respiration (encircled). After the addition of mitochondria only (State 1), various substrates can be added (State 2). Here, respiration is slow as the proton circuit is not completed by H^{+} re-entry through ATP synthase. Thus, the slow respiration can be attributed to a slow proton leak across the membrane. When ADP is added (State 3), ATP synthase synthesises ATP coupled to proton re-entry across the membrane. When the added ADP is exhausted, respiration slows (State 4) which is comparable to State 2 respiration and finally anoxia is attained once substrates are exhausted (State 5). Adapted from Nicholls and Ferguson (2001).

2.6.2.1.1 Substrates used during polarographic measurements

Mitochondrial function can be studied by measuring the rates at which they consume oxygen on different substrates and how an exposure to reagents such as metabolic poisons affects oxygen consumption.

When mitochondria are isolated, their oxygen consumption is slow (state 1). Mitochondria use oxidisable substrates (state 2) to produce NADH/$FADH_2$, which is used by the electron transport chain to produce an electrochemical potential of protons (Munnich et al., 1995).

Although several substrates (reducing equivalents) can be used for respiration, the substrates that were used in this study were pyruvate + malate, glutamate + malate and succinate.

2.6.2.1.1.1 Pyruvate and malate

Pyruvate is decarboxylated to acetyl-CoA as illustrated in Equation 2.2 by the pyruvate dehydrogenase complex; the acetyl-CoA is subsequently oxidised in the Krebs cycle. Reduced NADH is then produced. Malate is used to speed up the TCA as illustrated in Equation 2.9.

Equation 2.9: Malate is used to speed up the TCA cycle

$$\text{Malate} + \text{NAD} + \text{oxaloacetate} \rightarrow \text{NADH} + H^+$$

2.6.2.1.1.2 Glutamate and malate

Glutamate oxidation in the absence of malate depends largely on the oxidation of 2-oxoglutarate to generate oxaloacetate for transamination. Malate dehydrogenase is the predominant source of electrons for the respiratory chain and the role of glutamate is mainly to keep oxaloacetate from accumulating (Nicholls and Ferguson, 2001).

NADH, which is produced in the cytoplasm, does not have direct access to complex I, whose NADH binding site is located in the inner membrane. The malate-aspartate shuttle, as indicated in Figure 2.10, is used to transfer NADH into the mitochondrion so that it can bind to complex I (Nicholls and Ferguson, 2001).

Cytoplasmic NADH is oxidised by cytoplasm malate dehydrogenase. Malate enters the matrix in exchange for 2-oxoglutarate. Malate is then reoxidised in the matrix by the

malate dehydrogenase-generating matrix NADH. Matrix oxaloacetate transaminates with glutamate to form aspartate and 2-oxoglutarate. Subsequently, 2-oxoglutarate transaminates in the cytoplasm with transported aspartate to generate cytoplasmic oxaloacetate, producing cytoplasmic glutamate, which re-enters the matrix by proton symport in exchange for aspartate (Nicholls and Ferguson, 2001).

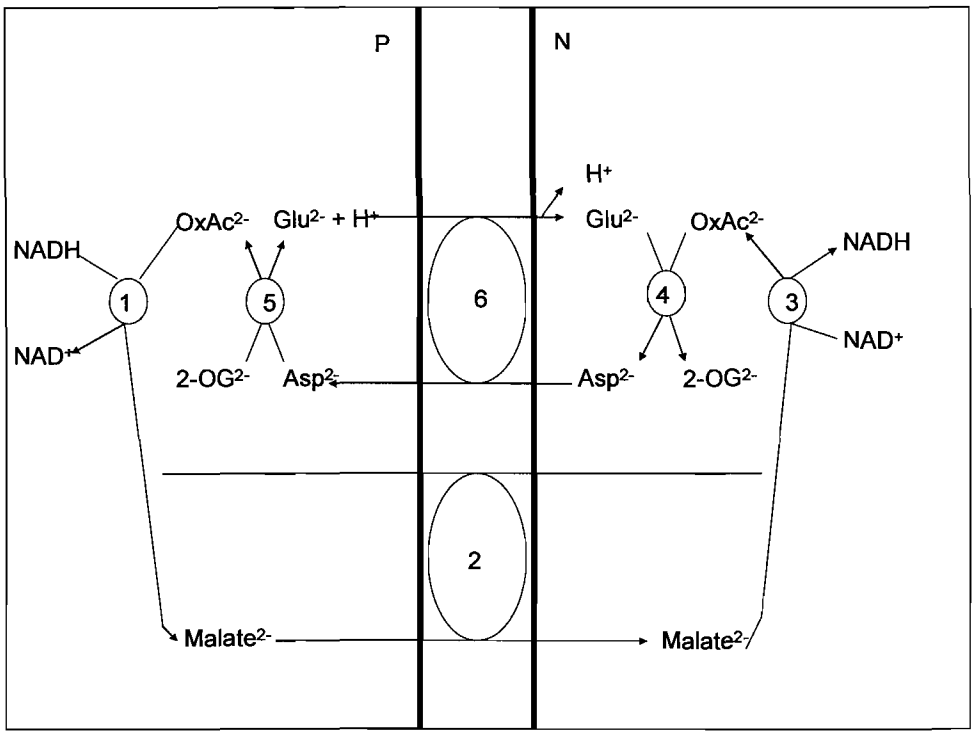


Figure 2.10: Malate-aspartate shuttle.

Cytoplasmic NADH is oxidised by cytoplasmic malate dehydrogenase (1); malate enters the matrix in exchange for 2-oxogluratate (2); malate is reoxidised in the matrix by malate dehydrogenase, generating matrix NADH (3); matrix oxaloacetate transaminates with glutamate to form aspartate and 2-oxoglutarate (4); 2-oxoglutarate transaminates in the cytoplasm with transported aspartate to regenerate cytoplasmic oxaloacetate-producing cytoplasmic glutamate (5), which re-enters the matrix y proton symport in exchange for aspartate (6). Adapted from Nicholls and Ferguson (2001).

2.6.2.1.1.3 Succinate

Addition of pyruvate and glutamate indicates the efficiency of complex I, whereas succinate tests the activity through complex II.

Rotenone is used to block electron transfer between the complex I-associated iron-sulphur clusters and ubiquinone pool by binding to a single high-affinity domain in the PSST subunit (Palmer, 1968; Schuler and Casida, 2001). Succinate molecules bind to the

enzyme complex called succinate dehydrogenase (complex II). The energy carrier FAD is also part of the succinate dehydrogenase complex. Because the enzyme and FAD are both part of the same complex, the only step needed to initiate succinate oxidation is the binding of succinate to the enzyme. Succinate is oxidised to fumarate as illustrated in Equation 2.4(a), and NADH is further produced in the TCA cycle (Nicholls and Ferguson, 2001).

2.6.2.2 ATP synthesis (OXPHOS)

The last complex in the inner mitochondrial membrane is responsible for the formation of ATP. The complex is an intrinsic membrane protein, ATP synthase, and consists of two major subunits, F_1, the catalytic subunit, and F_0, a complex of integral membrane protein that mediates proton transport (Nicholls, 2001). As discussed in Section 2.4.4 a proton gradient is built up as a result of NADH feeding electrons into an electron transport system. The protons return to the mitochondrial matrix through channels in the ATP synthase enzyme complex. The RC coupled to ATP synthase activity (OXPHOS) can be measured as a function of general mitochondrial activity, depending on the substrate that enters mitochondria. Adenosine triphosphate is usually measured by HPLC (high-performance liquid chromatography) or luminescence.

2.6.2.3 Pyruvate oxidation (NAD/NADH)

Pyruvate oxidation is the step which connects glycolysis to the TCA cycle. The bulk of ATP used by many cells to maintain homeostasis is produced by the oxidation of pyruvate in the TCA cycle. During this oxidation process, reduced NADH is generated. The NADH is principally used to drive the processes of OXPHOS, which are responsible for converting the reducing potential of NADH into the high energy phosphate in ATP (West, 2004).

In the literature Jansen et al. (2006) measured the mitochondrial energy-generating system's (MEGS) capacity by measuring $^{14}CO_2$ production rates from the oxidation of [1-^{14}C] pyruvate. Pyruvate oxidation is measured in the presence of malate or carnitine, which is added to remove acetyl-CoA to prevent inhibition of PDHc by accumulation of its product. PDHc is regulated by the ATP/ADP, NADH/NAD^+ and acetyl-CoA/CoA rations. From the results a defect in the TCA cycle and RC will lead to a decreased oxidation rate for [1-^{14}C] pyruvate + malate and an increase in the acetyl-CoA/CoA or NADH/NAD^+ ratio.

2.6.3 Respiratory chain enzyme assays

Activity measurement of respiratory chain enzyme activities, separately or by groups, is most commonly used for evaluation of the presence of mitochondrial deficiency (Thorburn et al., 2004). These assays are almost exclusively spectrophotometric and use specific electron acceptors and donors. A small amount of material for enzyme assays is required and muscle samples can be frozen until analysis (van den Heuvel et al., 2004). Spectrophotometric assays have been widely used, but this method has two limitations. Firstly, there may be interference from nonspecific reactions in the mixture and, secondly, it does not measure the relative stoichiometry (relative amount of the individual components) of the RC (Jung et al., 2000). The activities of the individual enzymes are mostly compared (normalized) to the activity of the TCA cycle enzyme, citrate synthase, and to other enzymes in the RC, e.g. complex IV, or protein content (Thorburn et al., 2004).

Biochemical pitfalls for the determination of RC enzyme defects are listed below (Thorburn et al., 2004):

- Tissue specificity
- Difficulty in defining realistic normal ranges
- Inability of enzyme assays to detect some functional defects
- Variation in assay protocols and lack of widely accepted diagnostic criteria
- Quality assurance scheme

Fischer et al. (1986), Zeviani et al. (1988), Wallace et al. (1988); Trijbels et al. (1993) ; Rustin et al. (1994) and Taylor and Turnbull (1997) contributed towards the development of suitable methods for measurements of the respiratory chain complexes (RCC) in human tissues, but there have been continuous attempts to optimise the protocols (Birch-Machin et al., 1994; Estornell et al., 1993; Wiedemann et al., 2000; Mayr and Sperl, 2000; Birch-Machin and Turnbull, 2001). Today numerous variants for the enzymatic methods and handling of biopsies are in use.

The use of different protocols may lead to different results and complicate the diagnostic process. In 2004 the Mitochondrial Medicine Society and MITONET initiated the re-evaluation of the use of different protocols. Gellerich et al. (2004) prepared standard mitochondria homogenate (SMH) from bovine skeletal muscle and aliquots were sent to the 14 participating laboratories. The results differed to a large extent, but Lenaz found amongst all the different results that CoQ1 was the best electron (e^-) acceptors for the

study of complex I activity (Estornell et al., 1993). Mayr and Sperl (2000) compared 0.2% Tween 20 and 0.025% Lauryl Maltoside as detergents, and with both the detergents similar rates were detected, but Tween 20 allowed for longer stationary rates and should therefore be preferred. Gellerich et al. (2004) found that using Triton x 100 for citrate synthase allowed data with higher values due to a complete release of the enzyme.

In the literature different protocols for RC enzyme assays are used. Total muscle homogenate, 600 x g supernatant and isolated mitochondria were used. Linked RC enzymes assayed like complex I+III, while others assayed the individual complexes. From the results that Gellerich et al. (2004) compared, they found that the preparation of supernatants from muscle homogenate advanced the technique, since it removes unspecific proteins from the samples, which decreases the light scattering in the photometer.

Realistic reference ranges for RC enzymes, which are sufficiently sensitive to recognize real enzyme defects without the risk of diagnosing false positives, remain difficult to define. Wide variations of normal ranges were described by different centres.

In a tissue biopsy Thorburn et al., (2004) defined major and minor enzyme criteria as RC enzyme ratios of <20 and <30% of the control mean, respectively. Detailed diagnostic criteria were described by Bernier et al., (2002). For a definite diagnosis one required at least two types of evidence. Identification of two major or one major and two minor criteria allowed a definite diagnosis.

Morris et al. (1996), Zheng et al. (1990), Pitkanen et al. (1996) and Birch-Machin et al. (1994) quoted normal ranges based on the mean ± 2SD, giving lower limits for normal skeletal muscle activity. Kirby et al. (1999), Bentlage et al. (1996) and Rotig et al. (1997) quoted observed ranges with the minimum value as the limit.

2.7 OTHER METHODS FOR DETERMINATION OF ENZYME ACTIVITIES

Spectrophotometric assays have been widely used, but this method has two limitations. Firstly, non-specific reactions may interfere in the mixture and, secondly, the relative stoichiometry (relative amount of the individual components) of the RC may not be measured (Jung et al., 2000). The activities of the individual enzymes were mostly compared (normalized) to the activity of the TCA cycle enzyme, citrate synthase, to other enzymes in the RC, e.g. complex IV, or to the protein content (Thorburn et al., 2004).

An alternative for enzymatic measurements is the use of proteomic procedures like Blue-Native polyacrylamide gel electrophoresis (BN-PAGE) and MitoSciences Dipstick Assay technology for measuring protein quantity or enzyme activity.

2.7.1 Blue-native polyacrylamide gelelectrophoresis

Blue-native polyacrylamide gel electrophoresis (BN-PAGE) is a technique that has recently received much attention for its use in structural analysis of native (non-denatured) OXPHOS complexes (Zerbetto et al., 1997, Schagger and Jagow, 1991, Nijtmans et al., 2002). BN-PAGE was originally developed by Schagger and colleagues to resolve electron transport complexes (Schagger, 1996). The method was specifically developed for isolation of enzymatically active membrane proteins from biological membranes for the production of antibodies, N-terminal protein sequencing and the analysis of molecular masses and oligomeric states of native complexes (Schagger, 1996). This technique will probably be included in future diagnostics, since it has the advantage that practically all mitochondrial proteins are detectable.

The existence of a protein provides no information regarding its activity and kinetic properties, and because of this enzymatic methods will always still be required.

2.7.2 Dipstick Assays

MitoSciences Dipstick Assay technology is a fast, reproducible, quantitative method. The dipstick assays are easy to perform and isolation of mitochondria is not necessary. As little as a few micrograms of whole cells or tissue extract are required, thus these assays are versatile and do not require much time to perform, between 30 and 120 minutes.

The dipstick assays for enzyme activity are based on measuring the activity of an immunocaptured enzyme directly on the dipstick. An immobilized captured antibody on the dipstick binds the enzyme, and activity is measured by providing enzyme substrates and a precipitating reporter dye for signal visualisation. The signal intensity correlates with the enzyme activity level and is measured by a dipstick reader (MitoSciences' Hamamatsu ICA-1000) or by any other imaging system, such as a flat-bed scanner. Figure 2.11 illustrates this method by giving an example of the assay for the enzyme activity of complex I.

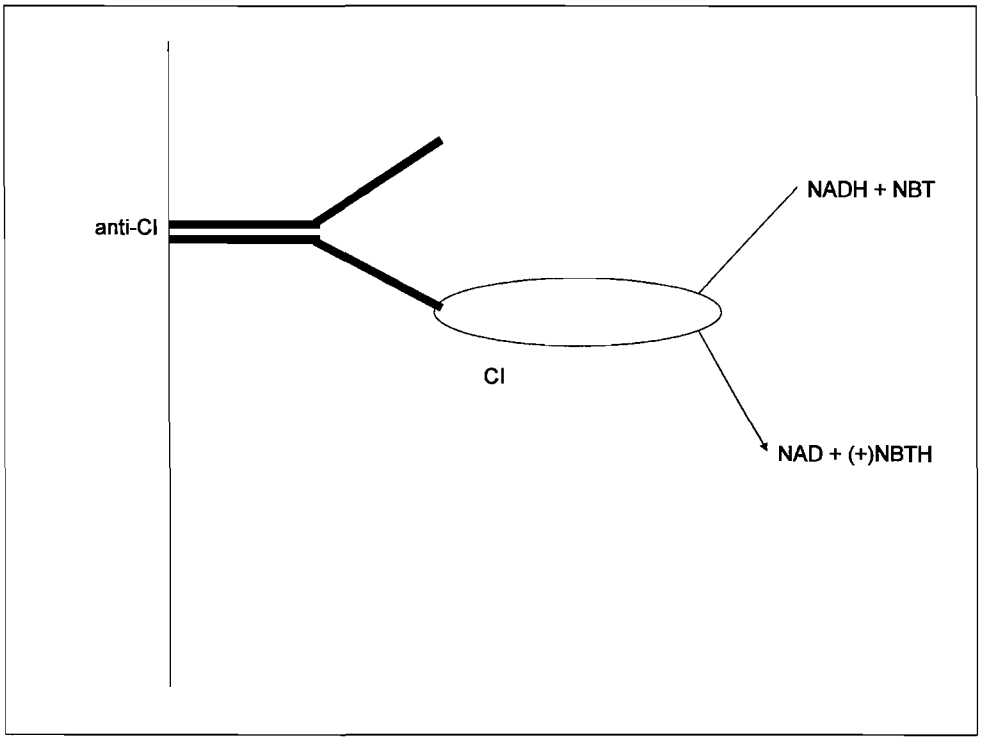


Figure 2.11: Enzyme activity of complex I using dipstick assay.

Complex I is immunocaptured onto the dipstick. The dipstick is immersed in complex I activity buffer containing NADH as a substrate and nitrotetrazolium (NBT) as the electron acceptor (reporter dye). Immunocaptured complex I oxidizes NADH; the resulting H^+ reduces NBT to form a blue-purple precipitate on the dipstick. Adapted from MitoScience (2007).

2.8 PROBLEM STATEMENT

Clinical suspicion of a mitochondrial disorder is typically based on the presence of suggestive clinical features and consideration of family history and the results of imaging or metabolic studies, particularly the finding of elevated lactic acid in cerebrospinal fluid or in blood. Over the past decade, mitochondrial disorders in South Africa have been clinically recognised and characterised, primarily by Dr. I. Smuts of the Department of Paediatrics and Neurology, University of Pretoria. However, the biochemical identification of mitochondrial disorders of South African patients has only more recently (since ~2004) been extended to include not only metabolic screening but also crucial single enzyme assays (School for Physical and Chemical Sciences, North-West University, Potchefstroom). The methodology for these assays has been based on those reported by Rahman et al. (1996). Assays on muscle biopsies have been performed on an infrequent basis and without optimisation due to the unavailability of enough control material, for which ethics approval is required. Although it is currently regarded as one of the "golden standard" for identifying mitochondrial disorders, only single-enzyme assays have been

performed on isolated mitochondria from muscle biopsies without any functional or mutation analyses to support the enzyme data.

The interpretation of patient RC enzyme activities, until now, has been based on "reference" values obtained (the presumably normal range of activities). Important to note is that these reference values were retrospectively compiled, after analysis, categorising values as "normal" or not. This approach, which uses normal distribution analysis of all data, is commonly used and has the advantage that a great amount of data can be included to set up reference values. However, its greatest limitation is that the biopsies used are from suspected mitochondrially diseased patients, and the data thus cannot confidently be regarded as proper, healthy, "normal" controls (Thorburn et al., 2004).

2.9 AIM, OBJECTIVES AND STRATEGY

Considering the previous limitations of biochemical evaluation described in Section 2.8, the *aim of this study therefore was to further enhance and evaluate key biochemical analyses to identify deficiencies of the respiratory chain enzymes and pyruvate dehydrogenase complex in South African paediatric patients.*

To achieve the aim of this study, two key objectives were formulated. The first objective was to set up peer-reviewed procedures for enzyme assays and respiration (as functional assays) in mitochondrial preparations and evaluate their responsiveness to protein content in reactions. The second objective was to obtain reference material (muscle biopsies) from at least 16 healthy children and determine the (presumably) normal distribution of enzyme and respiration activities in these biopsies, which may then be used as reference values for the identification of mitochondrial disorders. The methodological strategy is illustrated in Figure 2.12.

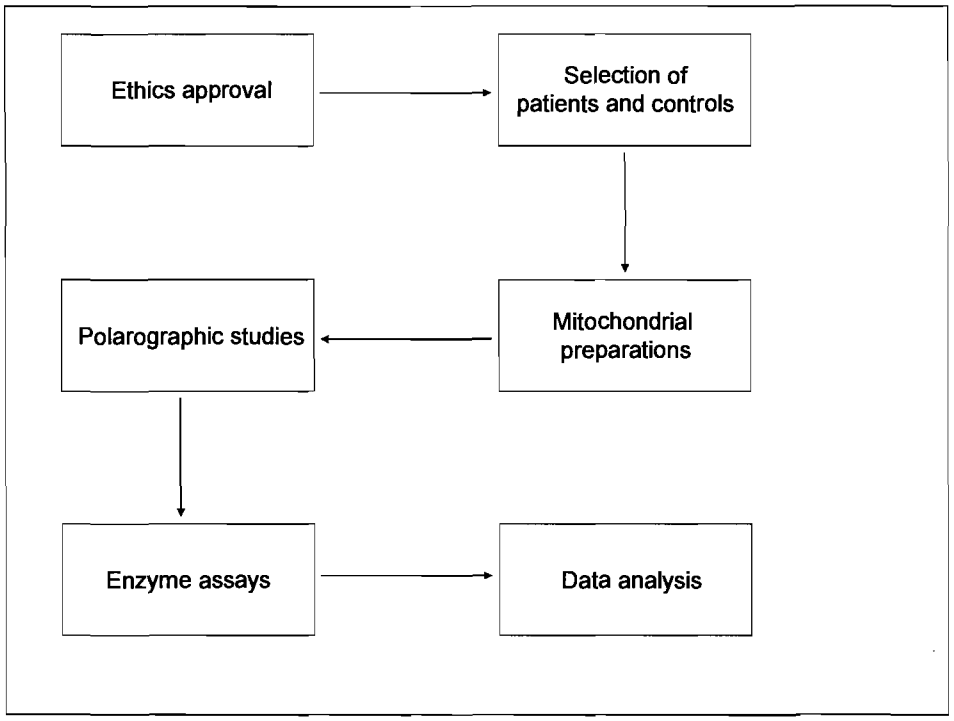


Figure 2.12: Step-by-step schematic illustration of the project strategy.

As shown in Figure 2.12, the first step was to apply for ethics approval for this study as it involves working with human material and all related ethical considerations. This ethics approval had to include the obtaining not only of muscle from clinically diseased patients, but also of (healthy) control muscle tissue, and the use of the material for the proposed biochemical procedures described in this study. Secondly, after obtaining ethics approval, paediatric patients (clinically identified as possibly having a mitochondrial disorder) and controls had to be selected and muscle biopsies obtained. This could be done exclusively by the paediatric neurologist and co-supervisor, Dr I Smuts, Paediatrics Neurology Unit at the Pretoria Academic Hospital.

Thirdly, the muscle biopsies were used to prepare enriched mitochondrial preparations after which (fourth step) respiration analysis was performed on this material. The fifth step in the strategy involved measuring RC enzyme activities using frozen mitochondrial preparations. These latter two steps in the strategy required setting up all the necessary facilities at two laboratories (UP and NWU) that would allow analyses of respiration and enzyme activities on a regular basis. The final step in the strategy was to analyse the data by statistical methods. To achieve the aim of the study, the data had to be evaluated to see what the distribution of data was between the various assays and to determine if respiration analysis added value to the process of identifying mitochondrial disorders. This

was done by determining if the trends observed from enzyme activities, as critical data for identifying the disorder, were reflected in the respiration data. Finally, a discussion and evaluation of the current approach for identifying mitochondrial disorders in South Africa, as was described in this study, is presented in Chapter 5.

Chapter 3
Materials and Methods

3.1 INTRODUCTION

In this study two specific analytical methods, namely mitochondrial respiration analysis (also called respirometry or polarography) and specific enzyme assays were applied in the identification of mitochondrial disorders. Research was done on a selection of patients and controls. The methods that were used were based on those that were published by leading scientists and clinicians in the area of mitochondrial disorders. The basic method that was used for the respiration measurements was based on those described by Rustin et al., (1993) and Aleardi et al., (2005), as well as on instructions by the manufacturer of the respirometer (Strathkelvin Instruments, Motherwell, Scotland). The method used for the enzyme assays is based on that described by Rahman et al., (1996). In this chapter descriptive methods are given, and in Appendix C more detailed methods are discussed.

The method for enzyme assays, using a spectrophotometric approach, has been used for a number of years (since 2004) in the Mitochondrial Research Laboratory at the School for Physical and Chemical Sciences, North West University Potchefstroom. However, the method was better standardised[4] for routine laboratory practice (see Appendix C), as well as better optimised for protein content. The respiration analysis was developed during this study as a mitochondrial function test and was optimised for protein content as well.

All analyses were performed *in vitro* at two different venues and required travelling between Potchefstroom and Pretoria on short notice. This logistical aspect is noteworthy since the practicability of this approach will be evaluated in Chapter 5. Due to the necessity to do respiration analysis on freshly isolated mitochondria, all apparatus and reagents for sample preparation and respiration analysis were installed or kept in a laboratory at the Pediatrics Neurology Research Clinic at the Pretoria Academic Hospital. The enzyme samples were then transferred to the Mitochondrial Research Laboratory at the School for Physical and Chemical Sciences, North West University, Potchefstroom, for enzyme analyses.

[4] The detailed methods in Appendix C describe practices that may appear trivial but have been developed better to enable routine use and make it more accurate for the specific laboratory where they are used.

3.2 PATIENTS AND CONTROLS

In this study, the co-supervisor, Dr. I. Smuts of the Paediatrics Neurology Research Clinic at the Pretoria Academic Hospital was responsible for the selection of patients and controls. Dr. Smuts applied for and obtained ethics approval (Protocol 91/98, with the latest amendments added July 2007) from the University of Pretoria for the making available and the use of muscle biopsies of patients, as well as controls, as described in this study. This approval allowed the use of these samples (after consent had been given) to carry out the respiration and enzyme analyses described in this study. Furthermore, the study was conditionally funded by a South African Medical Research Council (MRC) grant under the title "*The investigation of respiratory function in muscle of patients with possible mitochondrial disorders*".

The patients included in this study were mostly children (aged three months to 22 years, 9 months) with possible mitochondrial defects who had been referred to Dr. Smuts. These paediatric patients were clinically and biochemically examined (i.e. by body fluid tests), also for those features associated with mitochondrial disorders (see Nijmegen Mitochondrial Disease Criteria, Section 2.6). Patients whom she suspected to have a mitochondrial disorder (e.g. with NCMD score of 6 and higher) were selected to undergo a muscle biopsy for further biochemical tests. These patients underwent muscle biopsies (*vastis lateralis*) at the Paediatrics Neurology Research Clinic (Pretoria Academic Hospital). Such biopsies were used exclusively in this study.

The control muscle samples from children (aged two years, one month to 15 years five months) with assumingly normal mitochondrial functions were obtained from patients undergoing orthopaedic surgery at the Paediatrics Neurology Research Clinic (Pretoria Academic Hospital). All patients as well as their parents signed an informed consent (parents) and assent (patients) form that gave permission to use these muscle samples for, amongst others, the analyses described in this study. The signed forms are in the possession of Dr. I. Smuts but blank copie of this form are attached as Appendix B.

3.3 CALCULATION OF ENZYME ACTIVITIES

The principles in accordance with which the enzyme activities were determined in this study are based on the principles of steady-state kinetics described by Henri (1910) and Michealis and Menten (1912), as modified by Briggs and Haldane (1925). However, most of the enzyme assays in this study were two-substrate reactions and therefore the modifications of Alberty for two-substrate reactions, were used (Palmer, 2001). This

essentially means that to determine reaction velocities for the two-substrate reactions, substrates are kept at "very large" concentrations (Palmer, 2001). In all enzyme assays in this study, apparent maximum reaction velocity (V_{max}^{app}) was determined under published optimal reaction conditions and normalised for protein content or to a reference enzyme (specific activity).

In these reactions, enzyme and substrate combine to form an enzyme-substrate (ES) complex, which undergoes a further reaction to break down to enzyme and product. If the velocity is determined at the initial period of reaction, the product concentration is negligible and the formation of ES from product can be ignored as illustrated in Equation 3.1.

Equation 3.1: Enzyme and substrate combine to form a product.

$$E + S \leftrightarrow ES \rightarrow E + P$$

E = enzyme; S = substrate; P = product. Adapted from, Palmer 2001.

Initial velocity of a reaction is determined from a graph of product (or substrate) concentration against time, as illustrated in Figure 3.1. Alternatively, a direct instrumental reading known to be proportional to product (or substrate) concentration, an absorbance unit may be plotted against time (Palmer, 2001).

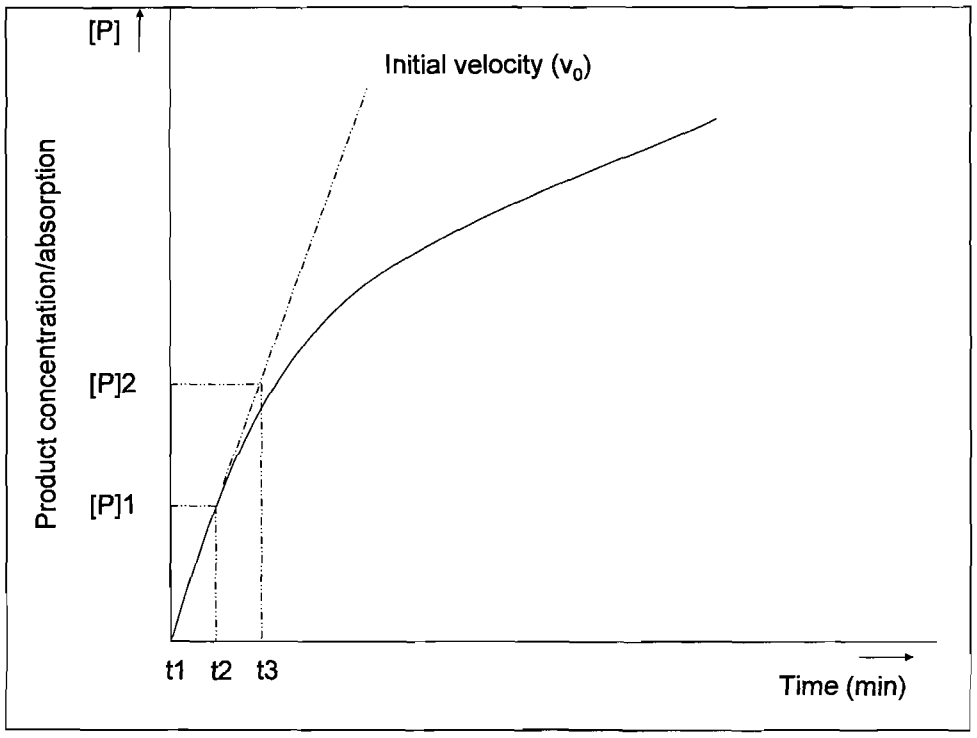


Figure 3.1: Example of measurement of initial reaction velocity.

The initial reaction velocity (v_o) can be determined by product formation (as indicated in this graph on the y-axis) or substrate use over time (x-axis). This is done using the linear slope as indicated by the period (*t1-t2*). The linearity of reactions can be evaluated by determining the R^2 value from the reaction R^2 = 1-SSR/SST, where SSR is the Sum Squared regression and SST is the Sum Squared Total. A value of 1 would indicate perfect linearity.

As indicated in Figure 3.1 the rate of the reaction at any time (t) is the slope of the curve at that point. This may be constant for a short time at the start of the reaction (steady state) and then decreases with the decreasing concentration of the reactant as the reaction proceeds, finally falling to zero. At that point much of the reactants have been converted into products, or much more commonly, a chemical equilibrium has been set up. The initial velocity of the reaction may be determined by using Equation 3.2.

Equation 3.2: Determining the initial velocity of a reaction

$$v_o = \Delta[S]/\Delta t = \Delta[P]/\Delta t$$

v_o = initial velocity; t = time. Adapted from, Palmer 2001.

If there is a difference between substrate and product in the absorbance of light of a particular wavelength, spectrophotometric techniques may be used to calculate molar concentration as well as reaction velocity and specific activity. In that case Equation 3.3

can be used, which has been used in principle in all enzyme assays in this study (compare Equations. 3.7, 3.9, 3.11, 3.13, 3.15 and 3.17)

Equation 3.3: Spectrophotometric techniques to calculate specific activity

$$\text{Specific activity} = \left[V / \left(\varepsilon \times d \times v \times c_{protein}\right)\right] \times \left(\Delta E / \Delta t\right)$$

V = assay volume (ml); ε = extinction coefficient (cm^2/µmole); d = light path (cm); v = volume of sample used in assay (ml); $c_{protein}$ = protein concentration (µmole/ml), (µg/ml); ΔE = extinction change; Δt = interval between measurements (min). Adapted from Bergmeyer (1963).

3.4 MATERIALS

The best available quality (mostly so-called "ultra pure" (99.9%) forms) of the following reagents were purchased from Sigma-Aldrich (St. Louis, Mo., USA): mannitol, sucrose, Tris.Cl, EDTA, KCl, Tris-phosphate, ADP, pyruvate, malate, glutamate, succinate, rotenone and potassium cyanide (KCN), NADH, antimycin A, bovine serum albumin (BSA), coenzyme Q1, ethanol, cytochrome c, n-dodecylmaltoside, KH_2PO_4, K_2HPO_4 and reagents for protein determination, using the bicinchoninic acid (BCA) method.

Water that was used to prepare all reagents and reactions has been purified using the MilliQ system from Millipore (Billerica, MA (USA) according to the procedures described in Appendix C, referenced to "water", except when ice was used to cool samples. This report implies water that was prepared as described. All the plastic consumables such as pipette tips and micro-centrifuge tubes as well as water were sterilised by autoclaving. Buffers and substrates for respiration were sterilised by filtration using a 0.2 µm filter (Pall Corporation, Acrodisc Syringe Filters).

3.5 MUSCLE SAMPLE PREPARATION

Mitochondria were isolated from fresh muscle biopsies within at most three hours after the biopsy had been taken from patients or controls. The muscle biopsies were collected and kept on ice in Medium A during the whole isolation procedure. After receiving samples, fatty tissue was first removed. The remaining muscle was then weighed and finely cut with a sterile blade on a cold glass plate which was placed on top of watery ice. The muscle sample was then suspended in Medium A (210 mM manitol, 70 mM sucrose, 10 mM EDTA, 50 mM Tris.Cl, pH 7.4) to a concentration of 10% (w/v) and homogenised in watery ice using a Potter Elvehjam homogeniser using 15 strokes with the pestle turning at 500 rpm. The homogenate was transferred to a micro-centrifuge tube and centrifuged at 1000 x

g for 5 min. The supernatant was transferred to a clean micro-centrifuge tube and centrifuged at 7000 x g for 10 min. The resulting pellet was resuspended in Medium B (225 mM manitol, 75 mM sucrose, 0,1 mM, EDTA, 10 mM Tris.Cl, pH 7.4) and centrifuged at 7000 x g for 10 min. This buffer contains much less EDTA compared to Medium A. The remaining mitochondrial pellet was carefully resuspended into 1/20 of the original volume using Medium B (Rustin et al., 1994; Aleardi et al., 2005). Protein concentration was measured by the bicinconinnic acid (BCA) method using bovine serum albumin as standard (Smith et al., 1985).

3.6 RESPIRATION ANALYSIS

Mitochondrial respiration analysis must be done on freshly isolated intact mitochondria because the inner mitochondrial membrane needs to be intact (coupled). Immediately after mitochondria had been isolated, the respiration analysis was done. The method used was based on that of Aleardi et al., (2005). The optimisation of the protocol for respiration and in particular the protein and ADP concentration was done in a preliminary study that was published in a previous research report (du Toit, 2006). A detailed procedure for this method is given in Appendix C.

3.6.1 Calibration of respirometer

Before each day's respiration analyses were done, the respirometer (see Figure 2.7), which consisted of a thermostatically controlled chamber equipped with a Clark-type oxygen electrode (MT200A system from Strathkelvin Instruments, Motherwell, Scotland) was calibrated as instructed by the supplier. The electrode was also calibrated when the measurement units were changed. After connecting the respirometer to a water bath (set at exactly 30 °C) and to the recording unit, which was connected to a computer, calibration commenced. The software that was used for all respiration measurements and analyses was the 782 Oxygen System (version 3.0) from Strathkelvin Instruments, Motherwell, Scotland. Calibration was done by firstly calibrating the electrode using an oxygen-free solution. This solution is prepared using sodium sulphite (20 mg/ml) which reacts with oxygen according to Equation 3.4:

Equation 3.4: Removal of oxygen from water using sodium sulphite

$$2Na_2SO_3 + O_2 \rightarrow 2Na_2SO_4$$

Na_2SO_3 = anhydrous sodium sulphite.

The result after this calibration was that the current read 0 pAmp in the oxygen-free solution. This solution was then removed and the chamber rinsed three times with water. The high-point calibration was done by adding air-saturated water (water that was stirred with a magnetic stirring bar at high speed for about 10 minutes) to the chamber. The plunger was then placed on top of the chamber, the stirring bar activated and high-point calibration initiated. The pAmp reading of this solution had to be greater than 300. After this step the respirometer would record this solution as 199.0 µmol/l oxygen in the solution, which is the concentration of oxygen-saturated water at 30 °C. After this step the calibration was completed and all reactions could then start at exactly the same temperature.

3.6.2 Respiration analysis of mitochondrial samples

Mitochondrial oxygen consumption was monitored at 30° C in volumes of 70 µl (continuously stirred using a magnetically driven stirring bar). The respiration buffer consisted of 75 mM mannitol, 125 mM sucrose, 100 mM KCl, 50 µM EDTA, 10 mM Tris-phosphate and 10 mM Tris.Cl (pH 7.5), as well as respiratory substrate combinations. These substrate combinations included either 10 mM pyruvate combined with 10 mM malate (P+M), 10 mM glutamate combined with 10 mM malate (G+M), or 10 mM succinate and 1 µM rotenone (S+R). The mitochondrial protein concentration used for respiration measurements was 0.25 µg/µl and state 3 respiration was obtained by addition of either 2.0 mM (mostly) or 0.2 mM ADP. The sequence of the reaction progressed basically as follows:

- Addition of buffer, water and mitochondria (excluding substrates), equilibration of temperature, followed by baseline respiration measurement for approximately 5 sec [state 1 respiration (mitochondria in presence of only phosphate)].
- Addition of a substrate combination (either P+M, G+M or S+R) followed by respiration measurement for approximately 15 sec [state 2/4 respiration (mitochondria in the presence of phosphates and substrate, without ADP)].
- Addition of 2 mM ADP, followed by respiration measurement for approximately 15 sec [state 3 respiration (mitochondria in the presence of phosphates, substrate and ADP)].
- Stopping the reaction and saving data under a specific file name.
- Rinsing sample holder three times with water before next reaction.

The rate of respiration was calculated using the 782 Oxygen System software. The linear rate of state 2/4 and 3 was manually selected and the software then converted these

absolute rates to a protein-normalised respiratory rate of either state 2/4 or state 3, expressed in pmol/min/µg protein. The respiratory control ratio (RCR), which is defined as state 3 respiratory rate divided by the state 2/4 respiratory rate, was also calculated. This value reflected the integrity or coupling of the inner mitochondrial membrane and thus gave an indication of the quality of the isolated mitochondria (Nicholls and Ferguson, 2001). The remaining mitochondrial samples were immediately frozen at -70 °C for enzyme analyses and transported on dry ice when required.

3.7 MITOCHONDRIAL RC AND PDHc ENZYME ASSAYS

The activities of each of the enzymes in the respiratory chain as well as PDHc and citrate synthase were measured using specific assays. These enzyme assays can be performed on frozen tissue or frozen, isolated mitochondria and appears to be stable for over a decade if the sample had been frozen and stored at -70 °C (Thorburn et al., 2004). Before mitochondria could be used for these assays the membranes first had to be disrupted. Freezing and thawing as well as sonification of the enriched mitochondria preparation disrupt the membranes and exposes the membrane- and matrix enzymes. The activities of complex I, complex I+III, complex II+III, complex IV and PDHc in skeletal muscle collected from the control and patients were calculated. Citrate synthase, an enzyme of the TCA cycle, was also assayed as a reference enzyme to indicate the relative amount of mitochondria present in each sample (Trounce et al., 1996; Thorburn et al., 2004). In addition, the protein concentration of each control and patient mitochondrial preparation was determined and utilised when calculating the activities of these enzymes.

The methods for RC enzymes that were used were based on original methods developed by Rahman et al., (1996). These methods, or more accurately the operating procedures, were modified since 2004 in the Mitochondrial Research Laboratory, NWU, to suit the apparatus used there (e.g. different spectrophotometer and kinetics software is used). Furthermore, the protein content for these assays were optimised since isolated mitochondria instead of whole homogenate supernatants as described in the original method were used (Rahman et al., 1996).

3.7.1 Partial optimisation of enzyme assays and use of reference controls

To optimise the assays for protein content and to obtain reference controls as indicators of day-to-day variation of assays, human tissue could not be used for ethical reasons. Therefore, rat muscle tissue was obtained and used for these purposes. Ethics approval

for this was obtained from the Ethics Committee of the NWU under the title,"*Die gebruik van proefdierweefsel as referensiemateriaal vir funksionele analises van mitochondriale oksidatiewe fosforileringsdefekte*" (06D03).

Rat (Sprague Dawly, male) muscle was obtained from the Research Animal Centre at the NWU and mitochondria were isolated as described in Section 3.5. Isolated mitochondria were disrupted and their protein concentration determined as described in Section 3.7.2. For each of the enzyme assays described in this section the protein content was varied in a series of reactions using rat mitochondria to determine the protein concentration range where the increase in enzyme activity gave a linear correlation with protein content. In other words, the protein concentration range where the specific activity (reaction velocity normalised to protein content) remained constant was determined for each enzyme assay. This allowed better sensitivity of the assays and would ensure that differences in the enzyme activities between different samples would be detected irrespective of small changes in protein content. Furthermore, due to the limited amount of muscle that was received, which resulted in small amounts of mitochondria, the minimum amount of protein required for the assays needed to be determined.

In this study the addition of reference controls were included as controls for day-to-day variation. These samples, which were frozen at -70 °C in aliquots, were prepared from a single large sample of rat muscle mitochondria. A single aliquot was used whenever a batch of human samples was assayed for all enzymes described here.

3.7.2 Protein concentration assay

The bicinchoninic acid (BCA) method for protein determination was used in this study (Smith et al., 1985), using bovine serum albumin (BSA) as standard. The BCA method in principle is the same as the Lowry assay, but requires only a single step. The BCA method uses the peptide-bond induced conversion of copper II (Cu^{2+}) to copper (Cu^{+}) ions under alkaline conditions. The formation of Cu^{+}, which is directly proportional to protein content, interacts with BCA and leads to the formation of a purple-coloured chromogen, which is measured at 560 nm. The protein content of the samples was inferred from the known concentration of the BSA standard (Smith et al., 1985).

For the assay, a reagent mix was prepared containing BCA and copper (II) sulphate pentahydrate ($CuSO_4.5H_2O$) in a ratio of 50:1, respectively. A standard series, ranging from 0 to 20 µg, were pipetted onto a microtiter plate. Mitochondrial preparations were diluted (1:10) in water and 10 µl of this was added in duplicate to the microtiter plate.

Subsequently, 200 µl of the reagent mix was added to each well and the microtiter plate incubated at 37° C for 20 minutes in a preheated FL 600 Microplate Fluorescence Plate Reader (Bio-Tek Instruments, Neufarn, Germany). The absorbance of the reaction was measured at 560 nm and the protein content of the samples calculated from the protein standards using linear regression. Protein concentration was then determined using Equation 3.5. Samples were next diluted in Medium B (Section 3.5) to a final concentration of 1 µg/µl and stored at -70 °C until use.

Equation 3.5: Determining protein concentration

$$[\mu g/\mu l] = [\text{protein content } (\mu g) \text{ of sample xdilution}]/\text{sample volume } (\mu l)$$

3.7.3 Citrate synthase assay

Citrate synthase (CS) activity was used to normalise all other enzyme activities. This is a common practice to express enzyme activities and is based on the assumption that mitochondrial content is proportionally related to CS specific activity (Thorburn et al., 2004). The method used is based on one commonly used and described by Robinson et al. (1987) and is founded on the following principle:

Equation 3.6: Principle of the citrate synthase assay

$$\text{OAA} + \text{acetyl CoA} \rightarrow \text{citrate} + \text{CoA} \cdot \text{SH (linked to DTNB)}$$

OAA = oxaloacetate. Adapted from Thorburn et al. (2004).

CS activity was measured at 412 nm in a preheated sample holder at 30 °C, using disposable cuvettes. The complete reaction in a volume of 500 µl consisted of 100 mM Tris.Cl (pH 8.0), 0.1 mM DTNB, 0.008 % (v/v) Triton X100, 60 µM acetyl-CoA, 10 mM oxaloacetate and 5 µg mitochondrial protein. The measurement of the reaction was initiated in the absence of OAA and this substrate-free rate was recorded for 1 minute (A1). The OAA was then added and the increase in the linear rate was recorded for 2 minute (A2). The specific activity of CS was calculated using Equation 3.7.

Equation 3.7: Calculating the specific activity of CS

$$\mu\text{mol/min/mg(UCS)} = [(A2 - A1)/13.6\text{mM}^{-1}) \times 0.5/(/([\text{protein}] \times 5\text{ul}/1000)]$$
$$\text{nmol/min/mg} = \text{UCS} \times 1000$$

13.6mM^{-1} = extinction coefficient for TNB. A1 = rate in absence of OAA, A2 = rate in presence of OAA. Protein concentration was measured in mg/ml. Adapted from Robinson et al. (1987).

3.7.4 Complex I (NADH: UBIQUINONE oxidoreductase)

Spectrophotometric assays of complex I can be followed either by the reduction of ubiquinone or its homologues or by the rotenone-sensitive oxidation of NADH (Trounce, 1996). To determine rotenone-sensitive activity, which relates to the specificity of complex I in crude preparations, the enzyme assay must be done in the presence and in the absence of rotenone. This specific activity of complex I can be calculated by subtracting the activity in the presence of rotenone from the activity in the absence of rotenone. The method used is based on that by Rahman et al. (1996) and is founded on the following principle:

Equation 3.8: Principle of the CI assay

$$NADH + Q_1 \rightarrow NAD^+ + Q1 \cdot H_2$$

Q_1 = coenzyme Q_1. Adapted from, Thorburn et al. 2004

Complex I activity was measured at 340 nm in a preheated sample holder at 30° C, using disposable cuvettes. The complete reaction in a volume of 500 µl consisted of 50 mM potassium buffer, 2 mM NADH, 1 mM KCN, 0.5 mM antimycin, 0.1 % (w/v) BSA, 10 mM CoQ1, 25 µg mitochondrial protein and 5 µl ethanol (A1) or 5 µl rotenone (A2),. The measurement of the reaction was thus recorded in the absence of rotenone (A1) and in the presence of rotenone (A2). The linear decrease at 340 nm (NADH oxidation) was recorded for 2 min. The specific activity of complex I was calculated using Equation 3.9 and was finally normalised against CS activity.

Equation 3.9: Calculating the specific activity of CI

$$\mu mol/min/mg = \left((A1-A2)/6.22mM^{-1}\right) x\ 0.5 / \left([protein]\ x\ 25\mu l/1000\right)$$

$$nmol/min/UCS = \left(1000\ x\ \mu mol/min/mg\right)/UCS$$

$6.22mM^{-1}$ = extinction coefficient for NADH, which includes correction of the contribution of CoQ1 at 340nm; A1 = rate in absence of rotenone. A2 = rate in presence of rotenone. Protein concentration was measured in mg/ml. Adapted from Rahman et al. (1996).

3.7.5 Complex I + III (NADH-cytochrome c reductase)

The combined activity of complexes I+III can be measured as a coupled assay following the NADH-driven rotenone-sensitive reduction of cytochrome c. If a limited sample is available and respiration studies are not possible, this assay as well as a coupled complex

II+III assay are useful because of the limited amount of material required. Information on organelle fragility or altered inner membrane function can be provided through these coupling assays (Trounce et al., 1996). The method used is based on that described by Rustin et al., (1994).

Complex I+III activity was measured at 550 nm in a preheated sample holder at 30 °C, using disposable cuvettes. The complete reaction in a volume of 500 µl consisted of 50 mM potassium buffer, 2 mM NADH, 1 mM KCN, 0.1 % (w/v) BSA, 2 mM cytochrome c, 5 µg mitochondrial protein, 5 µl ethanol (A1) or 2.5 µM rotenone (A2). The measurement of the reaction was recorded in the absence (A1) and in the presence of rotenone (A2). The linear increase at 550 nm (cytochrome c reduction) was recorded for 2 minutes. The specific activity of complex I+III was calculated using Equation 3.10 and was finally normalised against citrate synthase activity.

Equation 3.10: Calculating the specific activity of complex I+III

$$\mu mol/min/mg = \left((A1-A2)/29.5mM^{-1}\right) \times 0.5 / \left([protein] \times 5\mu l/1000\right)$$
$$nmol/min/UCS = \left(1000 \times \mu mol/min/mg\right)/UCS$$

$29.5mM^{-1}$ = extinction coefficient for cytochrome c. A1 = rate of cytochrome c oxidation in absence of rotenone. A2 = rate of cytochrome c oxidation in presence of rotenone. Protein concentration was measured in mg/ml. Adapted from Rustin et al. (1994).

3.7.6 Complex II+III (Succinate-cytochrome c reductase)

The method of reduction of cytochrome c by complex III coupled to succinate oxidation through complex II was followed (Trounce et al., 1996). The method used is based on that described by Rahman et al. (1996) and is founded on the following principle:

Equation 3.11: Principle of the CII+III assay

$$\text{Succinate} + \text{cytothrome } c_{ox} \rightarrow \text{fumarate} + \text{cytochrome } c_{red}$$

Cytochrome c_{ox} = oxidised cytochrome c ; cytochrome c_{red} = reduced cytochrome c . Adapted from Thorburn et al. (2004).

Complex II+III activity was measured at 550 nm in a preheated sample holder at 30 °C, using disposable cuvettes. The complete reaction in a volume of 500 µl consisted of 50 mM potassium buffer, 10 mM succinate, 1 mM KCN, 0.08 % (w/v) EDTA, 0.1 % (w/v) BSA,

2.5 µM rotenone, 5 µg mitochondrial protein, 0.2 M ATP and 2 mM cytochrome c. The linear increase at 550 nm (cytochrome c reduction) was recorded for 2 minutes (A1). The specific activity of complex II+III was calculated using Equation 3.12 and was finally normalised against citrate synthase activity.

Equation 3.12: Calculating the specific activity of complex II+III

$$\mu mol/min/mg = \left((A1-A2)/29.5\ mM^{-1}\right) \times 0.5 / \left([protein] \times 5\mu l/1000\right)$$
$$nmol/min/UCS = \left(1000 \times \mu mol/min/mg\right)/UCS$$

$29.5mM^{-1}$ = extinction coefficient. A1 = rate in presence of cytochrome c; A2 = rate in the absence of cytochrome c. Protein concentration was measured in mg/ml. Adapted from Rahman et al. (1996).

3.7.7 Complex IV (Cytochrome c oxidase)

Cytochrome c oxidase is readily measured by following the oxidation of reduced cytochrome c (Trounce et al., 1996). The method used is based on that described by Rahman et al. (1996) and is founded on the following principle:

Equation 3.13: Principle of the complex IV assay

$$Cytc_{red} + O_2 \rightarrow Cyt\ c_{ox} + 1/2H_2O$$

Complex IV activity was measured at 550 nm in a preheated sample holder at 30° C, using disposable cuvettes. The complete reaction in a volume of 500 µl consisted of 50 mM potassium buffer, 125 mM n-dodecylmatoside, 20 µM $cytc_{red}$ and 2 µg mitochondrial protein. The increase in the linear rate was recorded for 2 minutes (A1). The specific activity of complex IV was calculated using Equation 3.14 and was finally normalised against citrate synthase activity.

Equation 3.14: Calculating the specific activity of CIV

$$\mu mol/min/mg = \left(A1/29.5\ mM^{-1}\right) \times 0.5 / \left([protein] \times 2\mu l/1000\right)$$
$$nmol/min/UCS = \left(1000 \times \mu mol/min/mg\right)/UCS$$

$29.5mM^{-1}$ = extinction coefficient. A1 = rate in the presence of reduced cytochrome c; Protein concentration was measured in mg/ml. Adapted from Rahman et al. (1996).

3.7.8 Pyruvate Dehydrogenase complex

Pyruvate dehydrogenase complex (PDH_C) is one of the largest enzymes known (Mw = ~8,000,000 Da) and is an example of a multi-enzyme complex. Pyruvate dehydrogenase enables pyruvate to enter the tricarboxylic acid cycle by catalysing its overall conversion to acetyl-CoA (Palmer, 2001). The method used is based on a method described by Chretien et al., (1995). A key aspect of this method is that the problematic interfering reduction of NAD^+ by L-lactate dehydrogenase (LDH) is prevented by the inclusion of an inhibitor (oxamic acid) of LDH. This method is based on the following principle:

Equation 3.15: Principle of the PDHc assay

$$\text{pyruvate} + \text{CoASH} + \text{NAD}^+ \rightarrow \text{Acetyl}-\text{CoA} + \text{CO}_2 + \text{NADH}$$

PDH_C activity was measured at 340 nm in a preheated sample holder at 30 °C, using disposable cuvettes. The complete reaction in a volume of 500 µl consisted of PDH_C buffer (0.6 M D-Manitol, 20 mM KCl, 10 mM $MgCl_2$.$6H_2O$, 20 mM KH_2PO_4 .$3H_2O$, 0.2% (v/v) Triton X100) 0.5 mM NAD^+, 0.2 mM CoA, 25 mM oxamic acid, 1 mM cystein, 0.2 mM TPP, 2 µg mitochondrial protein and 40 mM pyruvate (A1) or 5 µl water (A2). The linear increase at 340 nm (NAD^+ reduction) was recorded in two separate cuvettes for 2 minutes. The specific activity of complex IV was calculated using Equation 3.16 and was finally normalised against citrate synthase activity.

Equation 3.16: Calculating the specific activity of PDHc

$$\mu\text{mol/min/mg} = \left((A1-A2)/6.22\ \text{mM}^{-1}\right) \times 0.5 / \left([\text{protein}] \times 10\mu\text{l}/1000\right)$$
$$\text{nmol/min/UCS} = (1000 \times \mu\text{mol/min/mg})/\text{UCS}$$

$6.22mM^{-1}$ = extinction coefficient; A1 = rate in the presence of pyruvate; A2 = rate in the absence of pyruvate. Protein concentration was measured in mg/ml. Adapted from Chretien et al. (1995).

3.8 STATISTICAL ANALYSIS OF DATA

The data that were generated in this study apply specifically to our conditions, and to compare absolute data with published data may be inaccurate. However, a comparison of the activities obtained is given in Chapter 5. The control group (C), which consisted of 18 controls, was used to set up a reference range for enzyme activities and respiration rates. Descriptive statistics were done with Statistica (Statsoft Inc.,version 7.1) software to

determine the distribution of the data. The following statistics were calculated: mean (average), median (middle number of a group of numbers that have been arranged in order by size), minimum, maximum, 5^{th} percentile, 10^{th} percentile, Std.Dev (the average amount by which a number varies from the average number in a series of numbers).

The approach to calculate a reference value (or reference point) from the data of control material where activities (when equal or lower) can be regarded as "lower than normal" was to use current approaches from established centres that perform the same tests. The statistical approach was as follows:

1. Using the 5^{th} and 10^{th} percentile of a data range as reference point (Zheng et al., 1990);
2. Using the mean -2SD as reference point (Zheng et al., 1990);
3. Using the lowest value as reference point (Kirby et al., 1999; Bentlage et al., 1996; Rotig et al., 1997); and
4. Using the value that is 25% of the mean as reference point (Thorburn et al., 2004).

The 5^{th} and 10^{th} percentiles were calculated using the Transformation Kernel Density Estimation program (Sheather and Marron, 1990). The reason for using this calculation programs is due to the fact that we had very limited data at our disposal and the calculated 5^{th} and 10^{th} percentile values were the same in some cases when Statistica (Statsoft Inc.,version 7.1) was used. Different approaches exist for the calculation of quintile estimators in the literature, as can be see in Parrish, (1990). By using equation two in Garsd et al. (1983), a calculation necessitating the amount of control results was done as well, producing a more accurate diagnosis. The Mean ±2SD, 25% of Mean and minimum value were calculated using the descriptive statistics determined with Statistica.

To get an indication of the robustness of the methodology that was used, the reproducibility of the assays were tested using aliquots of the same sample over different periods of time or whenever enzyme assays were performed. Inter-assay variation was determined by repeating the assays a number of times on one particular day and calculating the relative percentage of variation by dividing the average through the standard deviation. Intra-assay (day-to-day) variation was determined by calculating the percentage change of the data that was obtained with assays performed on separate days.

Chapter 4
Results and Discussion

4.1 INTRODUCTION

The methodology of this study to investigate mitochondrial function was respiration analyses, which is a functional analysis of the RC, and single-enzyme analyses of complexes I-IV. The methods were selected on the basis of commonly used protocols, but have been modified and partially optimised. It should be emphasised that although the conditions that were adopted from original publications were mostly kept constant, the protocols (procedures, volumes, order of steps, etc.) were significantly changed and developed into a protocol that resembles a *standard operating procedure* (SOP, see Appendix C) that can be used in future. In this chapter these protocols are not evaluated and only the results of the protein optimisation are given, after which the results obtained from patient and control sample analyses are given and discussed.

4.2 RESPIRATION ANALYSES

The respiration analyses were done using a Clark oxygen electrode system at the Department of Paediatrics at the Pretoria Academic Hospital. Different substrate combinations were used to measure state 4/2 respiration and ADP added to measure state 3 respiration in freshly prepared mitochondria. As mentioned in Chapter 3, the protocol was optimised in a previous study (du Toit, 2006) and therefore was not described here. However, an example of the type of result obtained from human muscle mitochondrial sample is shown in Figure 4.1.

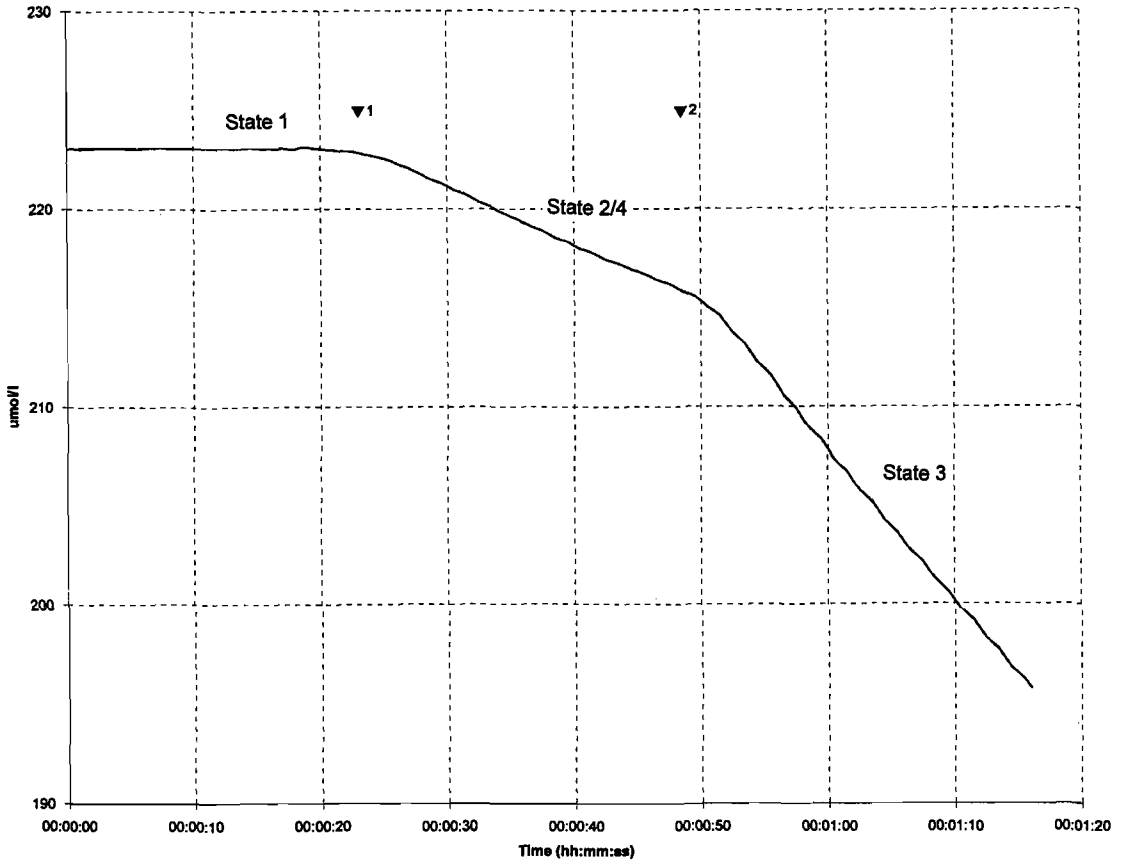


Figure 4.1: Example of respiration analyses by using succinate as substrate and rotenone to inhibit complex I in human muscle mitochondria.

The figure shows a scanned oxygen trace with oxygen levels (µmol/l, y-axis) against time (x-axis). At the first marker (▼1), before which state 1 respiration occurs, succinate (10 mM) has been added to a reaction already containing mitochondria, buffer and rotenone (see Section 3.6). State 2/4 respiration is then measured until addition of ADP (2 mM) at the second marker (▼2). State 3 respiration is then measured.

The respiration rates were measured in most samples where at least 42 mg of the isolated mitochondria were available after enough (~200 mg) had been stored for single-enzyme assays. This priority was given to enzyme assays because these analyses were regarded as more important/informative for identifying mitochondrial disorders. The type of result obtained from either G+M, P+M or S+R substrates and addition of ADP (2 mM) looks approximately like that showed in Figure 4.1.

Figure 4.1 (an oxygen trace) shows the levels of oxygen and their use (as a rate value in pmol/min) after the addition of mitochondria (without endogenous substrates, state 1),

with the addition of S+R as an example (state 2/4) and with the further addition of ADP to stimulate maximal respiration (state 3). The quality of the isolated mitochondria or their "coupling" (inner membrane integrity) could be measured from the ratio of states 3 and 2/4, which is also called the respiratory control ratio (RCR). Similar traces were obtained in most cases and duplicate values were mostly within 10% of each other. Respiration data are given in Sections 4.5, 4.6 and 4.8.

4.3 PARTIAL OPTIMISATION OF RC ENZYME ASSAYS

The enzyme assays were done using frozen mitochondria samples at the School for Physical and Chemical Sciences, North West University, Potchefstroom. A Bio-Tek UVIKON UV-Vis Double Beam Spectrophotometer was used to do all the enzyme assays. First the enzyme assays that were originally described by Rahman et al. (1996) and Rustin et al. (1994) were optimised for protein content. As will be observed from the data, the specific activities varied markedly at different protein concentrations, and for this reason it was important for the reactions of different samples to be done at the same protein concentrations. This was also the reason why the protein concentrations of samples were set at 1 mg/ml before enzyme assays were performed. Different amounts of mitochondrial protein were used to determine what amount of protein to use in activity measurements.

4.3.1 Citrate synthase

Spectrophotometric assays of citrate synthase were performed by the reaction of 5,5'-dithiobis 2-nitrobenzoic acid (DTNB), coupled to the reduction of CoA by the citrate synthase reaction in the presence of oxaloacetate (Robinson et al, 1987, Section 3.7.3). The activity is regarded as a reflection of the mitochondrial content of tissue and therefore is commonly used as a mitochondrial marker enzyme. The activity of citrate synthase was determined by Equation 3.7. Figure 4.2 shows an example of the progress curve for the enzyme reaction. The activity was measured before (A1) and after OAA was added (A2), and the activity expressed in nmole/min/mg.

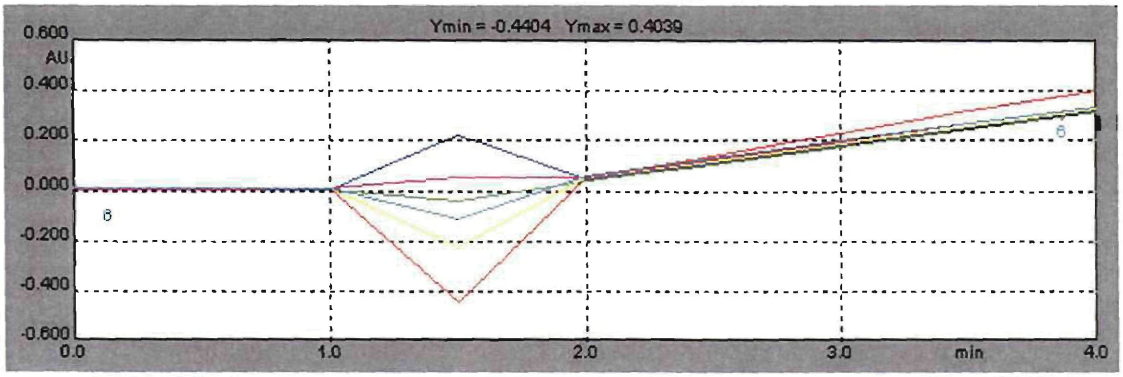


Figure 4.2: Illustration of measurement of citrate synthase activity using a spectrophotometric assay.

This figure illustrates AU (absorbance, y-axis) against time (min, x-axis). From 0–1 minutes no substrate (OAA) was added (A1). During the period 1–2 min, substrate was added and therefore readings were interrupted. From 2–4 minutes, substrate was present and the linear increase over time at 412 nm (TNB formation) was followed (A2). Six different reactions are shown in different colours.

To partially optimise the original assay described by Robinson et al. (1987), a series of reactions was analysed where the protein content had been changed. The tissue that was used to determine this protein range was skeletal muscle from Sprague Dawly rats. The protein concentration that was used in the protein range was 0–10 µg as shown in Figure 4.3. *It is important to note* that this and the similar following figures show *specific activities* and not absolute activities or rates (Figure 4.2) over a protein range. This means that the enzymes' reaction velocities (activities) over the protein range, which should and do increase when protein content increases, are normalised to protein content. Hypothetically, if the velocities increase in a perfectly linear way over this range, the specific activities as shown in Figure 4.3 should be the same at each protein concentration. This is the ideal, but it is important to investigate first.

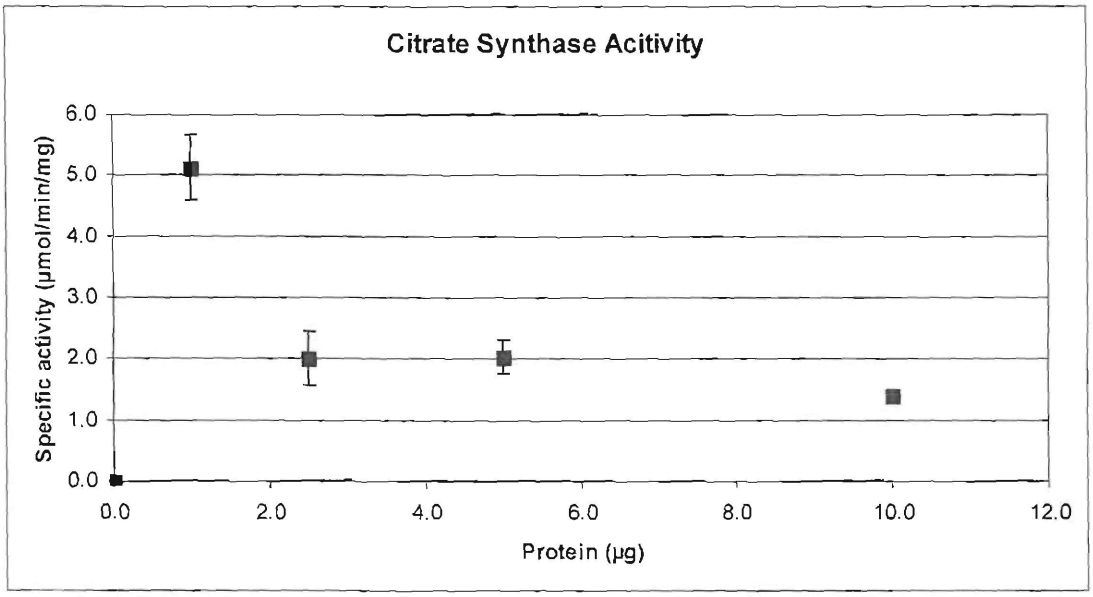


Figure 4.3: Graphic presentation of the effect of protein content on the specific activity of citrate synthase.

The data of the individual reactions are given in Appendix D. The specific activities (µmol/min/mg) were plotted against the amount of protein (µg) that was used. The mean activities (n = 3) and standard deviations are shown.

From the results shown in Figure 4.3 it can be observed that the background (0 µg) specific activity is negligible, which shows good stability of the assay. The specific activity then increases when, firstly, 1 µg protein is used, and then decreases and stabilises between 2.5 and 5 µg. The specific activity then further decreases at 10 µg.

This pattern demonstrated three important properties of enzyme reactions and why protein content needs to be optimised. Firstly, at very low protein content (e.g. 1 µg) the velocities that were measured almost always showed the highest specific activity because there were almost no substrate limiting factors, which occurred at high protein content. The problem was that around these low protein contents the detection neared its limits and it was therefore not ideal to use protein contents near these low values. Secondly, a protein content range was reached where the specific activities remain constant (e.g. 2.5–5 µg). In this range, the conditions were comparable and did not change much in the time that reaction took place. This was an ideal range to use because a slight change in protein content or enzyme activity will not change the specific activities, although velocities change. Thirdly, at higher protein content (e.g. 10 µg) the

specific activity decreased, probably because one of the substrates became limited or product inhibition occurred.

From these results it was decided that 5 µg protein would be used in this study to measure the activity of citrate synthase.

4.3.2 Complex I

Spectrophotometric assays of complex I were performed by measuring the oxidation of NADH (Rahman et al., 1996, Section 3.7.4). The activity of complex I was determined as described by Equation 3.9. Figure 4.4 shows the reaction of complex I when the activity was measured. The enzyme uses NADH at a linear initial rate at the start of the reaction and remains linear for at least two minutes. Later in this progress curve, the rate slows down as substrate becomes limiting or products accumulate. The reaction is performed in the absence (A1) and in the presence (A2) of rotenone, which inhibits complex I. The activity is expressed in nmole/min/mg and converted to nmole/min/UCS.

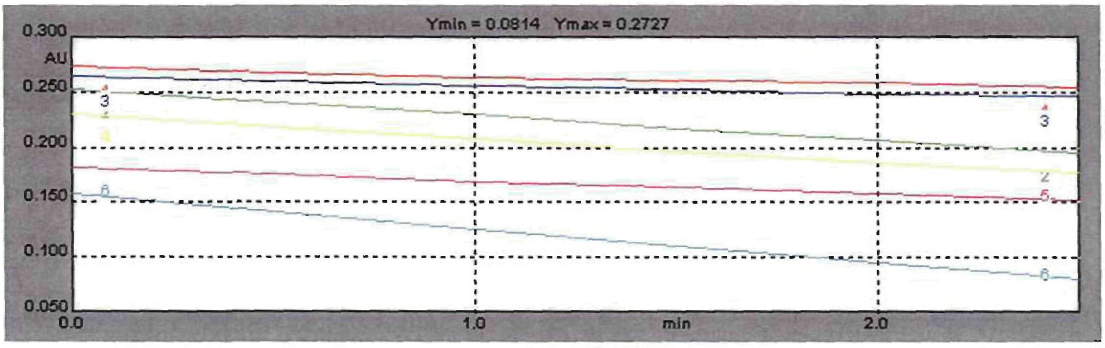


Figure 4.4: Illustration of measurement of complex I activity using a spectrophotometric assay.

This figure illustrates AU (absorbance, y-axis) against time (min, x-axis). The region 0–2.5 minutes shows the linear decrease over time at 340 nm (NADH oxidation) that was followed without (A1) and with (A2) addition of rotenone. Six different reactions are shown in a different colour each. Three of the reactions were followed without rotenone (higher slopes) and three with rotenone (lower slopes) in different colours.

To partly optimise the enzyme assay method for measuring the activity of complex I, a series of reactions was analysed where the protein content was changed. The protein concentrations that were used were 0–35 ug, using mitochondria isolated from Sprague Dawly rat muscle tissue. Figure 4.5 shows the results.

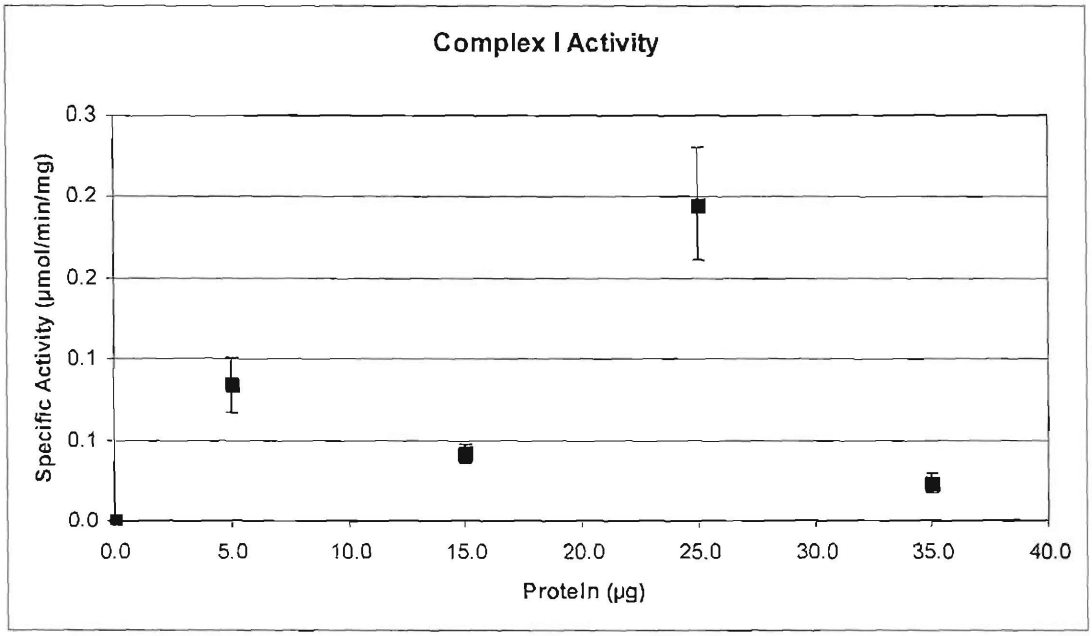


Figure 4.5: Graphic presentation of the effect of protein content on the specific activity of complex I.

The data of the individual reactions are given in Appendix D. The specific activities (µmol/min/mg) were plotted against the amount of protein (µg) that was used. The mean activities (n = 3) and standard deviations are shown.

In Figure 4.5 it can be observed that, with the obvious exception of 25 µg protein, that a similar pattern to the data from CS (Figure 4.3) occurred. After an initial undetectable activity at 0 µg, the specific activity increases at 5 µg and is similar albeit slightly lower at 15 µg, and then decreases at 35 µg. From the raw data (Appendix D) it can be observed that the experiment was duplicated and the values, while using 15 and 35 µg, were very low and while using 25 µg the values were constantly high. The same conclusions about the effect of protein content for CS were drawn here, with the exception of 25 µg, for which no convincing explanation except an experimental error could be given. For complex I reactions, 25 µg was chosen because lower protein content had significantly low rates that were considered inaccurate, while higher content had much lower specific activities.

4.3.3 Complex I + III

Spectrophotometric assay of complex I+III measures the NADH–dependent reduction of cytochrome c (Rustin et al., 1994). If limited samples are available and respiration studies cannot be done then the combination reactions of complex I+III and complex II+III are useful and can provide information on the organelle fragility or altered inner membrane and coenzyme Q function (Trounce et al., 1996). The activity of complex I+III was determined using Equation 3.10. Figure 4.6 shows the reaction of the combined complex I+III activity. The reaction occurs in the absence (A1) and in the presence (A2) of rotenone. The activity is expressed in nmole/min/mg and converted to nmole/min/UCS.

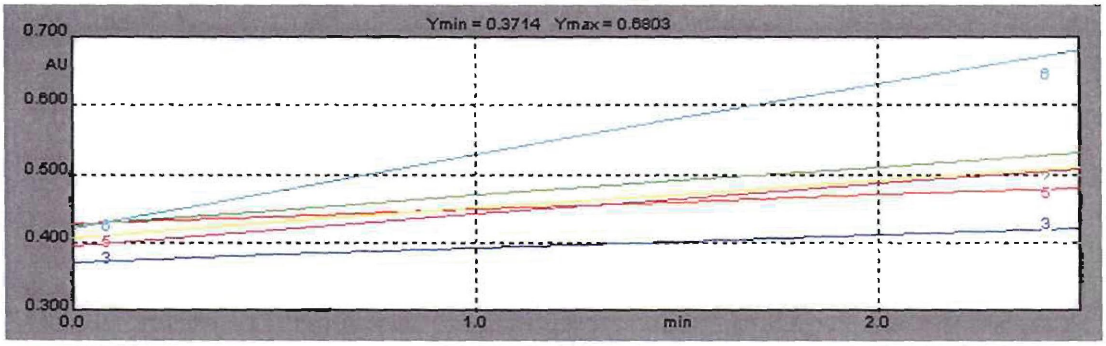


Figure 4.6: Illustration of measurement of complex I+III activity using a spectrophotometric assay.

This figure illustrates AU (absorbance) against time (min). The period 0–2.5 minutes shows the linear increase over time at 550 nm (cytochrome c reduction) that was followed without (A1) and with (A2) addition of rotenone. Three of the reactions were done without rotenone (higher slopes) and three with rotenone (lower slopes) in different colours.

To partly optimise the enzyme assay method for measuring the activity of complex I+III, a series of reactions was analysed where the protein content was changed. The protein concentrations that were used were 0–10 ug, using mitochondria isolated from Sprague Dawly rat muscle tissue. Figure 4.7 shows the results.

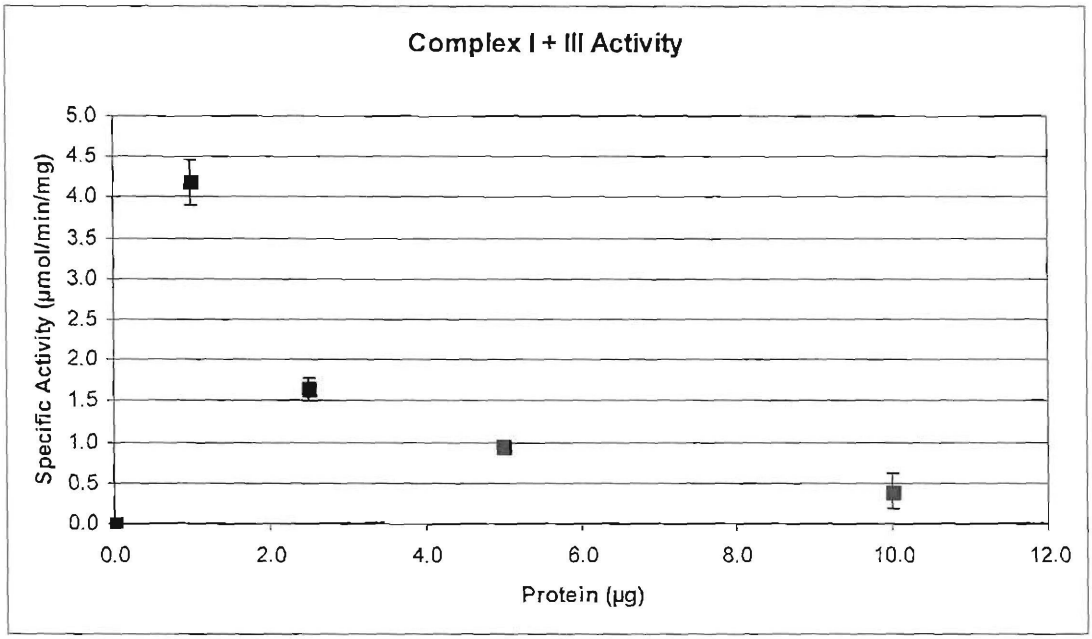


Figure 4.7: Graphic presentation of the effect of protein content on the specific activity of complex I+III.

The data of the individual reactions are given in Appendix D. The specific activities (µmol/min/mg) were plotted against the amount of protein (µg) that was used. The mean activities (n = 3) and standard deviations are shown.

In Figure 4.7 it can be observed that a pattern similar to that of CS (Figure 4.3) occurred. After an initial undetectable activity at 0 µg, the specific activity increases at 1 µg, decreases markedly at 2.5 µg, and then steadily at 5 and 10 µg, respectively. The same conclusions about the effect of protein content on CS were made here. Therefore, for complex I+III reactions, 5 µg was chosen, although a constant specific activity could not be obtained. This content was chosen to enable better detection.

4.3.4 Complex II + III

The spectrophotometric assay of complex II+III measured the succinate (complex II) driven reduction of cytochrome c by complex IIII (Trounce et al., 1996). The activity of complex II+III was determined by Equation 3.12. Figure 4.8 shows the reaction of the combined complex II+III when the reaction occurs in the presence of cytochrome c (A1) and in the absence of cytochrome c (A2). The activity is expressed in nmole/min/mg and converted to nmole/min/UCS.

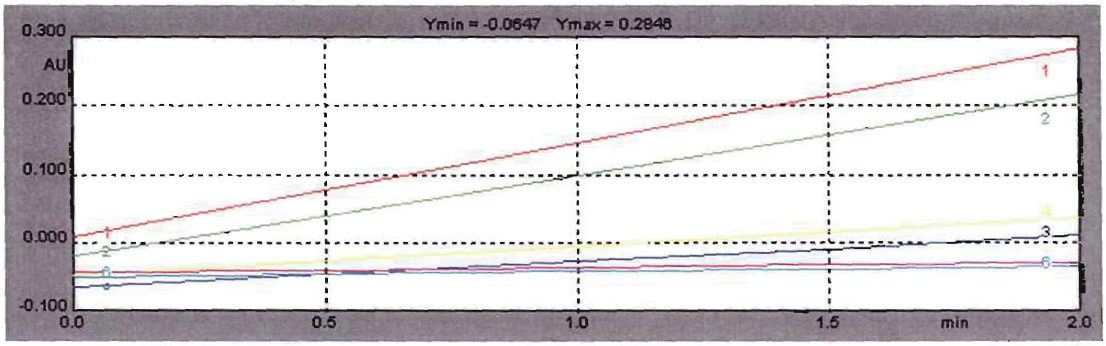


Figure 4.8: Illustration of measurement of complex II+III activity using a spectrophotometric assay.

This figure illustrates the AU (absorbance) against time (min). The period 0–2 minutes shows the linear increase over time at 550 nm (cytochrome c reduction) that followed with the addition of cytochrome c (A1). Six of the reactions are shown in different colours.

To partly optimise the enzyme assay method for measuring the activity of complex II+III, a series of reactions was analysed where the protein content was changed. The protein concentrations that were used were 0–10 ug, using mitochondria isolated from Sprague Dawly rat muscle tissue. Figure 4.9 shows the results.

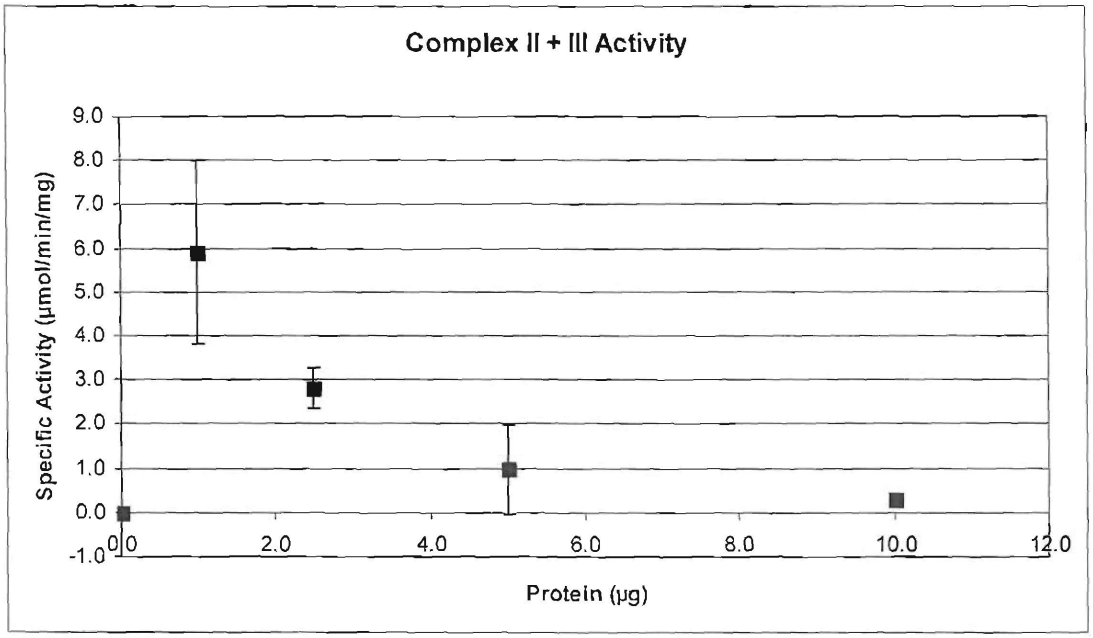


Figure 4.9: Graphic presentation of the effect of protein content on the specific activity of complex II+III.

The data of the individual reactions are given in Appendix D. The specific activities (µmol/min/mg) were plotted against the amount of protein (µg) that was used. The mean activities (n = 3) and standard deviations are shown.

From Figure 4.9 it can be observed that, as with previous enzymes, a pattern similar to that of CS (Figure 4.3) occurred. After an initial undetectable activity at 0 µg, the specific activity increases at 1 µg decreases at 2.5 µg and then again steadily decreases at 5 and 10 µg, respectively. Here also, the same conclusions were drawn about the effect of protein content on CS activity. Therefore, for complex II+III reactions, 5 µg was chosen.

4.3.5 Complex IV

Spectrophotometric assay for complex IV measures the oxidation of reduced cytochrome c (Trounce et al, 1996). The activity of complex IV was determined by Equation 3.14. Figure 4.10 shows the reaction of complex IV, and the activity is expressed in nmole/min/mg and converted to nmole/min/UCS.

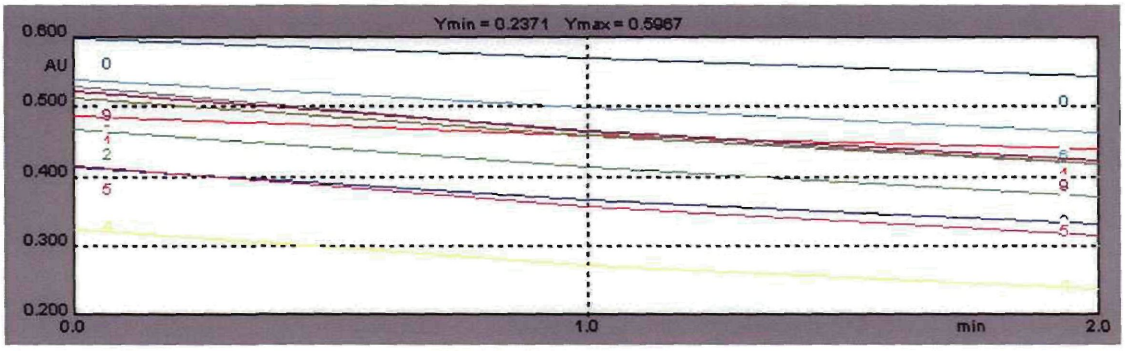


Figure 4.10: Illustration of measurement of complex IV using a spectrophotometric assay.

This figure illustrates AU (absorbance) against time (min). The period 0–2 minutes shows the linear decrease over time at 550 nm (cytochrome c oxidation) that followed. Twelve reactions are shown in different colours.

The enzyme assay was partly optimised using a series of reactions at different protein contents. The protein concentrations that were used were 0–5 ug, using mitochondria isolated from Sprague Dawly rat muscle tissue. Figure 4.11 shows the results.

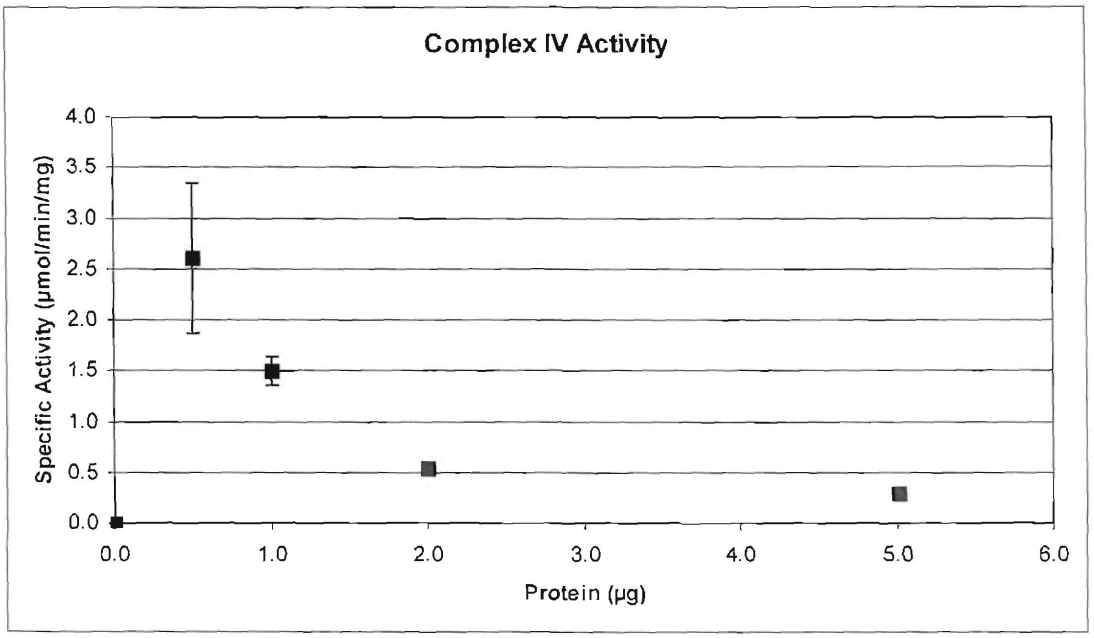


Figure 4.11: Graphic presentation of the effect of protein content on the specific activity of complex IV.

The data of the individual reactions are given in Appendix D. The specific activities (µmol/min/mg) were plotted against the amount of protein (µg) that was used. The mean activities (n = 3) and standard deviations are shown.

From Figure 4.11 it can be observed that again a pattern similar to that of CS (Figure 4.3) occurred. The specific activity increased at 0.5 µg after no activity had been detected at 0 µg, decreased at 1 µg and then again decreased at 2 and 5 µg respectively. For this enzyme, 2 µg was chosen for all further assays.

4.3.6 Pyruvate dehydrogenase complex

The activity of pyruvate dehydrogenase complex was measured directly (pyruvate-driven NAD reduction) and determined using Equation 3.16. Figure 4.12 shows an example of the progression of the reaction. As with the RC enzymes, the activity was expressed in nmole/min/mg and nmole/min/UCS.

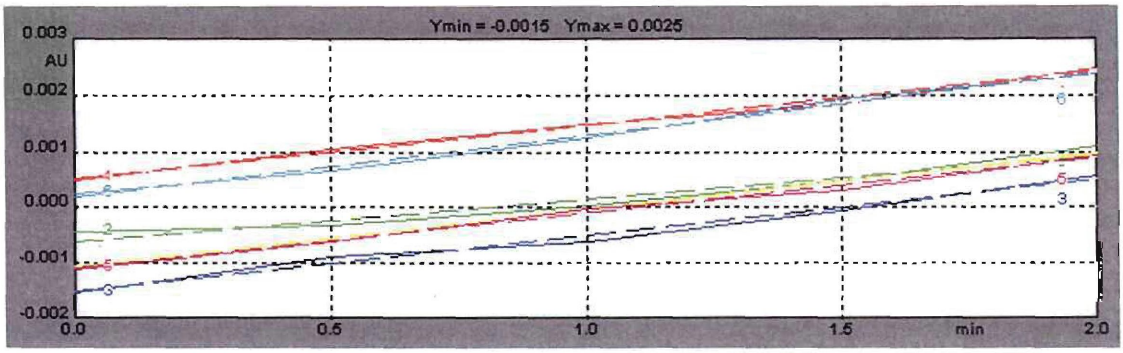


Figure 4.12: Illustration of measurement of PDHc using a spectrophotometric assay.

This figure illustrates AU (absorbance) against time (min). The period 0–2 minutes shows the linear increase at 340 nm (NAD reduction) that followed.

To partly optimise the enzyme assay as originally published by Chretien, et al. (1995), a series of reactions at different protein contents was performed. The protein concentrations that were used were 0–80 ug, using mitochondria isolated from Sprague Dawly rat muscle tissue. Figure 4.13 shows the results.

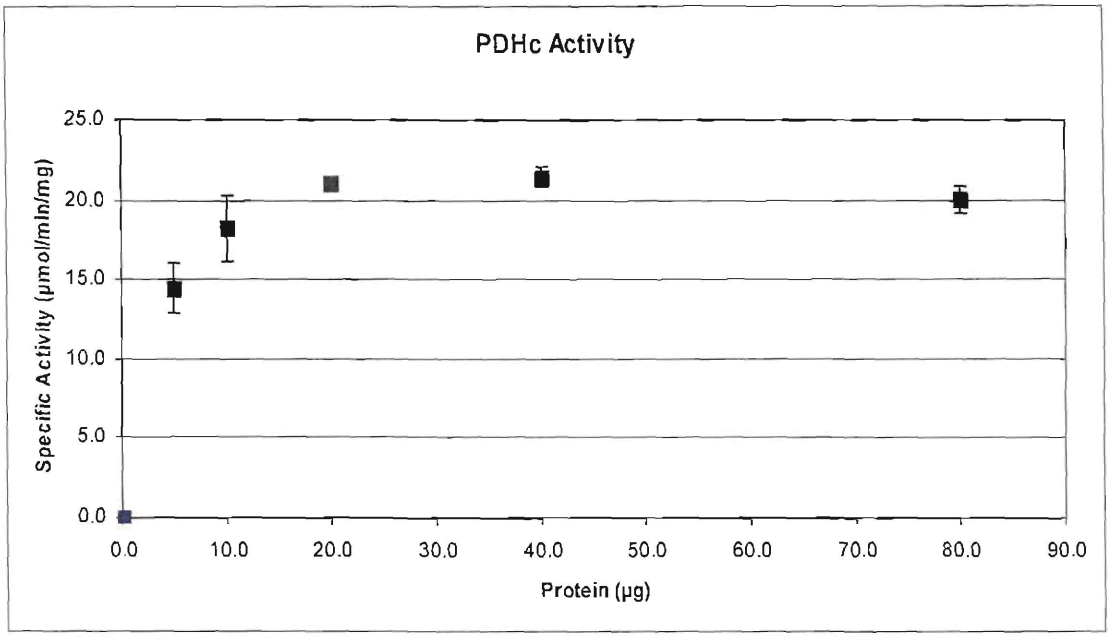


Figure 4.13: Graphic presentation of the effect of protein content on the specific activity of PDHc.

The data of the individual reactions are given in Appendix D. The specific activities (µmol/min/mg) were plotted against the amount of protein (µg) that was used. The mean activities (n = 3) and standard deviations are shown.

From the results in Figure 4.13 it can be observed that a different pattern occurred than with CS (Figure 4.3). The specific activity increased at 5 µg after no activity had been detected at 0 µg, increases again at 10 µg and 20 µg, and again steadily at 40, then decreases at 80 µg. For this enzyme, 10 µg was chosen for all further assays.

These partially optimised enzyme assays, in terms of protein content, were further used in all assays. The exact protocols including these protein content values are set out in Appendix D.

4.4 REPRODUCABILITY OF ENZYME ASSAYS

The reason for calculating reproducibility was to determine if the methods followed could be trusted to give the same values every time they are used. The ideal is to do al the control and patient sample enzyme assays at the same time, which will be more accurate. However, this is impossible as the patient results must be obtained as soon as

possible for diagnostic purposes. For testing reproducibility, aliquots of one rat heart muscle mitochondrial sample were included every time when a series of enzyme assays was done. The data from these control samples were used to determine the variation.

4.4.1 Inter-assay variation

The inter-assay variation analysis was used to evaluate the day-to-day variation between the same assays (Kramer, 2005). This could only be done for enzyme assays and not for respiration because fresh samples for respiration could not be stored. The results of this investigation at five different time points in the study using aliquots of one rat heart muscle mitochondrial sample are given in Table 4.1.

Table 4.1: Inter-assay variation for enzyme assays.

The average (n = 5) and standard deviation (Std. dev.) are given. The coefficient of variation (CV,%) is also shown, which indicates the relative (%) variation between time points.

	Inter-assay variation results					
	CS	CI	CI+III	CII+III	CIV	PDHc
	0.1316	0.0833	0.0520	0.0997	0.0259	0.0024
	0.1520	0.0680	0.0762	0.0849	0.0224	0.0023
	0.1500	0.0852	0.0403	0.0657	0.0214	0.0015
	0.1399	0.0905	0.0658	0.0760	0.0239	0.0015
	0.1110	0.0796	0.0788	0.0453	0.0253	0.0018
Average	0.1369	0.0813	0.0626	0.0743	0.0238	0.0019
Std. dev.	0.0166	0.0084	0.0163	0.0205	0.0019	0.0004
CV, %	12.2	10.4	26.1	27.5	8.0	22.6

The inter-assay variation of the RC enzymes, CS and PDHc, analysed at five different dates, revealed important information on the accuracy of data on any specific day. The lowest variation was observed for complex IV (8%), and the highest for CI+III (26.1%) and CII+III (27.5%). Although only five days' results were used and those specific days' results varied much more, these values mean that on any day a variation as indicated in the table could be expected. The variations of CI+III, CII+III and PDHc were alarming, as ideally values should be in the region of ± 10% of each other (Janssen et al, 2007).

4.4.2 Intra-assay variation

The intra-assay variation analysis was used to evaluate the variation of one sample analysed several times on one day (Kramer, 2005). The results of this investigation on

the same day using ten reactions of the same sample (rat heart muscle mitochondrial) are given in Table 4.2.

Table 4.2: Intra-assay variation for enzyme assays.

The average (n = 10) and standard deviation (Std. dev.) is given. The coefficient of variation (CV,%) is also shown which indicates the relative (%) variation between time points.

	Intra-assay variation results					
	CS	CI	CI+III	CII+III	CIV	PDHc
	0.1362	0.0939	0.0783	0.0615	0.0293	0.0011
	0.1379	0.0908	0.0847	0.0659	0.0169	0.0012
	0.1362	0.0920	0.0676	0.0635	0.0239	0.0012
	0.1397	0.1008	0.0896	0.0612	0.0258	0.0011
	0.1369	0.0979	0.0906	0.0760	0.0162	0.0013
	0.1382	0.0909	0.0895	0.0629	0.0260	0.0012
	0.1338	0.1012	0.0936	0.0633	0.0225	0.0013
	0.1342	0.1046	0.0900	0.0681	0.0250	0.0015
	0.1339	0.1070	0.0904	0.0687	0.0227	0.0013
	0.1358	0.1080	0.0891	0.0659	0.0171	0.0012
Average	0.1363	0.0987	0.0863	0.0657	0.0225	0.0012
Std. dev.	0.0020	0.0066	0.0078	0.0044	0.0044	0.0001
CV, %	1.4	6.7	9.0	6.8	19.7	9.5

The intra-assay variation of the RC enzymes, CS and PDHc, analysed in ten reactions, revealed important information on the reproducibility of data on one given day. The lowest variation was observed for complex I (1.4%), which is encouraging as in literature these are the most common enzymes to show deficiencies. Complex I is also the slowest (rate limiting) reaction of the RC. The highest variation by far was for CIV (19.7%), which is also the fastest reaction of the RC. These relative reaction rates can explain the variations as, generally, it is easier to follow a slower reaction over time than a faster reaction. Overall, the intra-assay variations of the enzymes were regarded as good because they were mostly below 10%. Ideally, values should be in the region of 2–8%, as reported by Janssen et al. (2007).

4.5 MITOCHONDRIAL RESPIRATION AND ENZYME ACTIVITIES IN HUMAN MUSCLE BIOPSIES

4.5.1 Muscle biopsies from healthy control and patients

Muscle biopsies from mostly *vastis lateralis* tissue were obtained after informed consent and assent had been obtained from patients and parents. During the course of the year of the study, surgery was performed on either assumingly healthy controls or (supposed) patients at the Pretoria Academic Hospital on 27 occasions where the possibility existed of obtaining muscle tissue. During these occasions, 18 control and 26 patient biopsies were obtained and processed to measure respiration and enzyme activities (see Sections 3.5 - 3.7).

In the subsections that follow, the descriptive data of the results are given and discussed. The data are shown in the form of box-plots that summarise the medians, ranges (minimums and maximums) and the 25th to 75th percentiles. The data of the controls are shown separately from those of the patients because the control data were used to obtain reference ranges. The data of the patients are also grouped in this section to show the comparison of the patient group with the control group. The patient group, however, includes "definite", "probable", "possible" as well as "unlikely" mitochondrial disorders, according to the paediatric neurologist and co-supervisor in this study, Dr. I. Smuts. Therefore, it is important to point out that the patients will be individually compared to the reference ranges and should not for the purpose of this study be compared as a group.

4.5.2 Descriptive data for respiration analyses

Respiration analyses were performed as described in Section 3.6 and are summarised in Figures 4.14–4.16 for the three substrates respectively. The figures show state 3 respiration activities for controls and patient as groups.

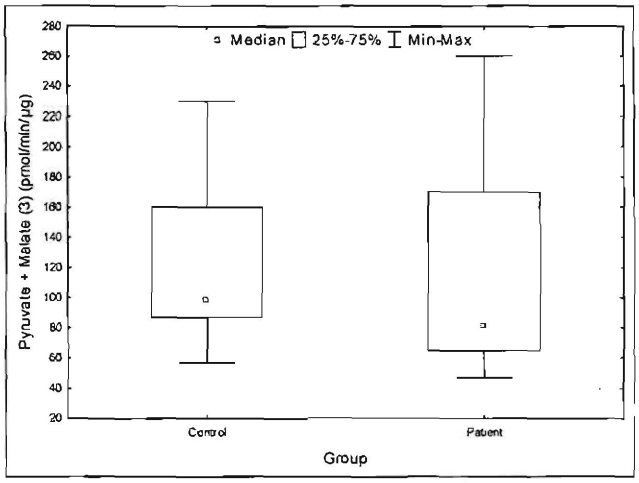


Figure 4.14: P+M state 3 respiration for control and patient groups.

The median, 25th and 75th percentiles and the minimum and maximum values are shown in the box plots.

As can be observed in Figure 4.14, the P+M respiration for the control group had a median value of ~98 pmol/min/mg, which was slightly (17.3%) higher than in the patient group. The range as well as the 25th and 75th percentiles for the control group were spread over a smaller region than that of the patient group. This was a common trend in the respiration data, which can be explained by the more homogenous nature (in terms of health) of the control group compared to the patient group. The quality of muscle samples from patients was also generally of poorer quality – they were smaller and contained relatively more connective tissue and fat. However, the RCR, which reflects the integrity of the mitochondrial samples, had a median of 1.9 in both groups.

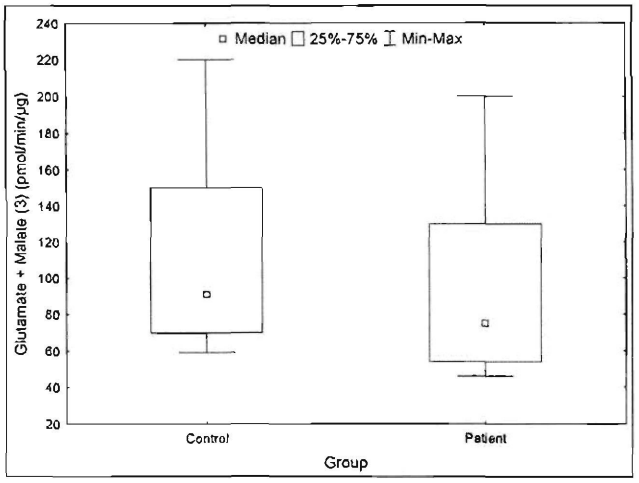


Figure 4.15: G+M state 3 respiration for control and patient groups.

The median, 25th and 75th percentiles and the minimum and maximum values are shown in the box plots.

Figure 4.15 illustrates that the G+M respiration for the control group also had a median value of ~91 pmol/min/mg, which was 17.6% higher than in the patient group. The range of the control group was slightly wider than that of the patient group. The 25th and 75th percentiles for the control group and patient group were spread over the same region size but those of the control group were at a higher values. The RCR had a median of 1.8 in the control group and 1.7 in the patient group.

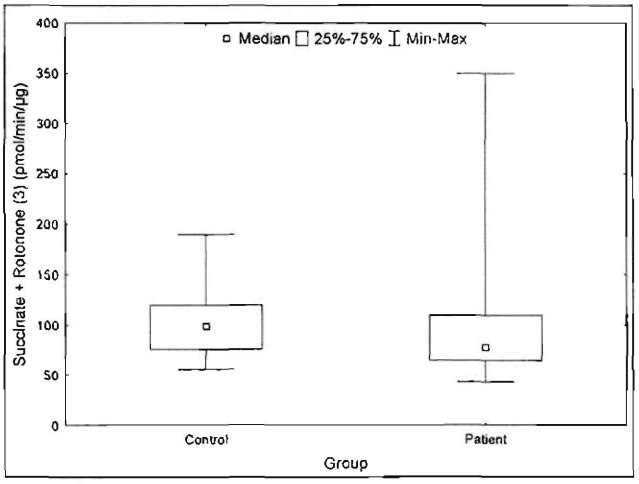


Figure 4.16: S+R state 3 respiration for control and patient groups.

The median, 25th and 75th percentiles and the minimum and maximum values are shown in the box plots.

As can be observed in Figure 4.16, the S+R respiration for the control group had a median value of ~99 pmol/min/mg, which was, as with the previous substrates, also

~21.2% higher than in the patient group. The range of the control group was spread over a much smaller region than that of the patient group. This was due to two values in the patient group that were 340 and 350 pmol/min/µg respectively, whereas all other values were spread around the median of 78 pmol/min/µg. The 25th and 75th percentiles for the control group and patient were spread over approximately the same region with the control group's region slightly higher, as with the other substrates. The RCR had a median of 2.6 in the control group and 2.9 in the patient group. This is notably higher than the RCR of P+M and G+M substrates. The reason for this is not clear but it was obvious that state 4/2 was relatively lower when using S+R.

4.5.3 Descriptive data for enzyme assays

The enzyme assays were performed as described in Section 3.7 and are summarised in Figures 4.17–4.23 for CS, RCC enzymes and PDHc. As was done in the case of respiration analyses, these figures show the specific activities for controls and patients as groups.

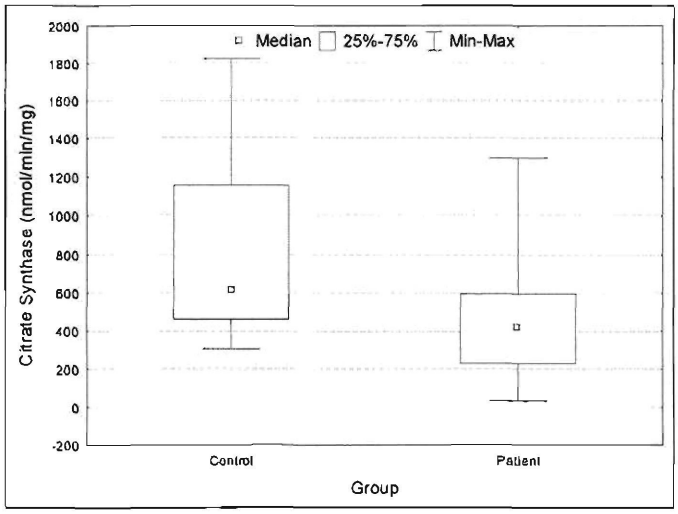


Figure 4.17: Citrate Synthase for control and patient groups.

The median, 25th and 75th percentiles and the minimum and maximum values are shown in the box plots.

The CS activity, as illustrated in Figure 4.17, had a median value of 608 nmol/min/mg for the control group, which was slightly (32.1%) higher than for the patient group. The range as well as the 25th and 75th percentiles for the control group were spread over a wider region than those of the patient group. This can be explained by the difference in

quality of isolated mitochondria from certain patients, as mentioned in Section 4.5.2. If the content of mitochondria was less during isolation, which happens when an affected muscle is used; it will reflect in the CS -specific activity. It is important to note that other enzyme activities are normalised to CS activity (UCS) and thus take the quality/mitochondrial load into account.

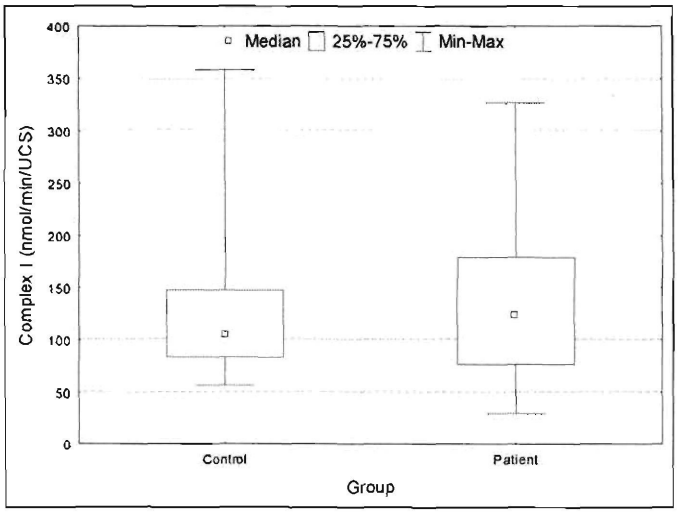


Figure 4.18: Complex I activity for control and patient groups.

The median, 25th and 75th percentiles and the minimum and maximum values are shown in the box plots.

As can be observed in Figure 4.18, the CI activity for the control group had a median value of ~105 nmol/min/UCS, which was slightly (17.2%) lower than for the patient group. The ranges of both the groups were of similar length but the minimum values of the control group were slightly higher than those of the patient group, which, as is shown at the end of the study, included CI-deficient patients. The 25th and 75th percentiles for the control group were spread over a narrower region than those of the patient group.

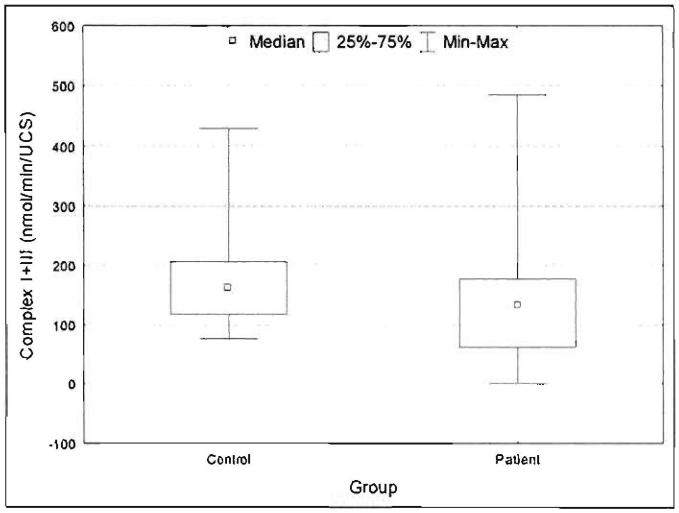


Figure 4.19: Complex I+III activity for control and patient groups.

The median, 25th and 75th percentiles and the minimum and maximum values are shown in the box plots.

Figure 4.19 indicates that the CI+III activities for the control group had a median value of ~161 nmol/min/UCS which was again slightly (18%) higher than the patient group. The range as well as the 25th and 75th percentiles for the control group were spread over a smaller region than that of the patient group, which, as mentioned before, included patients with CI+III deficiencies.

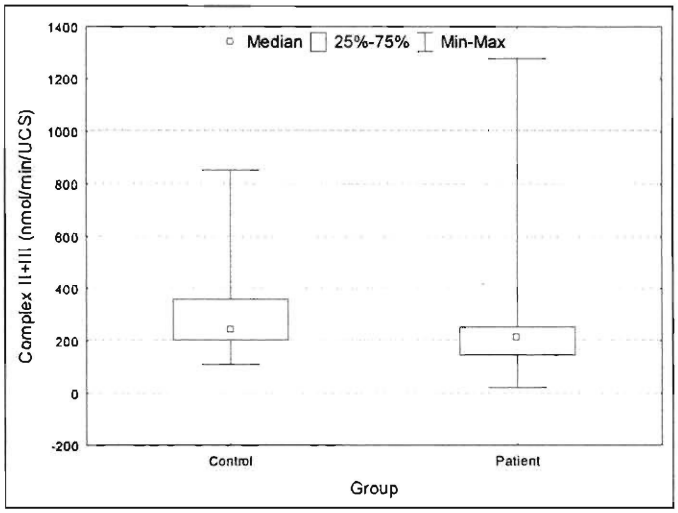


Figure 4.20: Complex II+III activity for control and patient groups.

The median, 25th and 75th percentiles and the minimum and maximum values are shown in the box plots.

As can be observed in Figure 4.20, the CII+III activity for the control group had a median value of ~244 nmol/min/UCS, which was slightly (14.3%) higher than in the case of the patient group. The range of the control group was spread over a smaller region than that of the patient group, which included three values that were very high (702, 853 and 1279 nmol/min/UCS). The 25th and 75th percentiles for the control group were therefore spread over a slightly larger region than those of the patient group.

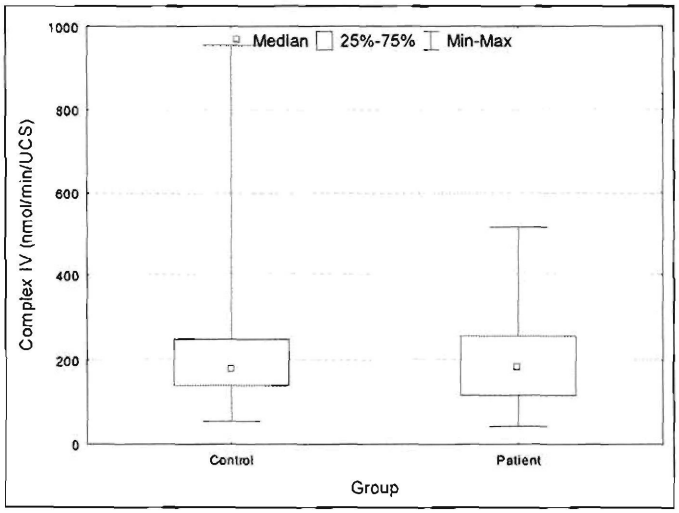


Figure 4.21: Complex IV activity for control and patient groups.

The median, 25th and 75th percentiles and the minimum and maximum values are shown in the box plots.

Figure 4.21 illustrates that CIV activity for the control group had a median value of ~175 nmol/min/UCS, which was similar to that of the patient group. Due to one very high value of 955 nmol/min/UCS, the range of the control group was spread over a greater region than that of the patient group. If this value were to be removed, the figures would have looked very similar. The 25th and 75th percentiles for the control group were spread over a slightly smaller but similar region than that of the patient group.

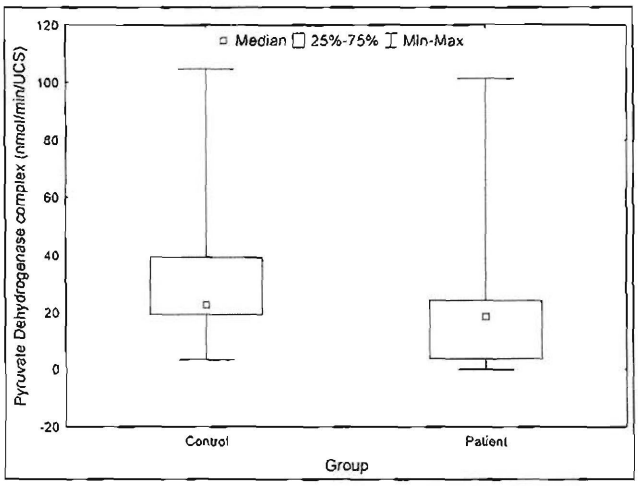


Figure 4.22: PDHc (CS) activity for control and patient groups.

The median, 25th and 75th percentiles and the minimum and maximum values are shown in the box plots.

As can be observed in Figure 4.22, the PDHc activity for the control group had a median value of ~23 nmol/min/UCS, which was slightly (17.4%) higher than that of the patient group. The maximum values of the two groups were similar, but the patient group contained several values of 0 nmol/min/UCS. Therefore, the 25th and 75th percentiles for the control group were much higher (compared to other enzyme data) than those of the patient group.

4.6 DETERMINATION OF 5TH AND 10TH PERCENTILES OF DATA USING THE TRANSFORMATION KERNEL DENSITY ESTIMATION PROGRAM

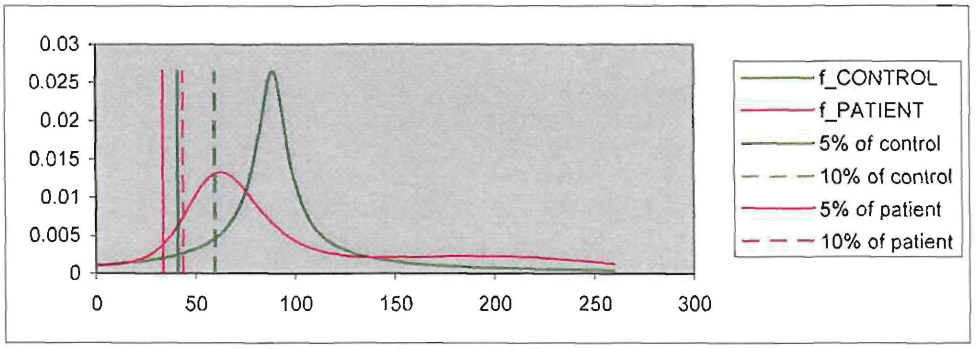
The biochemical data obtained, which were summarised in the previous section, were further processed. As discussed in Chapter 2 (Section 2.9), one objective was to use data from healthy controls to obtain reference values that can be used to diagnose patients with mitochondrial disorders. As described in Section 3.8, four different approaches to calculate the reference range had been used. The software programme, Statistica (version 7.1), was used to calculate the descriptive statistics, which were used to calculate the reference ranges. However, the 5th and 10th percentiles were calculated using the Transformation Kernel Density Estimation Program (TKDEP), because the amount of control data at the end of the study were not enough to obtain normal distribution; distribution, as can be seen in Section 4.5, was also skewed. The normality

analyses data are not shown, but none of the assays showed normal distribution. The low number of data points also meant that in some cases both the 5th and 10th percentile values were the same for the control group. To obtain a "smoother" distribution with the current number of controls, the TKDEP was used, which first transformed the data to a normal distribution and then calculated the 5th and 10th percentiles. In the following subsections, the TKDEP transformations and refined 5th and 10th percentile values are shown.

4.6.1 TKDEP for respiration analysis data

The TKDEP was done on the respiration data as described in Section 3.8. Figure 4.24 summarises the calculation of the 5th and 10th percentiles. In the figure it can be observed how the data was transformed to a normal distribution. The 5th and 10th percentiles are indicated using vertical lines. Although only the control group was used to calculate reference values, the patient group was also included for interest.

A



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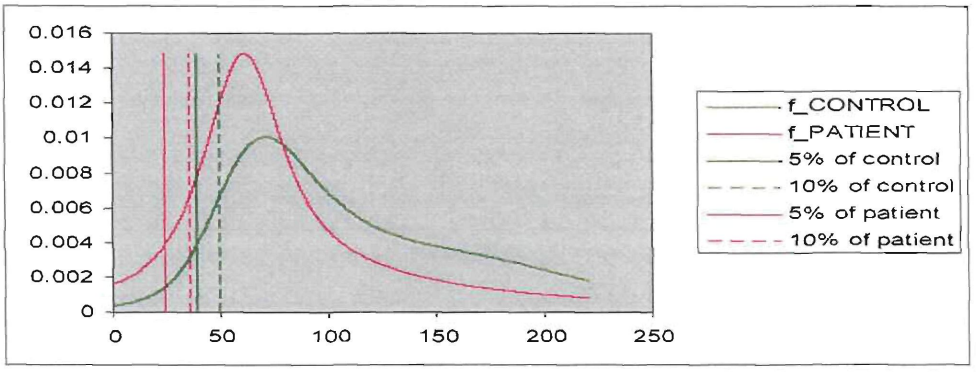


C

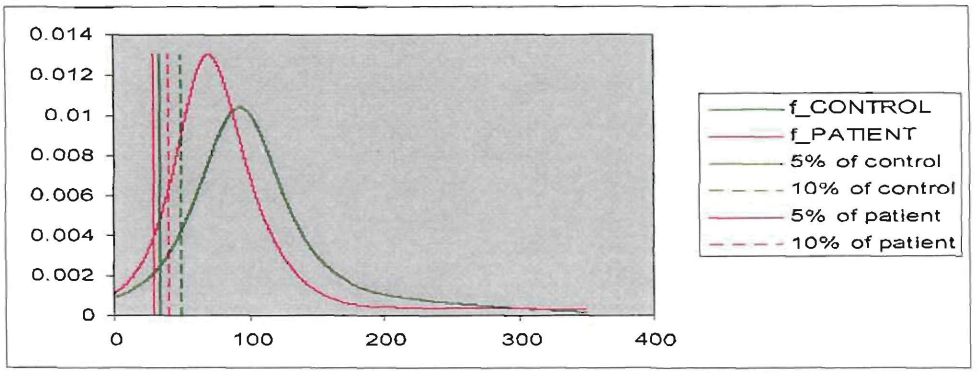


Figure 4.23: TKDEP transformation of state 3 respiration data.

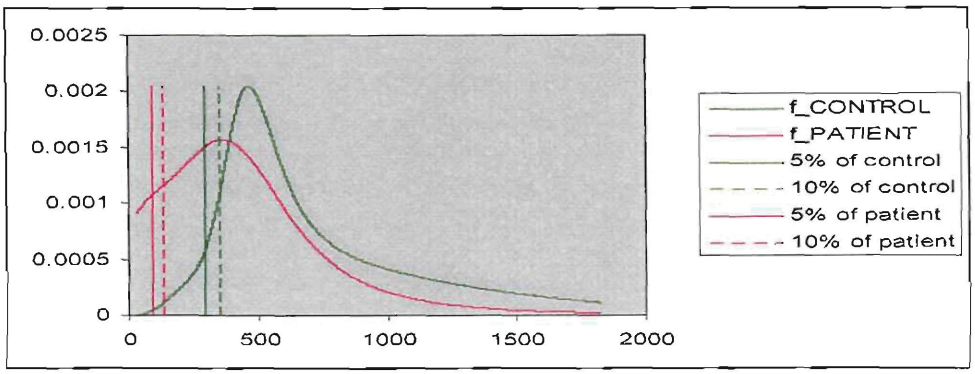
The transformation data for the three substrates for respiration are shown in Graph A (P+M), B (G+M) and C (S+R). The right-hand legends show the components of the graphs. Note the 5th (solid) and 10th (dashed) vertical percentile lines for the control and patient groups.

As illustrated in Figure 4.23 the normal distribution of the patient group for all three substrates was higher than the control group. This was also the case before transformation (see Section 4.5.2). Also, the 5th and the 10th percentiles of the patient group are lower than those of the control group. The 5th and 10th percentiles of the control group from the transformed data are given in Table 4.5, along with the other reference values of the control group.

4.6.2 TKDEP for enzyme activity data

The TKDEP was done on the enzyme assay data as described in Section 3.8. Figure 4.24 summarises the calculation of the 5th and 10th percentiles of transformed data. As with the respiration data, this figure shows the data transformed to a normal distribution and the 5th and 10th percentiles as indicated by the vertical lines. Again, the patient group was also included for interest.

A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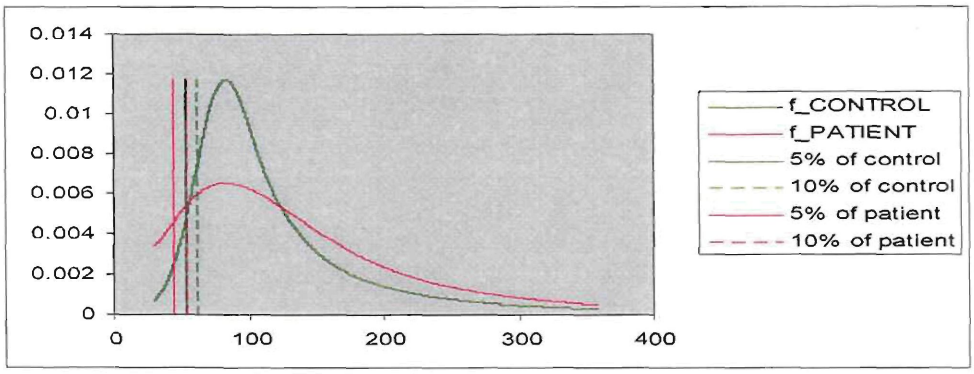
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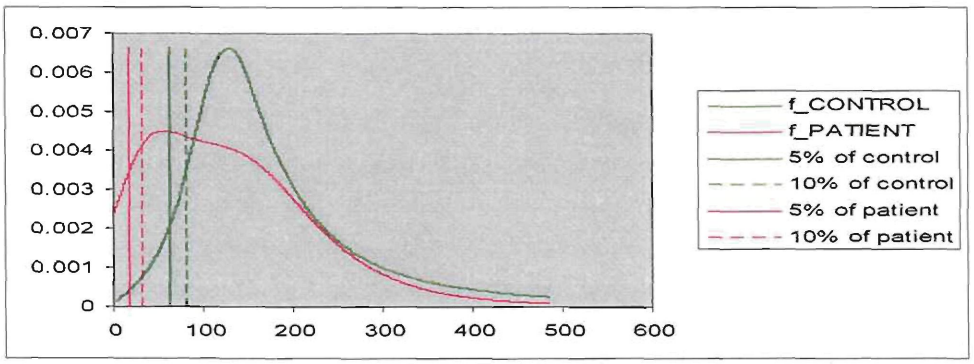
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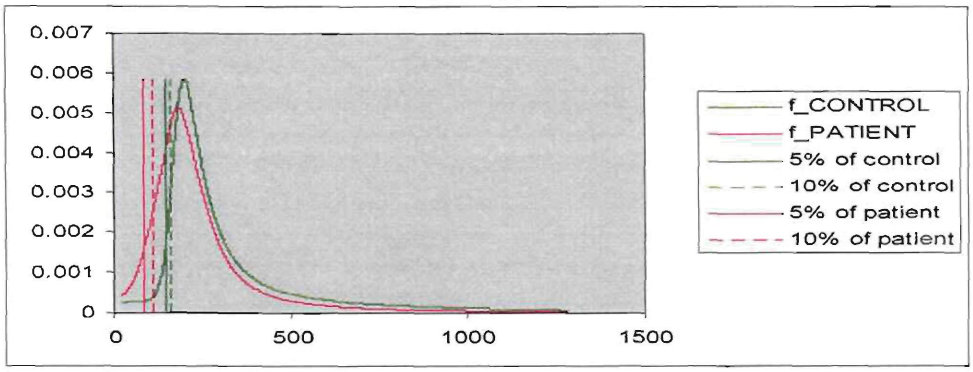
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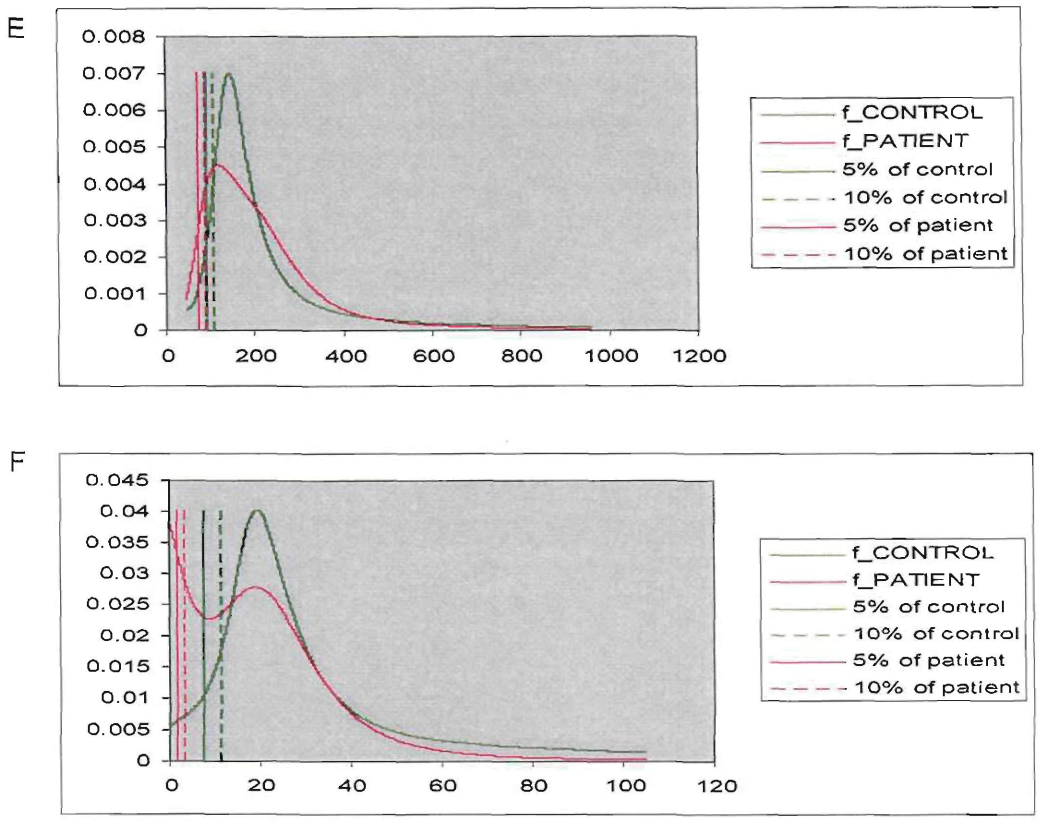

Figure 4.24: TKDEP transformation of CS, RCC and PDHc.

The transformation data for the enzyme analysis data are shown in Graph A (CS), B (complex I), C (complex I+III), D (complex II+III), E (complex IV) and F (PDHc). The right-hand legends show the components of the graphs. Note the 5th (solid) and 10th (dashed) vertical percentile lines for the control and patient groups.

As illustrated in Figure 4.24 the normal distribution of the control group for the entire enzyme analysis was higher than the patient groups. This was also the case before transformation (see Section 4.5.2). Also, the 5th and the 10th percentiles of the patient group were lower than those of the control group. The 5th and 10th percentiles of the control group from the transformed data are given in Table 4.5, along with the other reference values of the control group.

4.7 ESTIMATION OF CONTROL GROUP SAMPLE SIZE FOR ACCURACY OF REFERENCE RANGE

To develop a reference range that has enough data to form a normal distribution, the number of controls is important. This also increases the accuracy or confidence of the values and ranges. During this study 18 healthy control biopsies had been collected, which were not enough to give a normal distribution. To obtain enough data for normal distribution, the estimated sample size can be predicted, based on current data. The method of Sheather and Marron (1990) was used to estimate the sample size of the control group. This estimation of sample size is a statistical method for estimating dataset size requirements. The goal is to use existing classification results to estimate dataset size requirements for future classification experiments and to evaluate the gain in accuracy and significance of classifiers built with additional data (Sheather and Marron, 1990). Table 4.3 shows an example of sample size estimation based on the current control group data.

Table 4.3: Sample size estimation for control data

(Sheather and Marron, 1990)

Sample Size determination			
Enzyme	Sample Size	d (percentile 5)	d (percentile 10)
CS	18	186.78	128.21
	41	124.52	85.47
CI	18	22.64	19.72
	41	15.09	13.14
CI+III	18	53.01	41.91
	41	35.34	27.94
CII+III	18	67.84	41.20
	41	45.23	27.46
CIV	18	45.91	36.53
	41	30.61	24.36
PDHc (CS)	18	11.00	8.58
	41	7.34	5.72
PDHc (mg)	18	3.63	3.74
	41	2.42	2.49

d = desired precision

The sample size estimation was only done for the enzyme analysis. Table 4.3 illustrates the 5th and 10th percentile accuracy while a certain sample size is used. The sample sizes used in Table 4.3 are 18 and 41, and with the different enzymes the accuracy (desired precision), while using more control data, improves.

4.8 MITOCHONDRIAL RESPIRATION AND ENZYME ACTIVITY DATA OF PATIENTS CONTROL REFERENCE RANGES

The control data, including TKDEP transformed data, were used to calculate reference ranges, using four different approaches, as reported in the literature (see Section 3.8). The patient data were compared to these reference ranges. The summarised data are given in Tables 4.4 (respiration data) and 4.5 (enzyme data). The patient data that fell below any of these reference values were identified and marked. The various approaches to calculate reference values were not similar and therefore values from patients that were below different reference value were marked differently.

4.8.1 Respiration results of patients compared to reference ranges

Table 4.4 illustrates all the patient respiration analysis data that were compared to the reference range data of the four different approaches (A–D). The control group (C) consisted of 18 healthy controls and the patient group (P) consisted of 26 possible patients. Although one of the objectives of this study was to establish a reference range, using healthy control data, reference ranges using the patient group were also shown again to compare these ranges.

Table 4.4: Respiration data of patients compared with reference ranges from control- (C) and patient (P) group using four different procedures

	Mitochondrial respiration											
Reference data processing	P+M St 3 (pmol/min/µg)		P+M RCR		G+M St 3 (pmol/min/µg)		G+M RCR		S+R St 3 (pmol/min/µg)		S+R RCR	
	C	P	C	P	C	P	C	P	C	P	C	P
Procedure A												
5th percentile (A1)	44.9	29.0	1.18	1.18	39.1	24.4	0.93	1.10	34.8	29.2	1.82	1.51
10th percentile (A2)	58.2	41.6	1.28	1.25	49.7	35.9	1.04	1.24	50.7	41.4	2.12	1.72
Procedure B												
Mean – 2SD	15.6	N/A	N/A	0.45	7.4	N/A	0.75	0.59	23.8	N/A	1.64	1.30
Procedure C												
25% of Mean	29.8	27.3	0.54	0.48	27.9	23.0	0.47	0.42	26.1	27.8	0.66	0.69
Procedure D												
Minimum value	57.0	47.0	1.29	1.17	59.0	46.0	1.11	0.62	56.0	43.0	1.72	1.17
Patients												
P1	260		1.4		170		1.4		340		3.4	
P2	210		1.9		130		0.6 ($A1^{C,P}$,$A2^{C,P}$,B^{C}, $D^{C,P}$)		350		3.6	
P3	74		3.4		75		1.1 ($A1^{P}$,$A2^{P}$ D^{C})		78		3.5	
P4	210		1.2 ($A2^{C,P}$,D^{C})		130		1.4		74		3.5	
P5	150		1.3		85		1.5		91		1.2 ($A1^{C,P}$,$A2^{C,P}$, $B^{C,P}$,D^{C})	
P6	170		1.4		200		1.8		190		2.1	
P7	170		2.1		140		1.5		160		1.9	
P8	65		2.1		53 (D^{C})		1.4		43 ($A2^{C}$,$D^{C,P}$)		2.4	
P9	57 ($A2^{C}$,D^{C})		1.8		53 (D^{C})		2.2		77		3.5	
P10	82		1.4		55 (D^{C})		1.4		57		3.0	
P11	68		1.5		48 ($A2^{C}D^{C}$)		1.9		65		3.6	
P12	57 ($A2^{C}$,D^{C})		2.2		90		1.8		110		2.1	
P13	81		1.3		80		1.6		100		2.6	
P14	56 ($A2^{C}$,D^{C})		1.8		46 ($A2^{C}D^{C,P}$)		1.7		49 ($A2^{C}$,D^{C})		3.1	
P15	47 ($A2^{C}$ $D^{C,P}$)		1.8		54 (D^{C})		1.5		55 (D^{C})		3.9	
P16	190		3.7		190		3.3		150		2.9	
P17	ND		ND		ND		ND		ND		ND	
P18	88		1.6		71		1.3		67		2.7	
P19	57 ($A2^{C}$,D^{C})		1.5		63		2.1		69		3.5	
P20	ND		ND		ND		ND		ND		ND	
P21	83		1.7		72		1.6		91		2.4	
P22	78		3.3		54 (D^{C})		1.2 ($A2^{P}$)		75		1.4 ($A1^{C,P}$,$A2^{C,P}$, B^{C},D^{C})	
P23	79		1.3		78		1.5		59		2.4	
P24	69		1.5		59 (D^{C})		2.7		93		2.2	
P25	ND		ND		ND		ND		ND		ND	
P26	110		3.2		120		1.9		110		3.1	

C = Control group; P = Patient group; A1 = 5th percentile; A2 = 10th percentile; B = Mean – 2SD; C = 25% of Mean; D = Minimum value

When using procedure A (5^{th} and 10^{th} percentiles), patient 8 had a value lower than the 10^{th} percentile of both groups C and P for S+R. Patient 9, 15 and 19 had a value lower than the 10^{th} percentile of group C for P+M. Patient 11 had a value lower than the 10^{th} percentile of group C for G+M and patient 12 and 14 had values lower than the 10^{th} percentile of group C for P+M, G+M and S+R.

Procedure B (mean ±2SD) could not be used due to all the reference range values that were lower than zero. When using procedure C (25% of mean), none of the patient data were below the reference ranges, which is due to the very low values of the reference ranges.

When using procedure D (minimum value), patient 8 had a value lower than the reference value for both groups C and P for S+R and G+M. Patient 9 had a value lower than the reference value for P+M and G+M. Patients 12, 14 and 15 had values lower than the reference value of group C for all three substrates. Patient 19 had a value lower than the reference value for P+M only.

From the respiration data it was apparent that none of the patient data had values equal to or below 5% of the reference ranges from the control or the patient groups. In total, eight of the patients had G+M and/or P+M respiration values (which both enter the respiratory chain at complex I) that were lower than any of the reference values or approaches (Patient 8, 9, 10, 11, 12, 14, 15 and 19). Three patients (Patient 8, 14 and 15) had S+R respiration values (which enter complex II) that were lower than any of the reference values or approaches. The correlation with enzyme data is discussed in the next section.

4.8.2 Enzyme assay data of patients compared to reference ranges

Table 4.5 illustrates all the patient enzyme assay data that are compared to the reference range data of the four different approaches (A–D). The control group (C) consisted of 18 healthy controls and the patient group (P) consisted of 26 possible patients. Although one of the objectives of this study was to establish a reference range using healthy control data, reference ranges using the patient group were also shown again to compare these ranges.

Table 4.5: Enzyme assay data of patients compared with reference ranges from control- (C) and patient (P) group using four different procedures

Reference data processing	Mitochondrial respiratory chain enzyme									
	CI (nmol/min/UCS)		CI+III (nmol/min/UCS)		CII+III (nmol/min/UCS)		CIV (nmol/min/UCS)		PDHc (pmol/min/CS)		PDHc (pmol/min/mg)	
	C	P	C	P	C	P	C	P	C	P	C	P
Procedure A 5th percentile (A1)	53.5	44.0	62.2	18.5	144	85.2	91.5	73.2	7.48	1.65	3.19	0.52
10th percentile (A2)	62.2	55.0	81.8	32.7	166	112	108	88.8	11.6	3.58	4.72	1.12
Procedure B Mean – 2SD	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Procedure C 25% of Mean	32.1	34.0	46.6	34.0	86.1	66.1	63.93	52.0	8.90	5.0	6.40	2.48
Procedure D Minimum value	56.6	29.8	76.2	0	110	21.6	54.8	42.9	3.50	0	4.02	0
Patients												
P1	105		23 ($A1^{C}$, $A2^{C,P}$,$C^{C,P}$, D^{C})		168		131		34.8		5.6(C^{C})	
P2	139		62 ($A1^{C}$, $A2^{C}$,D^{C})		206		123		24.4		4.0($A2^{C}C^{C}$ D^{C})	
P3	142		148		352		217		23.4		11.3	
P4	85		100		212		310		20.7		11.3	
P5	90		97		231		168		8.6 ($A2^{C}C^{C}$)		4.0($A2^{C}$,C^{C}, D^{C})	
P6	78		69 ($A2^{C}$, D^{C})		235		220		7.0 ($A1^{C}A2^{C}C^{C}$)		2.4($A1^{C}$, $A2^{C}$,$C^{C,P}$, D^{C})	
P7	179		59 ($A1^{C}$,$A2^{C}$,D^{C})		100 ($A1^{C}$, $A2^{C,P}$, D^{C})		102 ($A2^{C}$)		4.0($A1^{C}$, $A2^{C}$,$C^{C,P}$)		1.6($A1^{C}$, $A2^{C}$,$C^{C,P}$, D^{C})	
P8	152		485		126 ($A1^{C}$, $A2^{C}$)		267		0.0($A1^{C,P}$, $A2^{C,P}$,$C^{C,P}$, $D^{C,P}$)		0.0($A1^{C,P}$,$A2^{C,P}$,$C^{C,P}$,$D^{C,P}$)	
P9	30 ($A1^{C,P}$, $A2^{C,P}$,$C^{C,P}$, D^{C})		52($A1^{C}$, $A2^{C}$,D^{C})		206		92 ($A2^{C}$)		35.0		22.5	
P10	74		230		195		248		23.3		6.4 (C^{C})	
P11	275		242		222		508		15.3		1.6($A1^{C}$, $A2^{C}$,$C^{C,P}$, D^{C})	
P12	99		159		1279		89 ($A2^{C}$)		101.3		42.6	
P13	187		158		640		394		0.0($A1^{C,P}$, $A2^{C,P}$,$C^{C,P}$, $D^{C,P}$)		0.0($A1^{C,P}$, $A2^{C,P}$,$C^{C,P}$,$D^{C,P}$)	
P14	42 ($A1^{C,P}$, $A2^{C,P}$, D^{C})		21($A1^{C}$, $A2^{C,P}$,$C^{C,P}$, D^{C})		22 ($A1^{C,P}$, $A2^{C,P}$,$C^{C,P}$, D^{C})		43 ($A1^{C,P}$, $A2^{C,P}$,$C^{C,P}$, D^{C})		0.0($A1^{C,P}$, $A2^{C,P}$,$C^{C,P}$, $D^{C,P}$)		0.0($A1^{C,P}$, $A2^{C,P}$,$C^{C,P}$,$D^{C,P}$)	
P15	327		172		371		257		0.0($A1^{C,P}$, $A2^{C,P}$,$C^{C,P}$, $D^{C,P}$)		0.0($A1^{C,P}$, $A2^{C,P}$,$C^{C,P}$,$D^{C,P}$)	
P16	157		262		404		187		53.8		44.2	
P17	255		0 ($A1^{C,P}$, $A2^{C,P}$,$C^{C,P}$, $D^{C,P}$)		142 ($A2^{C}$)		107 ($A2^{C}$)		0.0($A1^{C,P}$, $A2^{C,P}$,$C^{C,P}$, $D^{C,P}$)		0.0($A1^{C,P}$, $A2^{C,P}$,$C^{C,P}$, $D^{C,P}$)	
P18	213		206		253		145		13.0		10.5	
P19	242		137		134 ($A2^{C}$)		148		21.4		7.2	
P20	77		63 ($A2^{C}$,D^{C})		213		109		21.1		8.0	
P21	121		72 ($A2^{C}$,D^{C})		233		175		21.6		13.7	
P22	63		30 ($A1^{C}$, $A2^{C,P}$,$C^{C,P}$, D^{C})		98 ($A1^{C}$, $A2^{C,P}$, D^{C})		117		8.8($A2^{C}C^{C}$)		4.0($A2^{C}$,C^{C}, D^{C})	
P23	71		177		145 ($A2^{C}$)		229		16.7		21.7	
P24	48 ($A1^{C}$, $A2^{C,P}$, D^{C})		127		155 ($A2^{C}$)		226		0.0 ($A1^{C,P}$, $A2^{C,P}$,$C^{C,P}$, $D^{C,P}$)		0.0 ($A1^{C,P}$, $A2^{C,P}$,$C^{C,P}$,$D^{C,P}$)	
P25	125		158		194		279		29.8		13.7	
P26	127		227		338		517		36.5		21.7	

C = Control group; P = Patient group; A1 = 5th percentile; A2 = 10th percentile; B = Mean – 2SD; C = 25% of Mean; D = Minimum value

When using procedure A (5^{th} and 10^{th} percentiles), the following results were obtained: Patient 1 had values lower than the 5^{th} percentile (C) and 10^{th} percentile (C, P) for complex I+III; Patient 2 had values lower than the 5^{th} percentile and 10^{th} percentile of groups C for complex I+III; Patient 5 had a value lower than the 10^{th} percentile of group C for PDHc; Patient 6 had a value lower than the 10^{th} percentile of C for complex I+III; Patient 7 had values lower than the 5^{th} percentile of C for complex I+III, complex II+III and PDHc; Patient 8 had values lower than the 5^{th} percentile of group C for complex II+III and of both group C and P for PDHc; Patient 9 had values lower than the 5^{th} percentile of both groups C and P for complex I and of group C for complex I+III, as well as values lower than the 10^{th} percentile of both groups C and P for complex IV; Patient 12 had values lower than the 10^{th} percentile of group C for complex IV; Patients 13 and 15 had values lower than the 10^{th} percentile of both groups C and P for PDHc; Patient 14 showed values lower than 5^{th} percentile of both groups C and P for all RC enzymes and PDHc; Patient 17 had values lower than the 5^{th} percentile of both groups C and P for complex I+III, PDHc, also had values lower than the 10^{th} percentile of group C for complex II+III and complex IV. Patients 19 and 23 had values lower than the 10^{th} percentile of group C for complex II+III. Patients 20 and 21 had values lower than the 10^{th} percentile of group C for complex I+III. Patient 22 had values lower than the 5^{th} percentile of group C for complex I+III and complex II+III, as well as values lower than the 10^{th} percentile of both groups for complex I+III and complex II+III, and of group C for PDHc. Patient 24 had values lower than the 5^{th} percentile of group C for complex I, and of both groups C and P for PDHc, as well as values lower than the 10^{th} percentile of group C for complex II+III.

Procedure B (mean ±2SD) could not be used due to all the reference range values that were lower than zero.

When using procedure C (25% of mean) the following results were observed: Patient 1 had values lower than the reference value for both groups C and P for complex I+III; Patients 5 and 6 had values lower than the reference value for group C for PDHc; Patients 7, 8, 13, 15 and 24 had values lower than the reference values for both groups C and P for PDHc; Patient 9 had values lower than the reference values for both groups C and P for all enzymes. Patient 14 also had values lower than the reference values for both group C and P for all enzymes. Patients 17 and 22 had values lower than the reference values for both groups C and P for complex I+III and PDHc.

When using procedure D (minimum value of group) the following results were obtained: Patient 1, 2, 6, 20 and 21 had values lower than the reference value for group C for complex I+III; Patient 7 and 22 had values lower than the reference values for group C

complex I+III and complex II+III; Patients 8, 13 and 15 had values lower than the reference values for both groups C for PDHc. As with the other approaches, Patients 9 and 14 had values lower than group C and P for all enzymes compared to the reference values; Patient 17 had values lower than the reference values for both groups C and P for complex I+III and PDHc. Finally, Patient 22 had values lower than the reference value for group C for complex I+III and complex II+III.

To evaluate if respiration analysis must be done together with the enzyme assay to diagnose a patient with a mitochondrial disorder, the results of the two assays were compared to one another. From the enzyme analysis data it can be observed that patient 1 has a complex I+III deficiency, but from the respiration analysis no defect was apparent. Patient 2 has a complex I+III enzyme deficiency, but also no defect according to the respiration analysis. In Patient 5, who showed deficient PDHc activity, no defect according to the respiration analysis was observed. Patient 6 had a complex I+III deficiency, but here also no defect according to the respiration analysis was observed. Patient 7 had complex I+III, complex II+III and PDHc deficiencies, but again respiration analysis showed values higher than the reference value. Patient 8 had a complex II+III deficiency based on enzyme data. In this patient, analysis using G+M and S+R showed low/deficient respiration levels. Patient 9 had a complex I and complex I+III enzyme deficiency, and according to the respiration analysis all substrate respirations were deficient. Patient 12 had a complex IV deficiency, and accordingly the respiration analysis showed deficient G+M respiration. Patient 13 had a PDHc defect, but no respiration deficiency was detected using P+M as substrates. Patient 14, who has a marked deficiency in all enzymes, also showed deficient respiration using all substrates. Patient 15 had a PDHc deficiency and also had deficient respiration with all substrates. Patients 19 and 23 had a complex II+III deficiency, but no problems with respiration of any of the substrates. Patient 21 had a complex I+III deficiency, but also had no reduced respiration. Therefore, from these results only Patients 9 and 14 showed some form of deficient respiration that could be matched with the enzyme data. From the respiration analysis data, Patients 10 and 11 had deficient respiration using G+M as substrate, but there were no problems for these patients according to the enzyme assay results. A possible explanation can be that no problem occurred in the OXPHOS but rather did in the TCA cycle, because all the substrates used in the respiration analysis first passes through the TCA cycle.

To summarise, it was observed that when procedure A was used as reference range, 18 patients could be identified with some form of an enzyme deficiency. When procedure C was used as a reference value, 11 of the above-mentioned 18 patients could be identified with an enzyme deficiency. When procedure D was used as a reference value, 13 of the above 18 patients could be identified with an enzyme deficiency.

Chapter 5
Conclusion and Future Perspectives

5.1 PROBLEM STATEMENT, OBJECTIVES AND METHODOLOGY

Biochemical identification of mitochondrial disorders in South African patients has only more recently (since ~2004) been extended to include not only metabolic screening but also crucial single-enzyme assays. The interpretation of patient RC enzyme activities, until now, has been based upon retrospectively compiled "reference" values (the presumably normal range of activities).

The first objective of this study was to further develop existing biochemical methods and procedures to identify patients with mitochondrial disorders. These procedures were aimed at being applicable for the existing collaboration between the Department of Paediatrics, Medical Faculty, University of Pretoria (for clinical evaluation) and the School for Physical and Chemical Sciences, North-West University, Potchefstroom (for biochemical analyses). The second objective was to establish reference values for biochemical analyses using muscle samples from presumably healthy individuals not having a muscle disorder.

The identification of mitochondrial disorders includes clinical data, measurement of the metabolites, histology, analysis of RC/OXPHOS function or enzyme activity and DNA studies (van der Heuvel et al., 2004). Unfortunately, different biochemical methods for the identification of mitochondrial disorders exist at different centres worldwide. The problem of inconsistent methodologies and a lack of universal controls has been expressed in literature by several centres. As illustrated in Table 5.1, by way of example, different conditions for enzyme assays exist at four prominent European laboratories (1–4). These conditions are also different from the conditions adapted for this study, which are mainly based on the methods used by the Murdoch Children's Hospital, Melbourne, Australia (Rahman et al., 1996). In Table 5.1 the conditions for the enzyme assays are listed and are compared to the conditions that were used in this study (5). However, all of these assays are spectrophotometric methods but, although conditions differed, are essentially similar in their approach to enzyme kinetics. The strategy of this study has been

summarised in Section 2.19 and, in addition to RC enzymes, also included a functional test for RC function, namely respiration.

Table 5.1: Variation in conditions for RC and CS enzyme assays in muscle samples at five laboratories

Enzyme	Protein (µg)	°C	Buffers and Additions	Substrates	Inhibitors	nm	ε
CI (1)	82	37	50 mM Tris, pH 8.1, 0.25% BSA, 0.3 mM KCN	100 µM NADH, 50 µM DUQ	5 µM rotenone	340	6.22
CI (2)	38 - 56	30	10 mM Tris, pH 7.4, 50mM KCL, 1 mM EDTA, 1 mM KCN	150 µM NADH, 100 µM CoQ	5 µM rotenone	340 380	5.5
CI (3)	31	37	25 mM Pi, pH 7.5, 0.25% BSA, 5 mM $MgCl_2$, 0.6 mM KCN	100 µM NADH, 130 µM DUQ	10 µM rotenone	340	6.29
CI (4)	21- 31	30	20 mM Tris, pH 7.4, 250 mM saccrose, 50 mM KCL, 5 mM $MgCl_2$, 0.05% Laurylmaltoside	200 µM NADH, 60 µM DUQ	1 µM DQA	340 400	6.1
CI (5)	25	30	50 mM KPi, pH 7.4, 0.1% BSA, 10µM Antimycin A, 1 mM KCN	50 µM NADH, 10 mM CoQ	2.5 µM rotenone	340	6.22
CI+III (1)	42	37	50 mM Tris, pH 8.0, 1% BSA, 0.3 mM KCN	800 µM NADH, 40 µM Cyt c	3 µM rotenone	550	19.1
CI+III (2)	41	37	50 mM Tris, pH 8.1, 0.1% BSA, 0.3 mM KCN	200 µM NADH, 50 µM Cyt c	3 µM rotenone	550	
CI+III (3)	12 - 17	30	50 mM Pi, pH 8.0, 0.1 mM EDTA, 2.5 mM KCN	200 µM NADH, 40 µM Cyt c	1 µM antimycin A	550	19.1
CI+III (4)	14	37	50 mM Tris, pH 8.1, 0.1% BSA, 0.3 mM KCN	100 µM NADH, 40 µM Cyt c	5 µM rotenone	550	19.6
CI+III (5)	5	30	50 mM KPi, pH 7.4, 0.1% BSA, 1 mM KCN	50 µM NADH, 2 mM Cyt c	2.5 µM rotenone	550	29.5
CII+III (1)	92 - 121	30	25 mM Pi, pH 7.2, 5 mM $MgCl_2$, 3 mM KCN, 2 µg/ml rotenone	20 mM succinate, 50 µM Cyt c		550	
CII+III (2)	103	37	50 mM Pi, pH 7.8, 2 mM EDTA, 0.1% BSA, 0.2 mM ATP, 0. 3 mM KCN, 3 µM rotenone	10 mM succinate, 50 µM Cyt c	10 mM malonate	550	19.1
CII+III (3)	68	37	100 mM Pi, pH 7.5, 0.6 mM KCN	20 mM succinate, 1% Cyt c		550	18.5
CII+III (4)	60	37	50 mM Pi, pH 7.8, 2 mM EDTA, 0.1% BSA, 0.2 mM ATP, 0.3 mM KCN, 3 µM rotenone	10 mM succinate, 40 µM Cyt c	10 mM malonate	550	19.6
CII+III (5)	5	30	50 mM KPi, pH 7.4, 0.08% EDTA, 0.1% BSA, 0.2 mM ATP, 1 mM KCN, 2.5 µM rotenone	10 mM succinatec, 2 mM Cyt c		550	29.5
CIV (1)	795 - 946	30	100 mM Pi, pH 7.4, 0.02% LM	200 µM Cyt c_{red}	NoneO	550	5.9
CIV (2)	491	37	40 mM Pi, pH 7.0, 0.1% BSA, 2,5 mM LM	25 µM Cyt c_{red}	None	550	19.6
CIV (3)	329 - 477	25	50 mM Pi, pH 7.4	70 µM Cyt c_{red}	None	550	21.1
CIV (4)	163 - 198	30	10 mM Pi, pH 7.0, 0.5% BSA	100 µM Cyt c_{red}	None	550	21
CIV (5)	2	30	50 mM KPi, pH 7.4, 12.5 mM LM	20 µM Cyt c_{red}	None	550	29.5
CS (1)	185	37	78 mM Tris, pH 8.1, 0.1% Triton X-100	0.5 mM OAA, 0.43 mM Acetyl-CoA, 0.1 mM DTNB	None	412	13.6
CS (2)	151 - 179	30	75 mM Tris, pH 8.1, 0.1% Triton X-100	0.5 mM OAA, 0.43 mM Acetyl-CoA, 0.1 mM DTNB	None	412	13.6
CS (3)	98 - 141	25	100 mM Tris, pH 8.1, 0.025% Triton X-100	0.1 mM OAA, 0.3 mM Acetyl-CoA, 1 mM DTNB	None	412	13.6
CS (4)	118 - 140	30	25 mM TRA, pH 8.1, 0.5 mM EDTA	2.5 mM OAA, 0.37 mM Acetyl-CoA, 0.1 mM DTNB	None	412	13.6
CS (5)	5	30	100 mM Tris, pH 8.0, 0.04% Triton X-100	10 mM OAA, 60 µM Acetyl-CoA, 0.1 mM DTNB	None	412	13.6

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From Table 5.1 it can be observed that, compared to the other four listed laboratories' conditions, the methods that are used in our laboratory requires less protein. The reason for this is that the other methods use homogenates of muscle samples and not enriched mitochondria, as in our case. Other differences include the temperature that ranges from 25 to 37 °C, with the temperature that was used throughout this study at 30 °C. The pH is also almost the same in all cases, as are the wavelengths used for each enzyme.

The methodology chosen for this study was based on existing methods used at our institution since 2004 and have been adopted from Rahman et al. (2004) and Rustin et al. (1994). To evaluate critically the appropriateness of assays is not within the scope of this study, although they compared well with other reported methods (Table 5.1). However, the assays in this study were partially optimised for protein content. Variation of the methods was also investigated and it was found that the intra-assay variation, done on one specific day, varied between 1.4% and 19.7%, but was mostly below 10%. The inter-assay variation, done on a day to day basis, varied between 8% and 27.5%.

With regard to spectrophotometric methods, several new or modified methods that use either completely new substrates or modified conditions have been described in literature. These modifications are mostly aimed at obtaining better sensitivity and thus using less material. For example, Janssen et al. (2007) developed a new assay for complex I, where 2,-6-dicholoindaphenol (DCIP) is used as a terminal electron acceptor, instead of co-enzyme Q, which according the publication results into a three- to five-fold increase in sensitivity, compared to current methods.

The current trend in other centres in the world is still to do the enzyme assays via spectrophotometric methods. Recently, however, other options have become available, which include the use of so-called "dipstick assays", which are colorimetric measurements based on immunological detection (Mitosciences Inc). Over the past ten years, another method that has been included in research and diagnostics to support the results of the enzyme assays on a molecular level is blue-native polyacrylamide gel-electrophoresis (BN-PAGE). BN-PAGE is a non-denaturing mobility assay in a gel matrix using antibodies to stain enzyme complexes. This method is similar to the western-blot method and can detect deficient native complexes that have become unstable due to mutations or deficient assembly of the complexes (Nijtmans et al., 2002).

In view of these possible improvements to the diagnostic strategy, muscle tissue in this study was also frozen, in order to isolate mitochondria for respiration and enzyme assays (see Appendix C). This will make it possible to include both molecular (DNA) analyses and BN-PAGE in future studies on controls and patients.

5.2 STUDY MATERIAL

Two types of muscle biopsies were collected: biopsies from healthy patients (not having muscle disorders) (control group), as well as from suspected mitochondrially deficient patients (patient group). Obtaining healthy muscle tissue to set up reference material is a big improvement on the previous approach, where reference values were chosen based on retrospective inclusion of "healthy" patients. This was the case in the Mitochondrial Research Laboratory, NWU, as well in many centres worldwide. Only recently, publications appeared where healthy tissue reference values were reported.

For these control tissue samples, a relatively accessible option was to obtain muscle from patients undergoing orthopaedic surgery (Bourgeois and Tarnopolsky, 2004). These patients included paediatric patients and, apart from problems due to injury or structural disorders, were otherwise healthy. To use this approach in this study (as would generally be the case), ethics approval had to be obtained from the host academic institution and consent from patients and parents. As this was a relatively big intervention, and the approval had to include how the material would be used and reporting of data done, it involved major ethical issues. Ethics approval was obtained by Dr. I. Smuts to acquire and use control tissue in addition to patient material in this study. This can be regarded as a major improvement in this study, and as the study may continue in future years where more control tissue can be obtained, *reference values have been calculated using only control data and not any retrospective patient data, even though the number of controls (18) was still limited*

Dr. I. Smuts at the Paediatric Neurological Research Clinic of the Pretoria Academic Hospital collected the controls and patients. The patients were classified in terms of existing clinical and biochemical information according to the Nijmegen Criteria for Mitochondrial Disease (Appendix A). If the patients were suspected to have a possible deficiency according to their clinical data, a muscle biopsy was taken and the biochemical test performed. The biochemical data played an important role in the diagnosis of a patient, because biochemical and molecular analyses still form the cornerstones of the diagnosis of mitochondrial disorders (Naviaux, 2004). The ideal would be to include molecular analyses of DNA as well, but at this stage of the study, the most crucial data for diagnosis had been the main focus.

5.3 RESPIRATION AND RC ENZYME REFERENCE VALUES, AND IDENTIFICATION OF POSSIBLE MITOCHONDRIAL DEFICIENCIES

To identify significantly low enzyme activities, a reference range had to be established. As mentioned before, reference ranges were in the past set up using only retrospective data from patients, but in this study control muscle samples were used to set up reference ranges. Different approaches have in the past been used worldwide to set up reference ranges. In this study four of the common approaches were followed, as illustrated in Table 5.2, to compare the values and to identify which are more selective. The patient data have subsequently been compared with these four reference ranges and possible deficiencies identified (Section 4.8).

Table 5.2: Four different approaches to determine reference values

Statistical approach to calculate the reference range	Reference
5^{th} and 10^{th} percentile as reference point	Zheng et al., 1990
Mean - 2SD as reference point	Zheng et al., 1990
Lowest value as reference point	Bentlage et al., 1996
25% of the mean as reference point	Thorburn et al., 2004

As illustrated in Figure 4.4 and 4.5, the four different approaches to determine reference values were evaluated to choose the one that was the most selective reference range to identify a patient with a mitochondrial disorder. The approach used by Zheng et al. (1990) where the mean - 2SD is used, is one approach that could be eliminated, as most of the values from this study's data were less than zero. This approach probably required more data in a very narrow range. The approach used by Thorburn et al. (2004) may be an approach that is prone to bias as it is not clear on what basis the selection of a specific percentage of the mean, such as 25% or 20%, was made. This approach probably requires long-standing knowledge of what percentage of the mean enzyme activities can be regarded as leading to pathology. The approach suggested by Bentlage et al. (1996), and which is also used by the Nijmegen Centre for Mitochondrial Disorders, The Netherlands (personal communication), is a common-sense approach because it includes all healthy control values as "normal" and anything below that as possibly deficient. However, it can happen that one of the controls result in a very low value due to an error of some sort or technical variation, as shown in this study, and then the reference point between defective and healthy will be too low and lead to false positives.

As illustrated in Figure 4.5, one of the patients (patient 17) had an enzyme activity (complex I+III) value of zero, which would then be the cut-off point, yet that obviously cannot be correct. The approach to use the 5^{th} (more selective) or 10^{th} (less selective)

percentiles as reference points also has some bias as the reason for choosing these percentiles also has no sound scientific basis. The advantage of this approach is that at least outliers from the control data, which have a variation of at least 10% due to variations in the assays (see Section 4.4), will be omitted. Therefore, using the 5^{th} or 10^{th} percentiles of the control data it is an approach where, although bias is still present, the sensibility of using only healthy control values is combined with a statistical way of allowing for the variation of the methods.

Based on this reasoning, the 5^{th} and 10^{th} percentiles as reference points may be the best-supported approach to use when calculating reference values. In this study, the limited number of controls required the use of an alternative approach (using the TKDEP) for calculating these percentiles. The inclusion of more controls in future may result in obtaining better distribution and more accurate reference ranges as showed in Section 4.7.

It should be noted, as discussed in Section 2.6, that the biochemical data, such as the enzyme and respiration data, provide but one part, albeit crucial, to the diagnosis of mitochondrial disorders. The data presented here therefore do not include or consider any clinical, histological or other metabolic data that form part of the diagnostic procedure. Inclusion of these data was also not part of the objectives of this study and a next step would be to compare the biochemical data of this study with the other data from patients.

5.4 PERSPECTIVES ON THE CURRENT AND FUTURE APPROACHES TO IDENTIFYING MITOCHONDRIAL DISORDERS IN SOUTH AFRICA

One major problem that we have in South Africa is the distances between the major health centres and poor transport between these centres. In this study this was also the case where the distance between the hospital where the biopsies were taken and the laboratory where the tests were done was approximately 180 km. To make use of delivery transport would not be possible as the muscle had to be processed within 2-3 hours after the biopsy had been done. Therefore, it was not possible to transport fresh biopsies to the laboratory at the NWU, which required setting up a satellite laboratory for sample preparation and respiration analysis.

The cost of travelling to Pretoria every time a biopsy was available was expensive (approximately R600) and time-consuming as it took up the whole day. Out of the possible 35 control muscle biopsies that became available during the year, only 18 were used because of inadequate muscle, illness before the operation, consent that was withdrawn or

cancellation of the operation due to problems at the hospital. Therefore, the value of the current approach to include a functional test, for which fresh samples is required, in addition to the required enzyme assays, which can be done with frozen samples, has to be re-evaluated based on the results of this study.

From the results obtained, it can be observed that the respiration analyses in some patients (11%) (comparable outcome) correlated with the results obtained in the enzyme assays. The correlation was not to such a degree as to recommend continuing with the respiration analysis in the future. Enzyme assays are regarded as the golden standard for diagnosing mitochondrial disorders, so available money and time could rather be expended on enzyme assays than on using it to do respiration analysis (Naviax, 2004).

Several centres worldwide use either frozen muscle tissue, which is homogenised (Rustin et al. 1994; Gellerich et al., 2003) and used as such, or crude mitochondrial samples (e.g. 600 x g supernatants) for enzyme assays (Rahman et al., 1996; Janssen et al., 2007; Mayr and Sperl, 2000; Fischer et al., 1986; Taylor and Turnbull, 1997). These approaches eliminate the difficulty of obtaining and using fresh muscle biopsies. It is suggested that in future the enzyme assays in South Africa could be done on frozen biopsies as well. The process of getting the enzyme assays done would be less time-consuming and the costs would be lower. In addition, BN-PAGE, which can also be used on the same material simultaneously to do several analyses, can be used to support enzyme data. Considering the conclusions of this study, a modified strategy for the near future in comparison with the current one is shown in Figure 5.1.

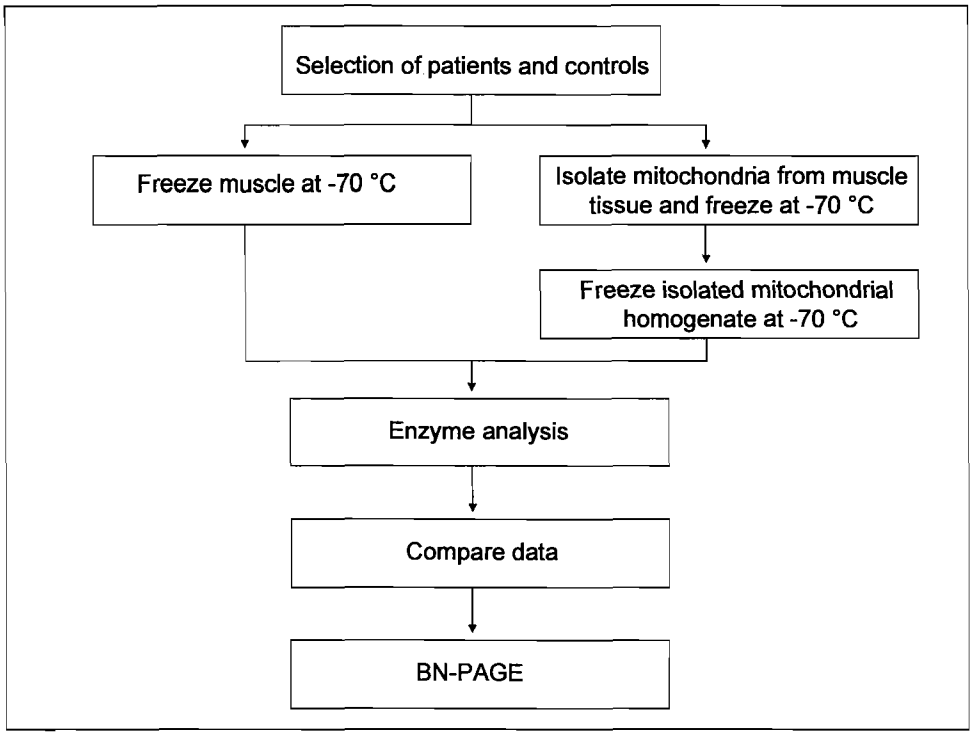

Figure 5.1: Modified strategy for the near future.

The strategy combines the current strategy, eliminating respiration analysis, with an additional sample preparation and BN-PAGE.

In conclusion, from previous work done on the identification of mitochondrial disorders at this institution and current literature, a problem was identified and a study formulated to meet the requirements for the degree of Master of Science (Biochemistry). Specific aims, as discussed in the strategy (Chapter 2), were achieved and reported (Chapters 3 and 4), using appropriate methodologies in an attempt to achieve the objectives of this study. The results were critically evaluated in this chapter and limitations were identified. An alternative strategy to develop this study further in future was proposed, which will hopefully add more value to the identification and research of mitochondrial disorders in South Africa.

Appendix

Appendix A: Nijmegen Criteria for Mitochondrial Disease

1.1 Muscular presentation		max 2 pt
plus CNS		max 1 pt
plus Multisystem		max 2 pt
		max 4 pt
PEO	2	
Ptosis, myopathic facies	1	
Exercise intolerance	1	
Reduced muscle power	1	
Rhabdomyolysis	1	

OR

1.2 CNS signs and symptoms		max 2 pt
plus muscle		max 1 pt
plus multisystem		max 2 pt
		max 4 pt
		1
DD/IQ<70	1	
Loss of aquired skills	1	
Epilepsy or abnormal EEG	1	
Myoclonus/Myoclonic epilepsy	1	
Cortical blindness	1	
Pyramidal tract involvement	1	
Extrapyramidal involvement	1	
Brainstem involvement	1	
Cerebellar involvement	1	

OR

1.3 Multisystem involvement		max 3 pt
plus muscle or CNS		max 1 pt
		max 4 pt
Haematology	1	
Sideroblastic anaemia		
Pancytopaenia		
Gastrointestinal tract	1	
ABN LFT		
FTT		
Exocrine pancreatic dysfunction		
Intestinal pseudoobstruction		
Unexplained chronic diarrhoea		
Endocrine	1	
Short stature		
delayed puberty		
DM I or II		
Hypoparathyroidism		
DI		
Heart	1	
Cardiomyopathy		
Conduction block		
Kidney	1	
Proximal tubular dysfunction		
FSG		
Eyes	1	
Cataract		
Retinopathy		
Optic artrophy		
Ears	1	
Sensorineural deafness		
Nerve	1	
Periphearl neuropathy		
General	1	
Exacerbation of symptoms with minor illness		
SIDS in family		

2 Metabolic or other investigations		**max 4 pt**
Elevated lactate > 2 mmol/l	2	
L:P > 18 if lactate is elevated	1	
Elevated alanine > 450 µmol/l	2	
CSF lactate > 1.8 mmol/l Score only if blood lactate is N	2	
Elevated CSF protein	1	
Elevated CSF alanine	2	
Urine: Lactate or TCA intermediates	2	
Ethylmalonic acid, 3-methylglutaconic acid or dicarbonic acid	1	
Other		
^{31}P-MRS Reduced Phosphocreatine/Pi	2	
Leigh (T2 hyperintense lesions)	2	
Strokelike picture, Leukodystrophy	1	
^{1}H-MRS: clear lactate peak	1	
3 Morphology		**max 4 pt**
RRF	2 to 4	
COX neg	2 to 4	
Stronly reduced overall COX-staining	4	
Reduced SDH-staining	1	
Strongly SDH reactive blood vessels	2	
EM: Abnormal mitochondria	2	

Evaluation	
1 point	Unlikely
2 to 4 points	Possible
5 to 7 points	Probable
8 to 12 points	Definite

Appendix B: Patients and Parents Permission Consent Forms

Amendment N: ASSENT FORM (CONTROL)

The investigation of the biochemical profiles of patients with possible mitochondrial disorders

---

We are going to explain the study to you. If you understand and want to take part, we ask you to sign the form.

WHAT DO WE WANT TO DO IN THE STUDY?

Mitochondrial disorders are diseases that may cause a problem in many parts of the body, because too little energy is made. It is difficult to tell when people have these diseases. One way to find out is to do special tests on their muscle *and urine*. The tests are very difficult. We hope to be able to have a centre one day in South Africa where patients with these problems could be helped. To be able to do so, we need muscle of patients who do not have this disease. The people in the laboratory can then compare the ill children's muscle to the healthy muscle *and urine* and then we can tell them what their problem is.

HOW LONG WILL THE STUDY BE?

This project will start in 2007 and carries on.

WHAT ARE WE GOING TO DO?

We will explain to you what we are going to do and give you time to ask your questions.

Your muscle is healthy, but you have a problem with your legs and the surgeons are going to operate your legs. We would like to have a tiny piece of the big muscle that the surgeon is going to cut. The people from the laboratory will then do special tests on your muscle. Next time when there is an ill patient we can compare their muscle tests to yours and then we can tell them what their problem is, although you are not going to benefit.

You are not going to feel anything, because you are going to sleep very nicely. The piece of muscle that you give is not going to make you weak later on.

We are also asking you to give us a bit of urine in a small bottle so that it can be tested.

WILL ANYBODY KNOW ABOUT YOU?

Only the doctors that know you and the people in the laboratory that work with muscle see the answers. Your hospital number and name will not be given to anybody. If we find some answers we would like to write about it in a medical journal, but nobody that will read the article will be able to know that you took part, only the answers will be given.

WHAT HAPPENS IF YOU DO NOT WANT TO TAKE PART?

You or your parent may at any time tell us if you do not want to take part any further at any time, but your parent must please write us a letter. We will not be cross with you.

THE ABOVE MENTIONED PROJECT WAS THOROUGHLY EXPLAINED AND THE FOLLOWING EXTRA POINTS WERE TOLD TO ME.

The muscle *and urine* are given for research and that I will not be paid for it.

I may decide if I do not want to participate anymore.

WHO SHOULD WE CONTACT IF WE HAVE QUESTIONS?

Dr I. Smuts, Paediatric Neurology Unit, Department of Paediatrics and Child Health, University of Pretoria, P.O. Box 667, Pretoria, 0001.

Enquiries regarding ethical aspects of the project can be addressed to the: Chairperson of the Ethics Committee, Pretoria Academic Hospital, Private Bag X169, Pretoria, 0001.

That I will receive a copy of assent form.

DECLARATION OF ASSENT

I, _____

(Print full name and surname)

Hereby assent to take part in the project as a control.

I confirm that I understand everything.

Signed at: _____ on _____.

(Place) (Date)

_____

(Signature of a minor)

Witness 1: _____ **Witness 2** _____

(Signature) (Signature)

_____

(Signature of responsible who explained the project and assent to the participant.)

Appendix C: Laboratory Protocols for Mitochondrial Respiration and Enzyme Analyses

1. Preparation of mitochondria from muscle sample

Rossignol, R et al., 2000. Tissue variation in the control of oxidative phosphorylation: implication for mitochondrial diseases. Biochem. J. 374, 45-53.

1.1 Reagents

1.1.1 Medium A:

1.1.1.1	Mannitol, 210 mM (Sigma M9546, Mr 182,2);	2.28 g/60 ml
1.1.1.2	Sucrose, 70 mM (Sigma S7903, Mr 342,3);	1.44 g/60 ml
1.1.1.3	Tris.HCl, 50 mM (Sigma A2395, Mr 121,1);	0.36 g/60 ml
1.1.1.4	EDTA.K, 10 mM (Fluka O3660, Mr 404,5);	243 mg/60 ml
1.1.1.5	Set pH to 7.4 using KOH.	
1.1.1.6	Filter medium using a 0.22 µm filter and store at -20 °C.	
1.1.1.7	Prepare fresh every three months	

1.1.2 Medium B:

1.1.2.1	Mannitol, 225 mM (Sigma M9546, Mr 182,2);	2.46 g/60 ml
1.1.2.2	Sucrose, 75 mM (Sigma S7903, Mr 342,3);	1.54 g/60 ml
1.1.2.3	Tris.HCl, 10 mM (Sigma A2395, Mr 121,1);	0.072 g/60 ml
1.1.2.4	EDTA.K, 0.1 mM (Fluka O3660, Mr 404,5);	2.4 mg/60 ml
1.1.2.5	Set pH to 7.4 using KOH	
1.1.2.6	medium using a 0.22 µm filter and store at -20 °C	
1.1.2.7	Prepare fresh every three months	

1.1.3 Water: Tap water initially passes through a Progard pre-treatment pack. It is designed to remove particles and free chlorine from the water. In addition, it helps to prevent mineral scaling in hard water areas. The water is pressurized with a pump and then is purified by reverse osmosis (RO). This produces intermediate quality water. The RO product water then passes through an electrodeionisation (E.D.I) module. This is the final purification stage used to reduce levels of organic and mineral contaminants. The water is then exposed to UV light at both 185 and 254 nm wavelengths. This oxidises organic compounds and kills bacteria. The function of the Quantum cartridge is to remove trace ions and oxidation by-products produced by the action of the UV light.
Purified water then passes through an Ultra filtration (UF) module. The UF module acts as a barrier to colloids, particles and organic molecules with a molecular weight greater than 5000 Daltons. The contaminants retained by the UF are periodically flushed out of the system via tubing to a drain.
A manual 3 way valve located in the point of use allows you to direct ultra pure water through a final filter made up of a 0.22 um membrane. The final filter

removes particles and bacteria greater than 0.22 um in size and prevents recontamination of the system from the point of use.
The A10 TOC monitor takes samples of ultra pure water to determine trace organic levels. Samples are taken periodically in product mode.

1.2 Procedure

1.2.1 Muscle tissue (± 300 mg) is collected in a clean plastic sample holder (type used for collection of urine) containing ±10 ml Medium A and placed immediately on ice. The sample has to be processed within 2 hrs after collection during which time it has to be kept on ice.

1.2.2 Remove fat tissue and weigh ± 50 mg muscle sample using a sterile blade on a glass plate (on ice).

- Mark sample name (patient initials and surname) + MB (for muscle biopsy) and date. Store at -70°C.

1.2.3 Weigh rest of sample and suspend in Medium A so that end concentration is 10% (w/v), e.g. 2500 µl for 250 mg sample.

1.2.4 Homogenise in a Potter Elvehjam homogenizer in ice water at 100 rpm using 15 strokes.

1.2.5 Transfer homogenate to a marked clean micro centrifuge tube.

1.2.6 Centrifuge at 1000 x g for 5 min at 4 °C.

1.2.7 Transfer supernatant to a marked clean micro centrifuge tube.

1.2.8 Centrifuge at 7000 x g for 10 min at 4 °C.

1.2.9 Remove supernatant and suspend pellet in the original volume Medium B

1.2.10 Repeat both 1000 and 7000 x g centrifuge steps.

1.2.11 Carefully suspend the remaining pellet in 1/20 of the original volume, e.g. 125 µl for original 2500 µl volume. Use a pipette and avoid making bubbles.

- Mark sample name (patient initials and surname) + 10KMMP (for 10K x g muscle mitochondrial pellet) and date.
- Keep sample on ice for respiration analysis whilst doing a protein determination (BCA method).

2. Protein assay (BCA method)

Reference: Smith P.K. et al. 1985. Analytical Biochemistry. 150, 76-85

2.1 Reagents

2.1.1 BCA protein assay kit containing BCA (Sigma B9643), $CuSO_4$, (Sigma 2284) and protein standard, bovine serum albumin (BSA) 1 µg/µl (Sigma P0914).

2.2.2 BCA reagent. Freshly prepare BCA:$CuSO_4$ solution at ratio of 50:1
e.g. for 6 ml add 120 µl $CuSO_4$ to 5880 µl BCA

2.2.3 Test sample as prepared in Section 1.2 (use undiluted).

2.2 Procedure

2.2.1 Prepare BSA standard (S) series as follows:

	S0	S1	S2	S3	S4	S5
µg	0	4	8	12	16	20
µl (BSA)	0	4	8	12	16	20
H_20	20	16	12	8	4	0
µl BCA* reagent (plates) **OR**	200	200	200	200	200	200
µl BCA* reagent (cuvettes)	600	600	600	600	600	600

2.2.2 Add (in duplicate) 10 µl undiluted homogenate to microtiter plate or tube as well as 10 µl H_20 (Medium B has no effect on assay compared to H_20).

2.2.3 *Add appropriate volume BCA reagent to standards and test samples *at the same time* and incubate at 37 °C for 30 min or longer (samples must become clearly purple).

2.2.4a When using a microtiter plate format use the programme "Proteïenbepaling" on the BioTek FL600 microplate reader (KC4 software) to read absorbance at 562 nm and to determine **protein content** in the 10 µl homogenate.

2.2.4b When using the 600 µl sample volumes at the University of Pretoria Paediatrics Laboratory, use the Excel programme "Proteïenbepaling" to determine the content.

2.2.5 Use the equation to determine **protein concentration**:
[µg/µl] = (protein content (µg) of sample x dilution)/reaction volume (Eq.1)

2.2.6 Dilute protein content to 1 mg/ml <u>using Medium B - carefully check your calculations using the equation C1 x V1 = C2 x V2)!</u>

2.2.7 Mark tube with initials and surname, + "KMMP" + date and use immediately for respiration analyses (keeping on ice water) or store at -80 °C for enzyme assays.

3. Respiration assay

Reference: Rossignol, R et al., 2000. Biochem. J. 374, 45-53.

3.1 Instrument

3.1.1 MT200A respirometry system and 782 Oxygen System (version 3.0) software from Strathkelvin Instruments, Motherwell, Scotland.

3.2 Set up

3.2.1 Connect water bath (set at 30 °C) to respirometer.
3.2.2 Connect respirometer to recording unit.
3.2.3 Connect recording unit to computer and open 782 Oxygen System (version 3.0) software.
3.2.4 Set experimental values at the following settings:

- Oxygen recording unit: µmol/l
- Respiration rate: µmol/min
- Water volume: 70 µl
- Biomass: 14 µg (if 14 µl of a 1 µg/µl sample is used)
- Temperature: 30 °C

3.3 Calibration

3.3.1 The electrode (recording unit) has to be calibrated every day or when measurement units have been changed.
3.3.2 After turning on the electrode or power to recoding unit wait 1 hour to stabilize the unit before calibrating.
3.3.3 During calibration the recoding unit displays electrode output in pAmp (pico amps).
3.3.4 While electrode stabilizes, prepare high and zero point calibration solutions as follows:

- High point calibration solution (HPCS): vigorously stir de-ionized and autoclaved ("MilliQ") water using a stirring magnet in a clean glass beaker.
- Zero point calibration solution (ZPCS): add ± 50 mg Na_2SO_3 (anhydrous) to 100 ml MilliQ water. Store at 4 °C and prepare fresh every month.

3.3.5 For calibration select "setup experiment menu" and select "calibration" option.
3.3.6 Perform zero point calibration (must be done first) as follows:

- Add 70 µl ZPCS to electrode and connect stirring bar.
- Select "calibrate zero". The recording unit should now move down to 0 pAmp before calibration time is complete.
- Select "Accept value" when calibration is complete and if reading has been 0 pAmp.
- Remove ZPCS and wash the chamber well with MilliQ water (three times with 200 µl.

3.3.7 Perform high point calibration (must be done after zero point calibration) as follows:

- Add 70 µl HPCS to electrode, connect stirring bar *as well as* the plunger.
- Select "calibrate high". The recording unit should now move up to over 300 pAmp before calibration time is complete.
- Select "Accept Value" and "Calibration Complete".
- The recording unit should now read 199 µmol/l (saturated O_2 at 30 °C)

3.4 Considerations when running a reaction

3.4.1 Make sure that no air bubbles are introduced to the sample during the procedure.

3.4.2 During the course of an experiment the substrate or inhibitors can be injected directly to the chamber, using the Strathkelvin modified syringe. The syringe has a spacer which ensures that the needle does not touch the membrane of the electrode. During injection keep the plunger in place to avoid introducing air bubbles.

3.4.3 Between runs, the content of the respiration chamber can be removed by suction (pipetting), followed by rinsing (3 x) with MilliQ water (200 µl).

3.5 Reagents

3.5.1 Respiration buffer (2x)

3.5.1.1	Mannitol, 150 mM (Sigma M9546, Mr 182.2);	1.64 g/60 ml
3.5.1.2	Sucrose, 50 mM (Sigma S7903, Mr 342.3);	1.03 g/60 ml
3.5.1.3	KCl, 200 mM (Sigma P9333, Mr 74.56);	0.894 g/60 ml
3.5.1.4	Tris.phosphate, 20 mM (Sigma T8655, Mr 219.1);	0.263 g/60 ml
3.5.1.5	Tris.HCl, 20 mM (Sigma A2395, Mr 121.1);	0.145 g/60 ml
3.5.1.6	EDTA.K, 100 µM (Fluka O3660, Mr 404.5);	2.4 mg/60 ml

3.5.1.7 Set pH to 7.4 using KOH.

3.5.1.8 Filter medium using a 0.22 µm filter and store at -20 °C.

3.5.1.9 Prepare fresh every three months.

3.5.2 ADP, 140 mM (Sigma A5285, Mr 501.3); 0.07 g/ml
Prepare fresh daily. Incubate tube at 30 °C when in use.

3.5.3 Piruvate, 1 M (Sigma P2256, Mr 110.0); 0.11 g/ml
Prepare fresh daily.

3.5.4 Malate, 1 M (Sigma M1125, Mr 156.1); 0.156 g/ml
When preparing start with minimum volume and first set pH to 7.4 using KOH pellets. Then set correct volume. Store in aliquots (200 µl) at -20 °C.

3.5.5 Glutamate, 1 M (Sigma G1501, Mr 203.2); 0.203 g/ml
pH is neutral, but set pH at 7.4 using KOH or HCl. Then set correct volume. Store in aliquots (200 µl) at -20 °C.

3.5.6 Succinate, 1 M (Sigma S7903, Mr 118.1); 0.118 g/ml
When preparing start with minimum volume and first set pH to 7.4 using KOH pellets. Then set correct volume. Store in aliquots (200 µl) at -20 °C.

3.5.7 Rotenone, 0.25 mM (Sigma R8875, Mr 394.4); 10 mg/ml

3.5.8 Piruvate/Malate (500 mM:250 mM) solution. Add piruvate (50 µl from 1 M stock) and malate (25 µl from 1 M stock) and 25 µl MilliQ water to a microcentrifuge tube. Mark tube "PM" and keep at 30 °C.

3.5.9 Glutamate/Malate (500 mM:500 mM) solution. Add glutamate (50 µl from 1 M stock) and malate (50 µl from 1 M stock) to a microcentrifuge tube. Mark tube "GM" and keep at 30 °C.

3.5.10 Succinate/Rotenone (500 mM:50 µM solution). Add succinate (50 µl from 1 M stock), rotenone (6.7 µl from 750 µM stock) and 43.3 µl MilliQ water to a microcentrifuge tube. Mark tube "SR" and keep at 30 °C.

3.5.11 MilliQ water. Use water from MilliQ system and autoclave for 30 minutes. Keep refrigerated and prepare fresh every month.

3.6 Reactions

3.6.1 Piruvate/Malate reaction.
Add to reaction chamber and in the following order:

3.6.1.1 2 x Respiration Buffer 35 µl (1 x)
3.6.1.2 MilliQ water* 18.6 µl
*The water volume should be corrected when components' volumes are changed or others added.
3.6.1.3 Mitochondrial protein 14 µl (0.25 mg/ml)
(freshly prepared at 1 mg/ml, see Section 2)

3.6.1.4 Incubate reaction for 1 minute to stabilize.

3.6.1.5 Select "start reaction" and record baseline (state 1) respiration for ± 15 sec.
3.6.1.6 Add PM solution (30 °C), 1.4 µl (10 mM piruvate, 5 mM malate)
3.6.1.7 Select "Mark" and type in "PM". Record state 2/4 respiration for ± 15 sec.
3.6.1.8 Add ADP (140 mM), 1 µl (2mM)
3.6.1.9 Select "Mark" and type in "ADP". Record state 3 respiration for ± 30 sec.

3.6.1.10 Select "Stop" and save file using patient Name (initials + surname) under "Respiration" folder in subfolder indicating date and add PM suffix, e.g. G.Cronje-PM.

3.6.2 Glutamate/Malate reaction.
Add to reaction chamber and in the following order:

3.6.2.1 2 x Respiration Buffer 35 µl (1 x)
3.6.2.2 MilliQ water* 18.6 µl
*The water volume should be corrected when components' volumes are changed or others added.
3.6.2.3 Mitochondrial protein 14 µl (0.2 mg/ml)
(freshly prepared at 1 mg/ml, see Section 2)

3.6.2.4 Incubate reaction for 1 minute to stabilize.

3.6.2.5 Select "start reaction" and record baseline (state 1) respiration for ± 15 sec.
3.6.2.6 Add GM solution (30 °C), 1.4 µl (10 mM glutamate/malate)
3.6.2.7 Select "Mark" and type in "GM". Record state 2/4 respiration for ± 15 sec.
3.6.2.8 Add ADP (140 mM), 1 µl (2mM)
3.6.2.9 Select "Mark" and type in "ADP". Record state 3 respiration for ± 30 sec.

3.6.2.10 Select "Stop" and save file using patient Name (initials + surname) under "Respiration" folder in subfolder indicating date and add GM suffix, e.g. G.Cronje-GM.

3.6.3 Succinate/Rotenone reaction.
Add to reaction chamber and in the following order:

3.6.3.1 2 x Respiration Buffer 35 µl (1 x)
3.6.3.2 MilliQ water* 18.6 µl
*The water volume should be corrected when components' volumes are changed or others added.
3.6.3.3 Mitochondrial protein 14 µl (0.2 mg/ml)
(freshly prepared at 1 mg/ml, see Section 2)

3.6.3.4 Incubate reaction for 1 minute to stabilize.

3.6.3.5 Select "start reaction" and record baseline (state 1) respiration for ± 15 sec.
3.6.3.6 Add SR solution (30 °C), 1.4 µl (10 mM succinate, 1 µM rotenone)
3.6.3.7 Select "Mark" and type in "PM". Record state 2/4 respiration for ± 15 sec.
3.6.3.8 Add ADP (140 mM), 1 µl (2mM)
3.6.3.9 Select "Mark" and type in "ADP". Record state 3 respiration for ± 30 sec.

3.6.3.10 Select "Stop" and save file using patient Name (initials + surname) under "Respiration" folder in subfolder indicating date and add SR suffix, e.g. G.Cronje-SR.

3.7 Calculations

3.7.1 µmol/min/µg x 1000000 = pmol/min/µg
3.7.2 state 3 respiration/state 2 respiration = RCR

4. Assays for Respiratory Chain and PDHc enzymes

Reference: Rahman S et al. 1996. Ann Neurol. 39, 343 – 351.
Dr D Thorburn, personal communication.

4.1 Instrument

4.1.1 BIO-TEK UVIKON XS (BIO-TEK Instruments, Neufarn, Germany) and LabPower Junior software (BIO-TEK Instruments, Neufarn, Germany).

4.2 Reagents

Stored in boxes marked "Mitochondriale ensiemanalises 4°C" or "Mitochondriale ensiemanalises -20°C"

4.2.1 KPi buffer, 0.5 M, pH 7.4. ((19 ml of 0.5 M KH_2PO_4 (68g/l) + 81 ml of 0.5 M K_2HPO_4 (87.1 g/l); pH 7.4, adjust with 0.5 M KH_2PO_4 (if pH > 7.4) or 0.5 M K_2HPO_4 (if pH< 7.4). Store at 0-10 C).

4.2.2 Bovine serum albumin (BSA), 10% (Roche 775 835); 0.1 mg/ml
Store in aliquots (200 at -20 °C.

4.2.3 CoQ1, 10 mM (Sigma C7956, Mr 250.3); 2.5 mg/ml ethanol
Store in dark bottle at -20 °C.

4.2.4 Cytochrome C (CytCox), 2 mM (Sigma C7752, Mr 12 500) 24.8 mg/ml
Store in aliquots (200 µl) at -20 °C.

4.2.5 Succinate, 1 M (sodium salt, Sigma S7903, Mr 118.1); 0.118 g/ml
For preparation see Section 3.5.6. Store in aliquots (200 µl) at -20 °C.

4.2.6 EDTA, 10%, pH 7.0 (Fluka 03660, Mr 404.5); 5 g/50 ml
Add 5g to ~40 ml MilliQ water, adjust pH to 7.0 with 2 M KOH and set volume to 50 ml. Store at 4 °C.

4.2.7 ATP, 0.1 M, pH 7.2 (disodium salt, Boehringer Mannheim 591987); 605.2 mg/10 ml
Add 605.2 mg in ~8 ml, set pH to 7.2 with 2 M KOH, set volume to 10 ml.
Store in aliquots (200 µl) at -20 °C.

4.2.8 Rotenone, 0.25 mM (Sigma R8875, Mr 394.4); 10 mg/ml ethanol
Dissolve rotenone in amount of ethanol, place in sonication bath for 2 hrs.
Store in dark bottle at -20 °C.

4.2.9 Antimycin A, 0.5 mM (Sigma A8674, Mr ~ 541); 0.27 mg/ml ethanol
Store in dark bottle at -20 °C.

4.2.10 KCN, 50 mM (Mr 65); 3.25 mg/ml in 50 mM Kpi, pH 7.4.
Store in aliquots (200 µl) at -20 °C.

4.2.11 Reduced cytochrome c (CytCred), 500 µM.
Prepare from CytC, 2 mM, by adding 10 mM sodium ascorbate (Sigma A4034 Mr 198, 2 mg/ml) and incubate at room temperature for 5 min (dark red colour

change to light red). Remove ascorbate by dialising (Pierce Slide-A-Lyzer Mini Dialysis unit, Cat # 69570) for 3 x 2 hrs in 200 ml 50 mM KPi buffer at 4 °C.

[$]Pipette 5 µl to 9995 µl KPi buffer (50 mM) in cuvette. Scan 500-600 nm to check reduction. If A550/A565 > 8 it is sufficiently reduced. Calculate CytCred using the following equation:

$$\text{CytCred (mM)} = \frac{\text{A550}}{29.5\ \text{mM}^{-1}.\text{cm}^{-1}} \times 200\ \text{(dilution)}$$

Dilute with KPi (50 mM) buffer to 500 µM. Aliquot in 200 µl volumes in microcentrifuge tubes, gas with N_2 for at least 60 sec. (quickly cap tube) and wrap with parafilm.
When using CytCred check[$] A550/A565 before analysis and afterwards gas with N_2 before closing tube.

4.2.12 PDHc-buffer (2X):

4.2.12.1	D-Manitol, 0.6 M (Sigma M9546, Mr 182.2);	10.9 g/100 ml
4.2.12.2	KCl, 20 mM (Sigma P9333, Mr 74.56);	0.15 g/100 ml
4.2.12.3	$MgCl_2$.$6H_2O$, 10 mM (Sigma M2670, Mr 203.30);	0.094 g/100 ml
4.2.12.4	KH_2PO_4 .$3H_2O$, 20 mM (Sigma P5504, Mr 228.23);	0.272 g/100 ml
4.2.12.5	Triton X100 0.2% (v/v), (BDH 30632)	0.2 ml /100 ml

Set pH to 7.8 with 2 M KOH and store in aliquots (1 ml) at -20 °C.

4.2.13 Oxamic acid, 1.25 M (Sigma O8754, Mr 127.1); 0.159 g/ml
Set pH to 7.8 with 2 M KOH and store in aliquots (200 µl) at -20 °C.

4.2.14 Cystein, 100 mM (Sigma C7880, Mr 175.6); 17.5 mg/ml
Set pH to 7.8 with 2 M KOH and store in aliquots (200 µl) at -20 °C.

4.2.15 Tris.HCl, 1M (mM (Sigma A2395, Mr 121.1); 12.1 g/100 ml
Set pH to 8.0 with cHCl and store at 4 °C.

4.2.16 Ethanol (100%) (Merck 1.00983.2500, Mr 46.07)

Reagents to be prepared fresh before analysis and kept on ice and away from light

4.2.16 Acetyl-CoA, 6 mM (trilithium salt, Roche 10 101 907 001, Mr 827.4); 4.96 mg/ml

4.2.17 Oxaloacetate (OAA), (Roche 107999, Mr 132.1); 1.32 mg/ml

4.2.18 5,5'-Dithio-bis[-2-nitrobenzoesaure] (DTNB), (Roche 104477, Mr 396.4); 0.4 mg/ml in 1M Tris.HCl, pH 8.0.

4.2.19 Triton X100, 10% (v/v) (BDH 30632); 1 ml/10 ml

4.2.20 NADH, 2 mM (disodium salt, Roche 107 727, Mr 709.4); 1.42 mg/ml

4.2.21 *n*-Dodecylmaltoside, 25 mM (Sigma D4641, Mr 510.63); 12.8 mg/ml

4.2.22 NAD, 25 mM (Sigma N1511, Mr 663.4); 16.6 mg/ml

4.2.23	CoA, 20 mM (Roche 103519, Mr 785.4);	15.7 mg/ml
4.2.24	Thiamin pirophosphate (TPP, Cocarboxylase), 20 mM (Sigma C8754, Mr 460.8);	9.2 mg/ml
4.2.25	Piruvate, 40 mM (Sigma P5280, Mr 110.0);	4.4 mg/ml

4.3 Citrate synthase

Reference: Robinson, J.B. 1987. In Mitochondria – A Practical Approach. P153–170.

4.3.1 Procedure

4.3.1.1 Thaw mitochondrial sample (1mg/ml) on ice and mix well.
4.3.1.2 Switch on spectrophotometer for citrate synthase programme (412 nm auto rate assay over 5 min with 30 sec intervals).
4.3.1.3 Connect water bath to spectrophotometer and set temperature at 30 °C.
4.3.1.4 Prepare a reaction master mix (on ice) as follows:

Table 1. CS Master mix

Reagent	1x (µl)	10x (µl)	20x (µl)	[end]
MilliQ water	413	4130	8260	
DTNB (1 mM)	50	500	1000	0.1 mM
Triton X100 (10%)	2	20	40	0.04%
Acetyl-CoA (6 mM)	5	50	100	60 µM
Total	470	4700	9400	

4.3.1.5 Place in disposable cuvette (Sigma (PlastiBrand) 759115) 470 µl of CS master mix.
4.3.1.6 Place cuvette into spectrophotometer sample holder and allow to pre-heat for 2 min. Select "Autozero".
4.3.1.7 Add 5 µl mitochondrial sample to the CS master mix. Mix well (by carefully shaking cuvette while holding at an ~45° angle).
4.3.1.8 Initiate reading and record rate A_{412} for 2 min (A1).
4.3.1.9 Add 25 µl OAA to each reaction and record linear rate increase* in A_{412} for 2 min (A2).

*Adjust protein content if A_{412} increase is not linear.

4.3.2 Calculation

µmol/min/mg (UCS) = $((A2-A1)/13.6mM^{-1}) \times 0.5/(mg/ml\ protein \times 5µl/1000)$

nmol/min/mg = UCS x 1000

4.4 NADH:Ubiquinone oxidoreductase (Complex I)

4.4.1 Procedure

Note 1: Activity of complex I in each sample is measured in presence and absence of rotenone (rotenone-sensitive complex I activity).
Note 2: Before performing assay preheat water and master mix by incubating tubes containing these reagents in water bath for at least 5 min.

4.4.1.1 Thaw mitochondrial sample (1mg/ml) on ice and mix well.
4.4.1.2 Switch on spectrophotometer for complex I programme (340 nm auto rate assay over 5 min with 30 sec intervals).
4.4.1.3 Connect water bath to spectrophotometer and set temperature at 30 °C.
4.4.1.4 Prepare a reaction master mix (on ice) as follows:

Table 2. Complex I Master mix

Reagent	1x (µl)	10x (µl)	20x (µl)	[end]
KPi buffer (0.5 M)	50	500	1000	50 mM
NADH (2 mM)	12.5	125	250	50 µM
KCN (50 mM)	10	100	200	1 mM
Antimycin A (0.5 mM)	10	100	200	10 µM
BSA (10%)	5	50	100	0.1 %
Total	87.5	875	1750	

4.4.1.5 Add, just before assay, into two separate cuvettes for each sample:
- Ethanol (A1) **or** rotenone (A2)* 5 µl

*Each sample has to be done with and without rotenone

4.4.1.6 Add into cuvettes 380 µl water.
4.4.1.7 Add 25 µl mitochondrial sample into each, place cuvettes into sample holder and allow to pre-heat for 2 min. **Select "Auto zero".**

4.4.1.8 Add to cuvette a pre-heated mixture (90 µl) of:

	1 x	11X
• Complex I master mix	87.5 µl	962.5 µl
• CoQ1 (10 mM)	2.5 µl	27.5 µl
Total	90 µl	990 µl

4.4.1.9 Mix well, initiate reading and record linear rate decrease at A_{340} for 3 min. (reactions has to start at an absorbance of ~ 0.4)

4.4.2 Calculation

µmol/min/mg = ((A1-A2)/6.22mM^{-1}) x 0.5/(mg/ml protein x 25µl/1000)

nmol/min/UCS = 1000 x µmol/min/mg/UCS

4.5 NADH-cytochrome c reductase (complex I+III)

4.5.1 Procedure

Note 1: Activity of complex I+III in each sample is measured in presence and absence of rotenone (rotenone-sensitive complex I+III activity).
Note 2: Before performing assay preheat water and master mix by incubating tubes containing these reagents in water bath for at least 5 min.

4.5.1.1 Thaw mitochondrial sample (1mg/ml) on ice and mix well.
4.5.1.2 Set on spectrophotometer for complex I+III programme (550 nm auto rate assay over 5 min with 30 sec intervals).
4.5.1.3 Connect water bath to spectrophotometer and set temperature at 30 °C.
4.5.1.4 Prepare a reaction master mix (on ice) as follows:

Table 3. Complex I+III Master mix.

Reagent	1x (µl)	10x (µl)	20x (µl)	[end]
KPi buffer (0.5 M)	50	500	1000	50 mM
NADH (2 mM)	12.5	125	250	50 µM
KCN (50 mM)	10	100	200	1 mM
BSA (10%)	5	50	100	0.1%
Total	77.5	775	1550	

4.5.1.5 Add, just before assay, into two separate cuvettes for each sample:

- Ethanol (A1) **or** rotenone (A2)* 5 µl

*Each sample has to be done with and without rotenone

4.5.1.6 Add 400 µl water.
4.5.1.7 Add 5 µl mitochondrial sample into each, place cuvettes into sample holder and allow to pre-heat for 2 min. **Select "Auto zero".**

4.5.1.8 Add to cuvette a pre-heated mixture (90 µl) of:

	1 x	11X
• Complex I+III master mix	77.5 µl	852.5 µl
• CytC (2 mM)	12.5 µl	137.5 µl
Total	90 µl	990 µl

4.5.1.9 Mix well, initiate reading and record linear rate increase at A_{550} for 3 min.

4.5.2 Calculation

µmol/min/mg = ((A1-A2)/29.5 mM^{-1}) x 0.5/(mg/ml protein x 5µl/1000)

nmol/min/UCS = 1000 X µmol/min/mg/UCS

4.6 Succinate-cytochrome c reductase (complex II+III)

4.6.1 Procedure

Note 1: Activity of complex II+III in each sample is measured in presence and absence of CytC.
Note 2: Before performing assay preheat water and master mix by incubating tubes containing these reagents in water bath for at least 5 min.

4.6.1.1 Thaw mitochondrial sample (1mg/ml) on ice and mix well.
4.6.1.2 Set on spectrophotometer for complex II+III programme (550 nm auto rate assay over 5 min with 30 sec intervals).
4.6.1.3 Connect water bath to spectrophotometer and set temperature at 30 °C.
4.6.1.4 Prepare a reaction master mix (on ice) as follows:

Table 4. Complex II+III Master mix.

Reagent	1x (µl)	10x (µl)	20x (µl)	[end]
KPi buffer (0.5 M)	50	500	1000	50 mM
Succinate (1 M)	5	50	100	10 mM
KCN (50 mM)	10	100	200	1 mM
EDTA (10%)	4	40	80	0.08%
BSA (10%)	5	50	100	0.1%
Rotenone 0.25 mM	5	50	100	2.5 µM
Total	79	790	1580	

4.6.1.5 Place for each sample into two disposable cuvettes:
- 398.5 µl water
- 79 µl CII+III master mix

4.6.1.6 Place cuvettes into sample holder and allow to pre-heat for 2 min.
4.6.1.7 Add 5 µl of mitochondrial sample into each of the two cuvettes and pre-incubate for another 5 min. to allow for activation of CII.
4.6.1.8 Add 5 µl ATP (0.2 M).
4.6.1.9 To start reactions add 12.5 µl CytC, 2mM (A1) and in control reaction 12.5 µl H_2O* (A2). **Select "Auto zero".**
*Each sample has to be done with and without CytC (this value however is always 0)
4.6.1.10 Mix well, initiate reading and record linear rate increase at A_{550} for 3 min.

4.6.2 Calculation

µmol/min/mg = ((A1-A2)/29.5 mM^{-1}) x 0.5/(mg/ml protein x 5µl/1000)

nmol/min/UCS = 1000 X µmol/min/mg/UCS

4.7 Cytochrome c oxidase (compex IV)

4.7.1 Procedure

Note 1: Check A550/565 ratio of CytCred as described in Section 4.1. It must be >8 (if not > 8 it cannot be used). Also re-calculate [CytCred] before assay to establish correct volume needed in reaction.
Note 2: Before performing assay preheat water and master mix by incubating tubes containing these reagents in water bath for at least 5 min.

4.7.1.1 Thaw mitochondrial sample (1mg/ml) on ice and mix well.

4.7.1.2 Set on Uvikon spectrophotometer for complex IV programme (550 nm auto rate assay over 5 min with 30 sec intervals).

4.7.1.3 Connect water bath to spectrophotometer and set temperature at 30 °C.

4.7.1.4 Place for each sample into one disposable cuvette a pre-heated mixture (478 µl) of:

	1 x	11 x
• KPi buffer (0.5 M)	90 µl	990 µl
• Water	378 µl	4158 µl
• n-dodecylmatoside (125 mM)	10 µl	110 µl
	478 µl	5258 µl

4.7.1.5 Place cuvettes into sample holder and allow to pre-heat for 5 min. **Select "Auto zero".**

4.7.1.6 Calculate volume of CytCred from stock solution (originally 500 µM) that is required to give an [end] of 20 µM.

e.g. 20 µl from 500 µM results in 20 µM [end]

4.7.1.7 Add 2 µl of mitochondrial sample (using a 10 µl pipette) into cuvette (A1). For one blank reaction use 2 µl water (A2).

4.7.1.8 Mix well, initiate reading and record linear rate decrease at A_{550} for 3 min.

4.7.2 Calculation

µmol/min/mg = ((A1-A2)/29.5 mM^{-1}) x 0.5/(mg/ml protein x 2µl/1000)

nmol/min/UCS = 1000 X µmol/min/mg/UCS

4.8 Piruvate dehydrogenase complex (PDHc)

Reference: Chretien, D et al. 1995. Clinica Chimica Acta. 240, 129-136.

4.8.1 Procedure

Note: Before performing assay preheat water and master mix by incubating tubes containing these reagents in water bath for at least 5 min.

4.8.1.1 Thaw mitochondrial sample (1mg/ml) on ice and mix well.
4.8.1.2 Set on spectrophotometer for PDHc programme (340 nm auto rate assay over 5 min with 30 sec intervals).
4.8.1.3 Connect water bath to spectrophotometer and set temperature at 30 °C.
4.8.1.4 Prepare a reaction master mix (on ice) as follows:

Table 5. PDHc Master mix.

Reagent	1x (µl)	10x (µl)	20x (µl)	[end]
PDHc buffer (2x)	250	2500	5000	1x
NAD (25 mM)	5	50	100	0.5 mM
CoA (20 mM)	10	100	200	0.2 mM
Oxamic acid (1.25M)	10	100	200	25 mM
Cystein (100 mM)	5	50	100	1 mM
TPP (20 mM)	5	50	100	0.2 mM
Water*	200	2000	4000	
Total	485	4850	9700	

*Water volume should be recalculated if mitochondrial sample volume is changed

4.8.1.5 Place 485 µl of PDHc master mix into a disposable cuvette.
4.8.1.6 Add 10 µl mitochondrial sample and place cuvettes into sample holder.
4.8.1.7 Allow to pre-heat for 2 min.
4.8.1.8 Add 5 µl piruvate (40 mM) to all samples (A1). For blank reaction use 5 µl water instead of piruvate (A2). **Select "Auto zero".**
4.8.1.9 Mix well, initiate reading and record linear rate increase at A_{340} for 3 min.

4.8.2 Calculation

µmol/min/mg = ((A1-A2)/6.22 mM^{-1}) x 0.5/(mg/ml protein x 10µl/1000)

nmol/min/UCS = 1000 X µmol/min/mg/UCS

Appendix D: Unprocessed Data

Table D.1: The unprocessed data used in Figure 4.3

This unprocessed data was used for a graphic presentation (section 4.3.1) of the effect of protein content on the specific activity of citrate synthase.

Volume (µl))	[prot] (mg/ml)	Citrate synthase (n=2) A1	A2	CS	UCS	AVG	SDEV
0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00
	1.00	0.00	0.00	0.00	0.00		
	1.00	0.00	0.00	0.00	0.00		
1.00	1.00	0.00	0.15	5551.47	5.55	5.13	0.55
	1.00	0.00	0.12	4511.03	4.51		
	1.00	0.00	0.15	5334.56	5.33		
2.50	1.00	0.00	0.12	1717.65	1.72	2.00	0.44
	1.00	0.00	0.17	2513.24	2.51		
	1.00	0.00	0.12	1773.53	1.77		
5.00	1.00	0.00	0.32	2337.50	2.34	2.04	0.27
	1.00	0.01	0.25	1819.85	1.82		
	1.00	0.00	0.27	1964.71	1.96		
10.00	1.00	0.01	0.38	1385.29	1.39	1.40	0.01
	1.00	0.01	0.39	1406.62	1.41		
	1.00	0.01	0.39	1395.96	1.40		

Table D.2 : The unprocessed data used in Figure 4.5

This unprocessed data was used for a graphic presentation (section 4.3.2)of the effect of protein content on the specific activity of complex I.

Volume (µl))	[prot] (mg/ml)	(Complex I, rotenone sensitive) (n = 2) A1 (-Rot)	A2 (+Rot)	umole/min/mg	AVG	SDEV
0	1.00	0	0	0.00	0.000	0.00
0	1.00	0	0	0.00		
5	1.00	0.007	0.0027	0.07	0.084	0.02
5	1.00	0.008	0.0021	0.10		
15	0.40	0.013	0.0044	0.12	0.077	0.05
15	1.00	0.0116	0.0045	0.04		
25	1.00	0.0756	0.0097	0.21	0.190	0.03
25	1.00	0.0605	0.0081	0.17		
35	1.00	0.0201	0.0108	0.02	0.021	0.00
35	1.00	0.0191	0.0105	0.02		

Table D.3: The unprocessed data used in Figure 4.7

This unprocessed data was used for a graphic presentation (section 4.3.3) of the effect of protein content on the specific activity of complex I+III.

Volume	[prot]	(Complex I and III, rotenone sensitive)				
(µl))	(mg/ml)	A1	A2 (rot)	umole/min/mg	AVG	SDEV
0.0	1.00	0	0	0.00	0.000	0.00
	1.00	0	0	0.00		
1.0	1.00	0.0557	0.006	4.00	4.188	0.27
	1.00	0.0607	0.0062	4.38		
2.5	1.00	0.0548	0.0065	1.55	1.646	0.13
	1.00	0.0604	0.0063	1.74		
5.0	1.00	0.0677	0.0077	0.96	0.960	0.01
	1.00	0.0667	0.0073	0.95		
10.0	1.00	0.0798	0.011	0.55	0.399	0.22
	1.00	0.044	0.0136	0.24		

Table D.4: The unprocessed data used in Figure 4.9

This unprocessed data was used for a graphic presentation (section 4.3.4) of the effect of protein content on the specific activity of complex II+III.

Volume	[prot]	(Complex II+III, malonate sensitive)				
(µl))	(mg/ml)	A1	A2	umole/min/mg	AVG	SDEV
0.0	1.00	0	0	0.00	0.000	0.000
	1.00	0	0	0.00		
	1.00	0	0	0.00		
1.0	1.00	0.065	0	5.20	5.92	2.099
	1.00	0.103	0	8.28		
	1.00	0.0531	0	4.27		
2.5	1.00	0.0951	0	3.06	2.814	0.451
	1.00	0.0961	0	3.09		
	1.00	0.0713	0	2.29		
5.0	1.00	0.1333	0	2.14	0.969	1.016
	1.00	0.1123	0	0.381		
	1.00	0.1134	0	0.384		
10.0	1.00	0.1832	0	0.311	0.317	0.005
	1.00	0.1887	0	0.320		
	1.00	0.1886	0	0.320		

Table D.5: The unprocessed data used in Figure 4.11

This unprocessed data was used for a graphic presentation (section 4.3.5) of the effect of protein content on the specific activity of complex IV.

Volume (µl))	[prot] (mg/ml)	(Complex IV) (n = 2) A1	umole/min/mg	AVG	SDEV
0.0	1.00	0	0.00	0.00	0.00
	1.00	0	0.00		
	1.00	0	0.00		
0.5	1.00	0.098	3.325	2.61	0.73
	1.00	0.0548	1.858		
	1.00	0.078	2.647		
1.0	1.00	0.094	1.600	1.50	0.13
	1.00	0.079	1.346		
	1.00	0.091	1.544		
2.0	1.00	0.067	0.567	0.54	0.03
	1.00	0.061	0.515		
	1.00	0.064	0.544		
5.0	1.00	0.087	0.295	0.30	0.01
	1.00	0.094	0.317		
	1.00	0.087	0.296		

Table D.6: The unprocessed data used in Figure 4.13

This unprocessed data was used for a graphic presentation (section 4.3.6) of the effect of protein content on the specific activity of PDHc.

Volume (µl))	[prot] (mg/ml)	PDHc A1 (-Pirovaat)	A2 (+Pirovaat)	nmole/min/mg	AVG	SDEV
0.00	1.00	0.0000	0	0.00	0.000	0.00
	1.00	0.0000	0	0.00		
	1.00	0.0000	0	0.00		
5.00	1.00	0.0000	0.0009	14.5	14.469	1.61
	1.00	0.0000	0.0008	12.9		
	1.00	0.0000	0.001	16.1		
10.00	1.00	0.0000	0.0025	20.1	18.221	2.02
	1.00	0.0000	0.002	16.1		
	1.00	0.0000	0.0023	18.5		
20.00	1.00	0.0002	0.0055	21.3	21.034	0.23
	1.00	0.0002	0.0054	20.9		
	1.00	0.0002	0.0054	20.9		
40.0	1.00	0.0007	0.0113	21.3	21.436	0.613957
	1.00	0.0007	0.0111	20.9		
	1.00	0.0007	0.0117	22.1		
80.0	1.00	0.0017	0.0208	19.2	20.029	0.805949
	1.00	0.0017	0.0224	20.8		
	1.00	0.0017	0.0217	20.1		

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