ABSTRACT

The mitochondrial myopathies are associated with an expanding list of mitochondrial DNA (mtDNA) abnormalities including point mutations, single and multiple deletions, tandem duplications, and generalised depletion of the genome. The existence of further mtDNA defects seems likely, but their detection relies either on the fortuitous loss or gain of an endonuclease restriction site, the loss of sufficient base pairs to cause a detectable size shift, or the sequencing of the genome. The correlation between these defects and the biochemical or clinical phenotype is obscure. Investigation of the mitochondrial translation products, either in vitro or in culture, in patients with these diseases, aimed to address both these issues.

An in vitro micro-technique for radiolabelling mitochondrially synthesised proteins was established. In 2 out of 5 cases with mtDNA deletions there was evidence for complementation for missing tRNAs. The possibility of complementation in the remaining cases was not excluded. No novel proteins correlating with translation of fusion genes were detected. Two cases with point mutations of tRNA^leu(UUR) had mitochondrial protein synthesis rates and polypeptide profiles comparable with that seen in controls, refuting the suggestion that such mutations might alter the ratio of ribosomal to messenger RNA transcription. Of five cases with no detectable mtDNA mutation, one had a abnormality of mtDNA expression correlating with the biochemical defect and consistent with a defect in either a ribosomal or transfer RNA gene.
Morphological, biochemical and genetic criteria for characterising the cultures were assessed. Myotubule cultures established from patients with mitochondrial myopathy associated with a heteroplasmic mtDNA deletion were found to lose the mutant mtDNA genomes within a few cell passages. Clonal myotube cultures showed differing proportions of wild type to mutant type mtDNA genomes in the different clones, which was maintained through a large number of cell divisions. The profile of mitochondrial translation products in the uncloned cultures did not differ from that obtained in controls.
ACKNOWLEDGEMENTS

I was fortunate to be in receipt of a Medical Research Council Training Fellowship for the duration of this project and I am most grateful for their generous support. Funding for the setting up and running of the tissue culture laboratory came from the Brain Trust.

I am grateful for the opportunity to work in this exciting and expanding field given to me by my supervisor, Dr Morgan-Hughes, and to Prof Marsden in whose Department Of Clinical Neurology I was working.

This work would not have been possible without the training and access allowed to me by several other departments. My appreciation extends not only to the heads of those departments but also to the individuals within who, even if not directly involved in my project were helpful and encouraging. Prof Walshe allowed access to his tissue culture facilities at the start of this project, and the use of his laboratories' batch tested fetal calf serum. Prof Clarke similarly allowed access to his department's biochemistry facilities, initially at St Bartholomew's Hospital Medical School, and latterly at the Institute of Neurology. Prof Harding's Department of Neurogenetics collaborated in the molecular biology studies required in this project. The electron-micrograph studies were kindly performed by Prof Landon's department. Dr E.J.Thompson and his Department of Special Chemical Pathology gave valuable technical advice and computer expertise on many occasions.
My personal thanks goes to several individuals. Dr Steven Moore patiently taught me the techniques of muscle tissue culture. Dr Ian Holt and Ms Mary Sweeney performed the mitochondrial DNA analysis. Personal encouragement and advice were given by many people but particularly Profs Clarke, Harding, and Schapira and Drs Ian Holt and Mary Davies.

A special thanks is due to my principle collaborator, Dr Mark Cooper, not least for the mitochondrial preparations essential for the in vitro translation studies, but for his helpful and continuing advice and support.

My appreciation is due to Marjory Ellison for her histocytochemistry and photographic skills. The lions share of the photography fell to George Kaim and Jackie Powell of the audiovisual department with some assistance from Camelia Kurutz.

Ms June Smalley gave generously of her secretarial and computing expertise.

Prof Attardi and Dr Chomyn generously donated a sample of their well characterised labelled HeLa mitochondria enabling direct comparision with my studies.
# TABLE OF CONTENTS

ABSTRACT ................................................................. i

ACKNOWLEDGEMENTS ............................................... iii

TABLE OF CONTENTS .................................................. v

LIST OF FIGURES ..................................................... xv

LIST OF TABLES ....................................................... xix

## PART 1: INTRODUCTION

CHAPTER 1: MITOCHONDRIA .......................................... 1

1. Morphology and Organization ................................. 1

2. Mitochondrial Metabolism ........................................ 4

3. The Respiratory Chain Complexes ............................. 7

  3.a. Complex I ................................................. 9

  3.b. Complex II ............................................. 11

  3.c. Ubiquinone ............................................. 11

  3.d. Complex III ............................................ 11

  3.e. Cytochrome c .......................................... 12

  3.f. Complex IV ............................................ 12

  3.g. ATP Synthetase Complex ............................... 13

4. The Chemiosmotic Theory ........................................ 13

5. The Genetic Origin Of Oxidative Phosphorylation System 14
6. Mitochondrial Biogenesis .................................................. 15
7. The Import Of Proteins Into Mitochondria ......................... 17

CHAPTER 2: MITOCHONDRIAL DNA .................................... 22
1. Structure And Replication .............................................. 22
2. Mitochondrial DNA Genes .............................................. 24
3. Control Of Transcription ................................................. 28
4. Control Of Translation .................................................. 31
5. The Role Of The Nuclear Genome .................................... 33
6. Physiological Control Of Mitochondrial DNA Expression .... 35
7. Maternal Inheritance Of Mitochondrial DNA ..................... 37
8. Mitochondrial DNA Mutation Rate .................................. 38

CHAPTER 3: THE MITOCHONDRIAL MYOPATHIES ............... 40
1. A Historical Review ..................................................... 40
2. Clinical Features Of The Mitochondrial Myopathies ........... 44
   2.a. Myopathy ............................................................ 46
   2.b. Chronic Progressive External Ophthalmoplegia / Kearns
       Sayre Syndrome .................................................. 46
   2.c. Major CNS Involvement ......................................... 48
       2.c.i. MERRF ...................................................... 48
       2.c.ii. MELAS .................................................... 49
       2.c.iii. Leigh syndrome .......................................... 49
       2.c.iv. Alpers' syndrome ....................................... 50
2.c.v. May White syndrome .................................................. 50
2.c.vi. NARP ................................................................. 50
2.c.vii. MNGIE ............................................................... 51
2.c.viii. Infantile lactic acidaemias ........................................ 51
2.c.ix. Multiple symmetrical lipomatoses ............................ 51

2.d. Other Syndromes .......................................................... 52

2.d.i. Cardiomyopathy .......................................................... 52
2.d.ii. Pearson's syndrome .................................................... 52
2.d.iii. Leber's Hereditary Optic neuropathy ....................... 52

3.a. Initial Investigations ...................................................... 53
3.b. Neurophysiology .......................................................... 53
3.c. Imaging Studies ............................................................ 54
3.d. Functional Studies ......................................................... 54
3.e. Histocytochemistry ......................................................... 55
3.f. Electron-microscopy ....................................................... 56
3.g. Biochemistry ............................................................... 56
3.h. Molecular Biology .......................................................... 58

4. The Aetiology Of The Mitochondrial Myopathies ............... 59
5. Defects Of The Nuclear Genome ...................................... 60
6. Bigenomic Defects .......................................................... 61
7. Defects Of The Mitochondrial Genome ............................. 62

7.a. Single deletions of mtDNA ............................................. 62
7.b. Multiple deletions of mtDNA .......................................... 66
7.c. Duplications of mtDNA .......................................................... 67
7.d. Depletion of mtDNA ............................................................ 67
7.e. Point mutations of mtDNA protein coding genes ............... 68
   7.e.i. Associated with LHON ............................................... 68
   7.e.ii. Associated with NARP or Leigh Syndrome ............... 69
7.f. Point mutations of mt tRNA genes ...................................... 70
   7.f.i. tRNA^leu<sup>UUR</sup> .................................................. 70
   7.f.ii. tRNA^leu<sup>UUR</sup> .................................................. 70
   7.f.iii. tRNA<sup>ile</sup> .......................................................... 71
8. General Properties Of Mitochondrial DNA Defects .................... 72
   8.a. Heteroplasm................................................................. 72
   8.b. Inheritance Of Defects .................................................. 73
   8.c. Association With Ragged Red Fibres .............................. 74
9. Defects Of Mitochondrial Protein Import ..................................... 74
10. Acquired Defects Of Mitochondria .......................................... 75
   10.a. Parkinson's Disease .................................................... 75
   10.b. Zidovudine Induced Myopathy ..................................... 76
   10.c. Autoimmunity ............................................................. 76
   10.d. Viral ....................................................................... 78
11. The Expression Of Mitochondrial DNA Defects .......................... 78
   11.a. Relationship between mtDNA defects and clinical features . 78
   11.b. Relationship between mtDNA defects and the biochemical abnormalities. ............................................. 81
11.c. The role of "complementation" ................................. 83
11.d. Expression of mtDNA deletions ............................... 85
11.e. The presence and effect of "fusion" proteins ............... 86
11.f. Mechanisms of disease expression applicable to point
mutations of tRNAs ............................................................ 87
11.g. Intragenic suppressor gene mutations .......................... 88

CHAPTER 4: IN VITRO MITOCHONDRIAL PROTEIN SYNTHESIS ...... 89
1. Introduction ................................................................. 89
2. Mitochondrial Preparations ............................................. 90
3. Energy Systems ............................................................ 92
4. Other Requirements ...................................................... 93
5. Bacterial Contamination ................................................... 95
6. Effects Of Inhibitors ....................................................... 95
7. Nuclear Influences .......................................................... 96
8. The Identity Of Synthesised Proteins ................................. 97
9. Species, Tissue, And Age Specific Differences .................. 100
10. Human Mitochondrial Protein Synthesis ........................... 101

CHAPTER 5: CELL CULTURES IN MITOCHONDRIAL DISEASES ........ 104
1. Muscle Cell Cultures ..................................................... 104
2. Human Muscle Disease In Culture ................................. 106
3. Respiratory Chain Deficiencies In Culture ....................... 107
   3.a. Animal Cell Lines ................................................... 107
PART 3: RESULTS

CHAPTER 7: PROTEIN SYNTHESIS IN ISOLATED RAT MUSCLE MITOCHONDRIA ................................................................. 138
1. Rate Of Incorporation ................................................................. 138
2. Sensitivity Of Incorporation To Inhibitors ............................... 141
3. The Effects Of Magnesium ......................................................... 143
4. Assessment Of Different Energy Systems ............................... 144
5. Variations To The Glutamate Energy System ......................... 149

CHAPTER 8: PROTEIN SYNTHESIS IN ISOLATED HUMAN MUSCLE MITOCHONDRIA ................................................................. 151
1. Patients ...................................................................................... 151
   1.a. Control patients ............................................................... 151
   1.b. Mitochondrial Myopathy Patients ........................................ 156
2. Mitochondrial Protein Synthesis: General Quantitative Aspects .... 161
   2.a. Controls ........................................................................ 161
   2.b. Mitochondrial myopathy patients ........................................ 162
   2.c. Relationship between mitochondrial protein synthesis and age ................................................................. 163
   2.d. Relationship between respiratory chain activity and mitochondrial protein synthesis ........................................ 164
3. Assignment Of Polypeptide Bands ............................................. 165
4. Mitochondrial Protein Synthesis In The Mitochondrial Myopathies 168
4.a. Group I (Point mutation) ............................................................. 168
  4.a.i. Patient VA ............................................................ 168
  4.a.ii. Patient DL .................................................................. 169

4.b. Group II (Deletions) ..................................................................... 169
  4.b.i. Patient CS ....................................................................... 173
  4.b.ii. Patient PS ....................................................................... 174
  4.b.iii. Patient MW .................................................................. 175
  4.b.iv. Patient MHO ............................................................... 176

4.c. Group III (No known mtDNA defect) ....................................... 178
  4.c.i. Patient DP ....................................................................... 178
  4.c.ii. Patient JL ....................................................................... 179
  4.c.iii. Other Group III Patients .......................................... 180

CHAPTER 9: MUSCLE CELL CULTURE STUDIES .......................................... 182

1. Patients Studied ............................................................................................. 182

2. The Expression Of Disease In Primary Muscle Cell Culture. ............ 183
   2.a. Growth Characteristics .................................................................. 183
   2.b. Morphological Characteristics .................................................... 183
   2.c. Biochemical Characteristics. ............................................................ 186

2.d. Mitochondrial DNA Analysis

3. Mitochondrial Protein Synthesis In Culture .......................................... 188
LIST OF FIGURES

Figure 1.1 Mitochondrial metabolism ................................................................. 5
Figure 1.2 The Respiratory Chain ................................................................. 8
Figure 1.3 Mitochondrial protein import ....................................................... 19
Figure 2.1 Replication of mtDNA ............................................................... 23
Figure 2.2 The mitochondrial genome ......................................................... 25
Figure 3.1 Substrates feeding into the respiratory chain ............................... 57
Figure 3.2 Linearised map of the mitochondrial genome indicating the extent
of the deletions in 30 cases. ................................................................. 63
Figure 3.3 Showing 10bp repeats flanking a mtDNA deletion ..................... 64
Figure 3.4 The slip replication model ......................................................... 65
Figure 3.5 Duplication of mitochondrial DNA ............................................. 67
Figure 3.6 Point mutation in the tRNA$^{ Tyr }$ ............................................. 70
Figure 3.7 Point mutation in the tRNA$^{ Leu(UUR) }$ ..................................... 71
Figure 3.8 Point mutation in the tRNA$^{ lLe }$ ................................................. 72
Figure 3.9 Mitochondria heteroplasmic for a mtDNA deletion ................. 84
Figure 3.10 Mitochondria homoplasmic for a mtDNA deletion ................. 84
Figure 7.1 The rate of $^{35}$S methionine incorporation into mitochondrial
proteins using isolated rat muscle mitochondria. .................................... 138
Figure 7.2 Autoradiograph showing the profile of mitochondrial proteins
synthesised after varying periods of incubation. .................................... 139
Figure 7.3 The effect of delay between the end of mitochondrial extraction and
the commencement of in vitro incubation on mitochondrial protein
synthesis. ............................................................................................................. 140

Figure 7.4 The effect of cycloheximide, chloramphenicol, and erythromycin,
compared with no inhibitor, on the level of $^{35}$S methionine
incorporation. ...................................................................................................... 141

Figure 7.5 Autoradiograph of the in vitro translation products of rat muscle
mitochondria without inhibitor and in presence of cycloheximide, and
chloramphenicol. ................................................................................................. 142

Figure 7.6 The effect of Mg$^{2+}$ concentration on $^{35}$S methionine incorporation
by isolated rat muscle mitochondria. ................................................................. 143

Figure 7.7 Autoradiograph of translation products of rat muscle mitochondria
illustrating the effects of Mg$^{2+}$ concentration. ................................................. 144

Figure 7.8 The rate of $^{35}$S methionine incorporation with and without added
ATP. .................................................................................................................... 145

Figure 7.9 Mitochondrially synthesised proteins seen when ATP is the sole
added energy source, as compared with when an added energy system
is present. ............................................................................................................ 145

Figure 7.10 Showing the efficacy of the three different energy systems used,
on the rate of in vitro mitochondrial protein synthesis and the effect of
the addition of rotenone (5μM) in each case. ................................................... 146

Figure 7.11 Autoradiograph of the mitochondrial translation products obtained
with the different energy systems and the effect of complex I inhibition
by 5μM rotenone. ............................................................................................... 147

Figure 7.12 The dose response curve obtained with increasing concentrations
of rotenone using the glutamate based energy system. ................................. 148
Figure 7.13 The translation products obtained with increasing inhibition of the glutamate based energy system by rotenone. .................................................... 149

Figure 7.14 The effect of variations to the glutamate based energy system on the rate of mitochondrial protein synthesis in isolated rat muscle mitochondria. ...................................................................................................... 150

Figure 7.15 Autoradiograph illustrating the translation products obtained with variations to the glutamate based energy system. ..................................... 150

Figure 8.1 Correlation of mitochondrial protein synthesis with age in the mitochondrial myopathies ................................................................. 164

Figure 8.2 The relationship between mitochondrial protein synthesis and glutamate oxidation rates. ................................................................................... 165

Figure 8.3 Autoradiograph comparing the mitochondrial translation products obtained in two in vitro studies with those obtained in HeLa cultures by the method of Chomyn et al 1983. ................................................................. 167

Figure 8.4 Autoradiograph of the labelled mitochondrial polypeptides in VA with a tRNA^{leu(3243)} mutation compared with that obtained from four controls. ........................................................................................................ 169

Figure 8.5 Linearised representation of the mitochondrial genome showing the extent of the deletion in the patients studied. ............................................. 170

Figure 8.6 Mitochondrial protein synthesis in cases with a mtDNA deletion. ................................................................. 172

Figure 8.7 Mitochondrial translation products for patient CS (Group II) with control JG. ........................................................................................................ 174

Figure 8.8 Autoradiograph of patient PS (Group II), and control AH. ........... 175

Figure 8.9 Mitochondrial translation products in patients DL (Group I), MW
(Group II) and MHE (Group III). .................................................. 176

Figure 8.10 Mitochondrial translation products of patient MHO (Group II)
compared with control JS. ............................................................. 177

Figure 8.11 Autoradiograph of mitochondrial translation products in patients SB
and DP (Group III) compared with those in controls AH, and HC. ... 178

Figure 8.12 Mitochondrial translation products in patient JL (Group III)
compared with control LB. ............................................................. 180

Figure 8.13 Mitochondrial translation products in patient PW (Group III)
compared with control MD. ............................................................. 181

Figure 9.1 Electronmicrograph showing examples of the abnormal
mitochondria seen in culture. .......................................................... 184

Figure 9.2 Detail of a single mitochondrion, obtained from cultured muscle. ... 184

Figure 9.3 Electronmicrograph of an abnormal mitochondrion published by
Askanas et al 1978. ................................................................. 185

Figure 9.4 Southern blot of mtDNA present in the muscle biopsy (M) and
muscle cell cultures of three patients with mitochondrial myopathy. ... 186

Figure 9.5 Southern blot of the mtDNA present in the muscle biopsy, fibroblast
cultures and muscle cultures, from patient MW. ............................. 187

Figure 9.6 Southern blot of the mtDNA present in eleven cloned muscle cell
culture lines from patient CS. ......................................................... 188

Figure 9.7 Protein synthesis in muscle cell cultures incubated with increasing
concentration of emetine .............................................................. 189

Figure 9.8 Mitochondrial protein synthesis in muscle cell cultures. ................ 190

Figure 9.9 Mitochondrial protein synthesis in muscle cell cultures. ................ 191

xviii
LIST OF TABLES

Table 1.1 Proteins of the inner membrane ............................................................... 3
Table 1.2 The respiratory chain complexes ............................................................ 10
Table 1.3 Stoichiometry of the respiratory chain .................................................. 13
Table 2.1 The mitochondrial genetic code .............................................................. 26
Table 2.2 Proteins encoded by mitochondrial DNA ............................................... 27
Table 2.3 Relative rates of mitochondrial polypeptide synthesis .......................... 32
Table 3.1 Clinical presentations of the mitochondrial myopathies ...................... 45
Table 3.2 Classification of polarographic results .................................................. 58
Table 3.3 Possible aetiologies for the mitochondrial myopathies ....................... 59
Table 3.4 Mitochondrial DNA defects ................................................................. 62
Table 3.5 Point mutations of mtDNA .................................................................... 69
Table 6.1 EDTA isolation medium ............................................................................115
Table 6.2 Dissociation solution for muscle .............................................................118
Table 6.3 Growth medium for muscle cultures ......................................................119
Table 6.4 Trypsin solution ......................................................................................120
Table 6.5 Differentiation medium for muscle cultures ..........................................121
Table 6.6 Stock buffer and salt solution .................................................................123
Table 6.7 Energy systems used ..............................................................................123
Table 6.8 Inhibitors used .....................................................................................124
Table 6.9 Composition of incubation mixture per single incubation (fV 100µl) . 124
Table 6.10 Histochemical stains used .................................................................126
Table 6.11 Substrates and inhibitors used for polarography ...............................127
Table 6.12 Classification of polarographic defects .................................................. 128
Table 6.13 Measurement of enzyme activities ....................................................... 129
Table 6.14 Sample buffer .......................................................................................... 130
Table 6.15 Composition of gels ............................................................................... 131
Table 6.16 Stock solutions for electrophoresis .......................................................... 132
Table 6.17 Electrode buffer ..................................................................................... 132
Table 6.18 TAE buffer............................................................................................... 135
Table 6.19 SSC buffer ............................................................................................... 136
Table 8.1 Clinical features of the control patients ................................................. 152
Table 8.2 Polarographic results in the control patients ......................................... 153
Table 8.3 Enzyme activities in the control patients .............................................. 154
Table 8.4 Cytochrome levels in the control patients ............................................... 155
Table 8.5 Clinical features of the mitochondrial myopathy patients ....................... 157
Table 8.6 Polarographic findings in the mitochondrial myopathy patients .... 158
Table 8.7 Enzyme activities in the mitochondrial myopathy patients ....................159
Table 8.8 Cytochrome levels in the mitochondrial myopathy patients ................. 160
Table 8.9 Mitochondrial protein synthesis in human controls ......................... 161
Table 8.10 Mitochondrial protein synthesis in mitochondrial myopathies .......... 163
Table 8.11 Molecular weights of protein bands ..................................................... 166
Table 8.12 Details of mtDNA deletions studied ....................................................... 171
Table 9.1 Mitochondrial myopathy cases studied in culture classified according to mtDNA findings. ................................................................. 182
Table 9.2 Details of additional controls used for muscle cultures .................... 183
CHAPTER 1: MITOCHONDRIA

1. Morphology and Organization

Eukaryotic cells are distinguished not only by the presence of a nucleus but also by the possession of mitochondria. Although the mitochondria fulfil many functions the major one is the process of oxidative phosphorylation which generates ATP from ADP. This considerably increased the evolutionary scope for these cells.

Mitochondria are traditionally represented as regular cylindrical organelles with a diameter of 0.2μm and up to 10μm in length making them large enough to be visualised by light microscopy. However time lapse microcinematography of living cells reveals a remarkable degree of plasticity of shape and mobility within the cytoplasm.

Their numbers can vary considerably in different cell types depending on their energy requirements. In human cells this variation ranges from zero in erythrocytes, which lose their mitochondria during maturation, to 1,000 in hepatocytes, where they comprise a substantial fraction of the cytoplasm. Even in a single cell type, such as vertebrate striated muscle, there can be an enormous variation in the mitochondrial content ranging from 40% of cell volume in cardiocytes of small mammals, to 2% of cell volume in type IIb skeletal muscle of larger mammals (Schaper et al 1985, Hoppeler et al 1981). Mitochondrial content is also responsive to developmental change, showing proliferation during myoblast fusion for example (Brunk 1981).
Environmental changes also induce variations in mitochondrial mass with increases being seen in striated muscle with endurance training (Saltin and Gollnick 1983, Holloscy and Coyle 1984) and electrical pacing (Eisenberg and Salmons 1981), which are reflected in an increase in oxidative phosphorylation capacity.

Mitochondria distribution and orientation within the cell also varies and they are generally located in sites of high ATP consumption. Thus they are seen packed between the adjacent myofibrils in cardiac muscle cells or helically wrapped around the flagellum of a spermatozoa.

The fundamental structure of all mitochondria is that of a pair of bilipid membranes which enclose and define two separate mitochondrial spaces: the intermembranous space and an inner matrix space. This creates four separate compartments which can be fractionated from isolated mitochondria by detachment of the outer membrane, using hypotonic buffer or digitonin, followed by centrifugation on a sucrose density gradient.

The outer membrane is generally smooth and featureless and has large aqueous channels formed by a specific protein, porin. This renders it freely permeable to all molecules, including peptides, of less than 10kD. It contains a functionally heterogeneous group of enzymes including monoamine oxidase, kynurenine hydroxylase, carnitine parmitoyl transferase 1, a fatty acyl CoA synthetase and a number of enzymes involved in phospholipid metabolism and fatty acid breakdown.

By contrast the inner membrane is usually highly convoluted forming a series
of invaginations, cristae, which greatly increase its surface area such that it can constitute five times the area of the outer membrane and up to a third of the total cellular membrane. The number and morphology of the cristae can vary in different cells reflecting their ATP demand, being threefold more numerous in cardiac than liver mitochondria. The inner membrane has a high proportion (10%) of a unique acidic phospholipid cardiolipin (diphosphatylglycerol) which makes it impermeable to all but small uncharged molecules such as \( \text{O}_2 \), \( \text{CO}_2 \), \( \text{NH}_3 \) and undissociated \( \text{H}_2\text{O} \). It contains three major groups of proteins:

<table>
<thead>
<tr>
<th>Table 1.1 Proteins of the inner membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Specific transport proteins</td>
</tr>
<tr>
<td>2) The electron transport chain</td>
</tr>
<tr>
<td>3) The ATP synthetase complex.</td>
</tr>
</tbody>
</table>

These proteins are embedded to varying degrees in the membrane, some projecting from either the matrix (M) side, cytosol (C) side, or both. Negative stained electronmicrographs show the presence of an array of regularly stacked particles on the M surface.

Due to the permeability of the outer membrane the intermembranous space has a composition similar to that of the cytosol. It contains several enzymes which use the ATP that passes out of the matrix to phosphorylate other nucleotides such as AMP.

The matrix space is granular in appearance and contains a highly concentrated mixture of numerous different enzymes. They include those required for the oxidation
of pyruvate and fatty acids and for the tricarboxylic acid (TCA) cycle. It also contains mitochondrial ribosomes, mitochondrial DNA and the enzymes required for the synthesis and expression of this genome. The enzyme superoxide dismutase responsible for rapid removal of free radicals derived from oxygen reduction is also present in the matrix.

2. Mitochondrial Metabolism

The mitochondria are involved in numerous metabolic processes with the TCA cycle and the β oxidation pathway being important sources of precursors for other metabolic processes and with the TCA cycle in particular, playing a central role in the interconversion of proteins, fats and sugars. However the predominant role of mitochondria is the replenishment of ATP stores via the process of oxidative phosphorylation. Oxidative phosphorylation is the result of the coupling of two distinct processes; the respiratory chain which oxidises a variety of substrates to generate protonic energy, and the ATP synthetase complex which uses this energy to phosphorylate ADP to ATP.

The respiratory chain acts as an electron transfer chain which takes electrons down a stepwise energy gradient using the energy released to vectorially translocate protons across the impermeable inner membrane into the intermembranous space. This generates an electrochemical proton gradient across the inner membrane (the proton motive force), which is harnessed to drive the phosphorylation of ADP by the ATP synthetase complex.
The source of electrons and protons for the respiratory chain is hydrogen, derived from the oxidized carriers, nicotinamide adenine dinucleotide (NADH) or flavin adenine dinucleotide (FADH₂). The regeneration of NADH and FADH₂ from the resulting NAD⁺ and FADH is achieved using acetyl CoA obtained by the oxidation of sugars and fats.

![Mitochondrial metabolism](image)

**Figure 1.1** Mitochondrial metabolism

The metabolism of glucose via the glycolytic pathway results in the formation
of pyruvate and can itself generate a net gain of two ATP molecules per glucose molecule by direct phosphorylation. Under aerobic conditions the pyruvate enters the mitochondria and is catalysed to acetyl CoA by the pyruvate dehydrogenase enzyme complex. In anaerobic situations, such as obtained in muscle undergoing strenuous activity, the pyruvate is instead converted to lactate by lactate dehydrogenase located in the cytosol.

Acetyl CoA is also the end result of the metabolism of fatty acids which occurs within the matrix of the mitochondria by the process of β oxidation. Long chain fatty acids are transported into the mitochondrion as fatty acyl CoA by an active transport system that uses carnitine as a carrier. Subsequent β oxidation involves the repetitive operation of a series of four enzymes each cycle of which sequentially cleaves off two carbon atoms as acetyl CoA. Eventually the fatty acyl CoA containing n carbon atoms is completely oxidised to n/2 acetyl CoA molecules. As well as producing acetyl CoA the β oxidation cycle directly reduces 1 molecule of FAD and NAD⁺ for each acetyl CoA produced.

The acetyl CoA derived from either glycolysis or β oxidation is finally coupled to the regeneration of NADH and FADH₂ using the tricarboxylic acid cycle (TCA cycle), also known as the citric acid or Kreb’s cycle. The acetyl CoA is condensed with the four carbon oxaloacetate to produce citric acid and a multiple enzyme sequence yields 2 CO₂, 3 NADH and 1 FADH₂ molecules.
3. The Respiratory Chain Complexes

For reviews see the monograph by Nicholls (Nicholls 1982), and reviews by Sherratt and Turnbull (Sherratt and Turnbull 1990, Sherratt 1991).

By the use of ionic detergents such as cholate or deoxycholate it is possible to fractionate four complexes from the mitochondrial respiratory chain leaving only ubiquinone and cytochrome c. The isolated complexes retain their individual electron translocating properties and when combined together in artificial lipid bilayers reconstitute the activity of the respiratory chain. They can therefore be considered as the smallest functioning subunits of the respiratory chain.

The electron transferring capability of the respiratory chain complexes is dependant on prosthetic groups which are electron carriers capable of undergoing oxidation or reduction by the removal of electrons or protons (hydrogen atoms). The different respiratory chain complexes utilise several such electron carriers including flavins, cytochromes and iron sulphur proteins. In most, the ability of iron to relay electrons by conversion from a ferrous ($\text{Fe}^{2+}$) to ferric ($\text{Fe}^{3+}$) state is utilised, but two copper atoms contribute to the electron transport capability of the cytochromes $\text{aa}_3$ in Complex IV.

The flavins include flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). The iron sulphur proteins have the iron atoms covalently bound to the protein by cysteine sulphurs, and to other iron atoms by sulphur bridges. There
Figure 1.2 The Respiratory Chain
are about 10 iron sulphur centres associated with mitochondria containing either four or two iron atoms. Despite the presence of multiple iron atoms each centre will only transfer a single electron: the need for so many iron sulphur centres in the respiratory chain is unknown. Iron containing porphyrins i.e. haems, form the prosthetic group of the cytochromes, and according to the precise structure of the porphyrin, these are designated cytochrome a, b and c. Mammalian mitochondria utilise cytochromes a, b, and c all of which may exist in more than one form and are therefore further subclassified. Cytochrome a can be measured as cytochromes a and a₃ but since these may not actually be separate entities they are often referred to as cytochrome aa₃. Cytochrome c can be resolved into cytochromes c and c₁.

3.a.Complex I

This is the largest of the respiratory chain complexes, comprising at least 26, perhaps as many as 30, polypeptide subunits which contain 1 covalently bound FMN and 8 to 9 FeS centres as prosthetic groups. It spans the inner membrane with its NADH site protruding from the M surface as one might logically expect given its interaction with matrix located enzyme systems. It accepts reducing equivalents (protons and electrons) from NADH and transfers electrons to ubiquinone (NADH ubiquinone reductase). This electron transfer is linked to the vectorial translocation of protons across the inner membrane.
## Table 1.2 The respiratory chain complexes

<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>Molecular weight (kD)</th>
<th>Polypeptides Total / mtDNA</th>
<th>Prosthetic Groups</th>
<th>Action</th>
<th>Proton Translocating</th>
<th>Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>NADH-ubiquinone reductase</td>
<td>750</td>
<td>c26</td>
<td>8-9 FeS centres</td>
<td>NADH oxidation</td>
<td>Yes</td>
<td>Rotenone</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 FMN</td>
<td>Ubiquinone reduction</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Succinate-ubiquinone reductase</td>
<td>200</td>
<td>4</td>
<td>3 FeS centres</td>
<td>Succinate oxidation</td>
<td>No</td>
<td>TIFA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 FAD</td>
<td>Ubiquinone reduction</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ubiquinol-cytochrome c reductase</td>
<td>300</td>
<td>11</td>
<td>FeS centre (Reiske)</td>
<td>Ubiquinol oxidation</td>
<td>Yes</td>
<td>Antimycin A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cyto. c&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Cytochrome c reduction</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cytos. b&lt;sub&gt;366&lt;/sub&gt;, b&lt;sub&gt;362&lt;/sub&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>Cytochrome c oxidase</td>
<td>160</td>
<td>13</td>
<td>Cytos. a, a&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Cytochrome c oxidation</td>
<td>Yes</td>
<td>Cyanide</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2-3 Cu atoms</td>
<td>Oxygen reduction</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.b.Complex II

This consists of four protein subunits with prosthetic groups including 1 covalently bound FAD, 3 FeS centres and a b type cytochrome different from that seen in Complex III. Two of the protein subunits are the succinate dehydrogenase components. Like Complex I its active site protrudes from the M side of the inner membrane. It transfers electrons from succinate to ubiquinone (succinate ubiquinone reductase). It is the only complex of the respiratory chain not to translocate protons.

3.c.Ubiquinone

This hydrogen carrier is a substituted benzoquinone with a polyisoprene side chain, of variable length, which ensures its solubility in the hydrophobic interior of the inner membrane. In mammalian mitochondria the sidearm has ten isoprene residues; so called Co-enzyme Q\textsubscript{10} or CoQ\textsubscript{10}. For in vitro assays the more water soluble single residue sidearm version is commonly used ie. CoQ\textsubscript{1}. As well as accepting electrons from NADH via Complex I, it can also accept them from FADH\textsubscript{2} via Complex II, and glycerol 3-phosphate dehydrogenase, and from the acyl CoA dehydrogenases via the electron transfer flavoprotein (ETF) and ETF dehydrogenases. These electrons are transferred to Complex III.

3.d.Complex III

Complex III contains 11 polypeptide subunits, 1 FeS centre (dubbed the Rieske FeS centre) and several haems including cytochromes b\textsubscript{566}, b\textsubscript{562} and c\textsubscript{1}. It accepts electrons from ubiquinol (ie reduced ubiquinone) and transfers them to cytochrome c (ubiquinol cytochrome c reductase). In so doing it translocates protons from the matrix
into the intermembranous space. It spans the inner membrane protruding 70Å from the M side and 30Å from the C surface.

3.e. Cytochrome c

Cytochrome c is located in the intermembranous space, loosely attached to the C face of the inner membrane and acts as an intermediate carrier of single electrons from Complex III to Complex IV.

3.f. Complex IV

Complex IV comprises 13 polypeptides with cytochromes a and a₃, and 2 or 3 copper atoms. It accumulates the single electrons from cytochrome c and combines four of them with dioxygen and 4 protons to produce water. It is located in the membrane with its active sites extending from the C surface and contributes to the generation of the proton motive force.

The concentration of the various components of the respiratory chain has been determined by spectral analysis, inhibitor binding or antibody titre studies and these give a stoichiometry for the elements of the respiratory chain as shown in Table 1.3.

Many of the components, such as Complexes I and IV, are believed to exist in dimeric form and are distributed randomly in the membrane. Electron transfer is thought to occur during random collisions between the relevant components this being facilitated by the carriers ubiqinone and cytochrome c having much faster lateral diffusion rates in the membrane as well as being more numerous stoichiometrically.
Table 1.3 Stoichiometry of the respiratory chain  
(Nicholls 1982)

<table>
<thead>
<tr>
<th>Complex</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex II</td>
<td>2</td>
</tr>
<tr>
<td>Complex III</td>
<td>3</td>
</tr>
<tr>
<td>Complex IV</td>
<td>6</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>6</td>
</tr>
<tr>
<td>Ubiquinone</td>
<td>36</td>
</tr>
</tbody>
</table>

3.g. ATP Synthetase Complex

The ATP synthetase polypeptide complex contains 13 subunits. It can be split by chaotropic agents into two fractions. One, designated the F₁ fraction, is water soluble and has ATPase activity but does not synthesise ATP and is not sensitive to oligomycin, as is the whole complex. The other fraction, F₀, is water insoluble, lipid containing and confers oligomycin sensitivity on the complex. The full characteristics of the in vivo ATP synthetase complex requires not only the presence of the above two fractions but also a section of the inner membrane containing highly hydrophobic polypeptides. These three elements make up the so called tripartite unit with F₀ inserted into the inner membrane, connected by a stalk to F₁ which protrudes into the matrix.

4. The Chemiosmotic Theory

The chemiosmotic theory first proposed by Mitchell in 1951 links the process of oxidation to the phosphorylation of ADP. The respiratory chain complexes are so
ordered that electron flow proceeds from one complex to the next down a gradient of decreasing negative redox potential. This electron flow is linked to the vectorial extrusion of protons, derived from the dissociation of water, out of the matrix at several points, as detailed above. This process generates a pH and electrochemical gradient across the inner membrane; the proton motive force (PMF). In the absence of ADP, electron flow through the respiratory chain will slow and eventually cease as it becomes counterbalanced by an increasing PMF (so called state 4 respiration). In the presence of ADP, the PMF drives protons through the ATP synthetase complex (Complex V) supplying the energy for the phosphorylation of ADP to ATP (state 3 respiration). Anoxia or respiratory chain inhibitors disrupt the generation of the PMF and under these circumstances the ATP synthetase complex is capable of the reverse process of hydrolysing ATP to ADP, with protons flowing in the opposite direction, there being a close equilibrium between the two reactions. Generation of the PMF is dependant also on the impermeability of the inner membrane, and this may be compromised by mechanical abuse or by the use of "uncoupling" agents such as dinitrophenol (DNP).

5. The Genetic Origin Of Oxidative Phosphorylation System

One of the unique features of the respiratory chain and the ATP synthetase complex is that it is the product of two separate genomes. Although most of the polypeptide subunits are encoded by the nuclear genome, thirteen of them are encoded by the mitochondrial genome. These 13 subunits include, seven Complex I subunits, one Complex III subunit, three Complex IV subunits and two Complex V subunits.
6. Mitochondrial Biogenesis

The similarity between mitochondria and bacteria, gave rise to the longstanding hypothesis that mitochondria might have evolved from the ingestion of bacteria by primitive prokaryocytes. The subsequent symbiotic relationship conferred considerable evolutionary advantage for the emergent eukaryocytes. Arising from this popular idea was the notion that, like their ancestral bacteria, mitochondria would be autonomously replicating.

The first suggestion that this might be possible came from the discovery of mutant yeast which were respiration deficient and inherited their mutation in a non-Mendelian fashion (Ephrussi 1950). It was postulated that this might indicate the presence of extra-chromosomal genetic factors present within mitochondria. Visualisation of mitochondrial DNA (mtDNA) was then achieved by electron microscopy (Nass and Nass 1963) and autoradiography (Guttes and Guttes 1964). More direct evidence for the existence of mtDNA came from its isolation from mitochondria, exploiting its lower buoyant density in a caesium chloride density gradient, compared with nuclear DNA (Borst et al 1967). Further electron micrographs seemed to show replication of the mtDNA and this proposition was strengthened by the discovery of a mitochondria specific DNA polymerase (Neubert et al 1967) and the demonstration that isolated mitochondria can synthesise DNA from the constituent nucleotides (Brewer et al 1967). Evidence for transcription of the mtDNA came from the isolation of a mitochondrial DNA dependant RNA polymerase (South et al 1968) and of the synthesis of mitochondrial RNA hybridising specifically to mtDNA.
(Wintersberger et al 1965, Wintersberger et al 1968). The localisation of ribosomes on the mitochondrial inner membrane, differing from cytoplasmic ribosomes (Kuntzel and Noll 1967), and the presence of tRNAs (Barnett et al 1967) meant that mitochondria also had their own translation apparatus. The trail turned full circle with the finding that the mutant respiratory deficient yeast did in fact have altered overall base composition of their mtDNA thus confirming that mtDNA was of relevance for the normal respiratory function of mitochondria (Mounolou et al 1966).

However, from a early stage it was apparent that even the larger mtDNA molecule of yeast, let alone the much smaller mammalian version, would not be able to code for all the numerous proteins present in the mitochondria. Clearly the bulk of the mitochondrial proteins would have be synthesised in the cytoplasm and imported into the mitochondria. So while the mitochondrion did have its own mtDNA with its distinctive protein synthesising system it was only semi-autonomous.

Initially mitochondria were thought to arise de novo from the invagination or evagination of other cellular membranes since "regeneration" of mitochondria was claimed in cells which had their mitochondria removed or destroyed (Harvey 1946, Zollinger 1948). Similarly yeasts grown in anaerobic conditions appeared to loose their mitochondria altogether, only to regain them when conditions allowed the resumption of aerobic metabolism (Luck and Reich 1964). Electron micrographs were also published appearing to substantiate this idea (Bell and Muhlethaler 1964). However the anaerobically grown yeast were subsequently shown to retain their mitochondria albeit in attenuated form as "promitochondria" (Criddle and Schatz 1969). The presence of
a separate and functioning mitochondrial genome and the distinctive nature of the mitochondrial inner membrane also led to re-evaluation of the idea of de novo biogenesis.

It is now felt that mitochondria derive from growth and division of pre-existing mitochondria, implying mitochondrial continuity through the generations. Consistent with this hypothesis were the choline labelling experiments of mitochondrial membrane resulting in uniform labelling of all mitochondria even after several cell divisions (Luck 1965). Direct evidence for mitochondrial division has been obtained in the unicellular flagellate Chromulina, whose single mitochondrion has been visualised undergoing binary fission, but such evidence is less forthcoming in the mitochondria of higher organisms. Morphological studies have failed to show whether mitochondrial population doubles before or after mitosis and in human HeLa cells, mitochondrial volume seems to be constant irrespective of cell cycle state (Posakony et al 1977). Mitochondrial numbers can also be regulated in fully differentiated non-dividing muscle cells (Eisenberg and Salmons 1981).

7. The Import Of Proteins Into Mitochondria

Over 90% of the mitochondrial proteins are coded for by nuclear DNA and are synthesised in the cytosol. They therefore have to be imported to their final destination in one of the four mitochondrial compartments. A common pattern of protein import appears to be that of translocation into the matrix space with eventual sorting of the proteins for their final locations from thence. This schema has been dubbed
"conservative sorting" since it is felt that it reflects the possible endosymbiotic origin of mitochondria. In the prokaryotic ancestor, the mitochondrial matrix would have corresponded to the cytoplasm and would have had the machinery for sorting of the proteins synthesised there. With transfer of the mitochondrial genes to the nucleus, additional machinery for the translocation of the proteins across the double membrane of the mitochondria into the matrix has been introduced. Thereafter the original prokaryotic type sorting mechanism takes over. (Pfanner et al 1988)

The cytosolic proteins are usually synthesised as precursor proteins with a positively charged amino-terminal peptide extension, or "pre-sequence", which targets the protein to the correct mitochondrial compartment. Fusion of such pre-sequences to non-mitochondrial proteins results in the transfer of these proteins into mitochondria. The efficiency of transport is however enhanced by the presence of a hydrophobic carboxyl-terminal portion of some of the mature genuine mitochondrial proteins (Pfanner et al 1987).

In order to facilitate the import of the precursor proteins and to keep their targeting presequences exposed these newly synthesised proteins are kept in a disaggregated and partially unfolded state by association with cytosolic ATP dependant "chaperonin" or "heat shock proteins" (because they accumulate during heat stress of cells) of 70kD (Hsp70) (Cheng et al 1989).

The general pattern of mitochondrial import involves interaction of the precursor protein with an exposed mitochondrial import receptor on the outer membrane.
Figure 1.3 Mitochondrial protein import
Subsequently the precursor presequence is inserted into the outer membrane at the
general insertion site (GIP, general insertion protein) and translocated across the outer

Several mitochondrial outer membrane (MOM) proteins have been analysed and
are designated according to their molecular weight. MOM19 represents the
mitochondrial import receptor preferentially used by most precursor proteins, though
others utilise MOM72 (see page 21). MOM7, MOM8, MOM30 and MOM38 are
components of the general insertion site while MOM22 has uncertain function (Sollner

Those precursor proteins destined for the outer membrane, such as porin,
diverge from those requiring further transport into the mitochondria. For the latter the
ensuing pathway is via contact sites where the inner and outer membrane are in close
apposition. These contact sites are stable structures associated with specific proteins
which in cooperation with the lipid result in a proteinaceous hydrophilic environment
favourable for precursor translocation. The insertion of the presequence of precursors
into these translocation contact sites is dependant on a membrane potential across the
inner membrane. This membrane potential, being positive outside, exerts an
electrophoretic effect on the positively charged regions of the precursor proteins.
Subsequent translocation of the major polypeptide chain does not require a membrane
potential or ATP and is driven by the high affinity of matrix located Hsp70s for the
extended polypeptide chain. ATP is however required for the eventual release of
Hsp70s from the polypeptides (Neupert et al 1990).
Once in the mitochondrial matrix the amino-terminal presequences are proteolytically cleaved by the cooperative action of a matrix processing peptidase (MPP) and a processing enhancing protein (PEP). In some cases the cleavage of the presequence is a two stage process with the first step often leaving an uncharged hydrophobic stretch of about 20 amino acids which further specifies its final location within the mitochondria. This particularly applies to those proteins destined for the intermembrane space (cytochrome $b_2$ and cytochrome $c$ peroxidase) or the C face of the inner membrane (cytochrome $c_1$ and the Rieske FeS protein of complex III) where export of the protein from the matrix across the inner membrane is required. The second cleavage step of the presequence of such proteins is performed by additional processing activities located at the eventual destination of the protein. Intermediates associated with matrix Hsp70s are transferred to Hsp60s for ATP dependant folding.

There are some exceptions to this general pattern of import. Cytochrome $c$ lacks a precleavable sequence and enters the mitochondria by a direct route not involving the GIP receptor to eventually locate in the intermembrane space. Other proteins such as the ADP/ATP carrier (AAC) and the uncoupling protein of mitochondrial inner membrane also lack a cleavable presequence and are thought to have their targeting signals within the mature protein structure. Translocation of proteins such as AAC into the outer membrane also utilises a different mitochondrial import receptor, a mitochondrial outer membrane protein of 72kD (MOM72), though thereafter the import route is via GIP (Sollner et al 1990). It is interesting to note that these latter proteins do not have a prokaryotic equivalent and might therefore be predicted to digress from the principle of conservative sorting.
CHAPTER 2: MITOCHONDRIAL DNA

1. Structure And Replication

In human cells mitochondrial DNA (mtDNA) generally comprises less than 1% of the total cellular DNA. It is a double stranded circular molecule containing 16,569 base pairs which have been sequenced (Anderson et al 1981). The two complementary strands are designated Light (L) or Heavy (H) strand reflecting their differing buoyancy in a caesium chloride density gradient which results from the latter's excess of G and C nucleotide bases.

Both the H and L strands of mtDNA are replicated uni-directionally from separate origins. H strand replication begins first proceeding in a clockwise direction until when 67% complete it initiates the replication of the L strand by exposing its origin of replication. While H strand replication continues to completion, L strand replication proceeds in an anticlockwise direction. The entire replicative cycle of mtDNA takes two hours, implying a very slow overall rate of polymerisation of around 270 nucleotides per minute per strand, which may be due to the relatively dehydrated state of the mitochondrial matrix (see Figure 2.1).

The basic monomeric genome can exists as a single supercoiled molecule but multimeric versions have also been demonstrated either as head to tail unicircular dimers, or as two or more monomeric circles interlinked to form structures.
known as catenanes. Not all newly initiated H strands continue to completion and in fact the majority terminate at a variable distance, up to 700 nucleotides downstream. The resultant short newly synthesised H strands remains associated with its original template thereby creating a triple stranded structure known as the displacement, or D, loop. This version of mtDNA termed D mtDNA represents the major form of the molecule in mammalian cells and leads to some relaxation in the parental supercoiled form.

Since about 20% of all nucleotides polymerised on mtDNA templates are in D loop strands \cite{Bogenhagen and Clayton 1978} there must be some advantage in maintaining this structure but its precise function is uncertain. It may be the initiator of mtDNA synthesis but this would not explain the high turnover of D strands. Alternatively it may be a binding site for some other entity such as protein, or it may have a role in regulating mtDNA transcription with its turnover relating to varying transcriptional demands \cite{Clayton 1982}.
Each mitochondrion may contain from two to ten copies of mtDNA but copy number does not appear to be tightly controlled in mammalian cells nor is it related to cell volume (Bogenhagen and Clayton 1974). The net amount of mtDNA replication is sufficient to maintain a constant copy number within each cell but some molecules may replicate more than others (Flory and Vinograd 1973). The relationship between mtDNA replication and mitochondrial division is unknown but replication can occur at any point in the cell cycle (Bogenhagen and Clayton 1978). The site of mtDNA replication within the organelle is also unknown.

2. Mitochondrial DNA Genes

The simultaneous publication of the human mtDNA sequence (Anderson et al 1981) and the analysis of mitochondrial transcripts (Montoya et al 1981) revealed a wealth of information on the organisation of this genome. Many of these features are novel and remain unique to this molecule.

It is a small genome, its 16.569 kilobases dwarfed by that of, say, the dystrophin gene which is over 2000 kilobases long. Yet despite this, it contains the genes for 2 ribosomal RNAs (rRNAs), 22 transfer RNAs (tRNAs) and 13 proteins. This unprecedented density of genetic information is achieved by the virtual absence of non coding regions or introns, and by the dispensing of certain features normally considered intrinsic to nuclear genes.
Figure 2.2 The mitochondrial genome
Its genetic code differs from the universal code with different start and stop codons and different codons for some of the amino acids, as detailed in Table 2.1 (Barrell et al 1979).

Table 2.1 The mitochondrial genetic code

<table>
<thead>
<tr>
<th>Triplet Codon</th>
<th>Universal Code</th>
<th>Mitochondrial Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>UGA</td>
<td>Stop</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>AGA or AGG</td>
<td>Arginine</td>
<td>Stop</td>
</tr>
<tr>
<td>AUA</td>
<td>Isoleucine</td>
<td>Methionine</td>
</tr>
<tr>
<td>AUA or AUU</td>
<td>-</td>
<td>Start</td>
</tr>
<tr>
<td>AUG</td>
<td>Start</td>
<td>-</td>
</tr>
</tbody>
</table>

Another major difference lies in the pattern of codon recognition by mitochondrial tRNAs which enables 22 of them to constitute a complete set for mitochondrial protein synthesis, in contrast to the minimum 32 required for nuclear transcription (Barrell et al 1980). This is achieved by; the tRNA anticodon and codon requiring only two out of the three nucleotides to match (Lagerkvist 1978), or U:N base pairing (Barrell 1980); by the absence of the AGA or AGG arginine codons in the protein coding genes and therefore of the corresponding tRNAs with these anticodons; and by the presence of only one tRNA\textsuperscript{met} (Attardi 1985).

The mitochondrial tRNAs are generally smaller than their nuclear equivalents and they also have numerous structural differences from non-mitochondrial tRNAs. Likewise the rRNAs are much smaller than their nuclear counterparts being 1,559
nucleotides (16S rRNA) and 954 nucleotides (12S rRNA) long.

Thirteen open reading frames were identified in the mtDNA sequence, of which five could be identified by their sequence homology with their equivalent yeast mitochondrial counterparts namely, subunits I, II, and III of cytochrome oxidase (COX I, II, and III), cytochrome b and subunit 6 of ATPase (Anderson et al. 1981). In addition for the first four of these subunits bovine protein sequence data was available which matched the bovine mtDNA nucleotide sequence (Steffens and Buse 1979). Of the remaining eight unidentified reading frames (URFs), the smallest was recognised as coding for an ATPase subunit on the basis of its homology to a later discovered yeast gene for subunit 8 of ATPase (Macreadie et al. 1983). The proteins encoded by the remaining seven URFs were identified in HeLa cell mitochondrial translation products by the use of antibodies prepared against chemically synthesised peptides predicted from the DNA sequence, (Mariottini et al. 1983, 1986; Chomyn et al. 1983, 1986) and functionally assigned to Complex I by their immunoprecipitation with Complex I antisera (Heron et al. 1979, Chomyn et al. 1985). They were therefore relabelled ND (for NADH dehydrogenase) subunits 1, 2, 3, 4, 4L, 5 and 6.

<table>
<thead>
<tr>
<th>Table 2.2 Proteins encoded by mitochondrial DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex I</td>
</tr>
<tr>
<td>Complex II</td>
</tr>
<tr>
<td>Complex III</td>
</tr>
<tr>
<td>Complex IV</td>
</tr>
<tr>
<td>Complex V</td>
</tr>
</tbody>
</table>

27
The H strand represents the main coding strand and contains the genes for both rRNAs, 14 tRNAs and 12 of the protein coding genes. The L strand contains the remaining 8 tRNAs and the gene for ND6. The different genes on the H strand are mostly butt jointed to each other or separated by only a few nucleotides. Many of the reading frames have only an abbreviated stop codon consisting of either a T or TA at their end, and rely on polyadenylation of the transcript to complete the termination codon (Anderson et al 1981, Ojala et al 1981). The tRNAs are scattered along the H strand and often function as "punctuation marks" separating the rRNA and protein coding genes.

It is interesting to speculate as to why these particular genes have been retained in the human mitochondrial genome. One theory has been that the hydrophobicity of the proteins they code for precludes the possibility of their synthesis and transport in the cytoplasm. But the gene composition of mitochondria of different organisms is far from invariate and if one surveys these distinctions, one finds that only the two rRNAs, a few tRNAs, COX 1 and cytochrome b are common to all. The "missing" genes are synthesised in the cytoplasm along with other equally hydrophobic subunits such as ATPase 9 (Attardi and Schatz 1988).

3. Control Of Transcription

Contrasting with the very asymmetrical distribution of genes on the two mtDNA strands is the remarkable symmetry of their transcription which encompasses the entire length of both strands in a polycistronic fashion. Variation in gene expression is largely
dependant on various post transcriptional mechanisms which differ for the different RNA species.

Transcription initiation sites for both the L and H strands are located in a small region near the origin for replication of the H strand. The L strand is transcribed as a single unit proceeding clockwise from its initiation site and this polycistronic transcript is processed to produce the 8 tRNAs and the mRNA for ND6 encoded by this strand. According to Attardi, there are two H strand initiation sites which generate two overlapping polycistronic transcripts proceeding in an anti-clockwise direction. The downstream initiation site transcript encompasses almost the entire H strand and is processed to generate all the mRNAs and tRNAs encoded by this strand. The upstream initiation site produces a transcript which terminates just beyond the 3' end of the 16S rRNA gene thus producing transcripts for both the rRNAs and for the tRNAVal and tRNAPhe (Attardi et al. 1987).

The resulting large polycistronic transcripts have to be precisely cleaved to produce the individual RNA species. This requires enzymes to cut the intervening tRNAs on their 5' and 3' sides and to poly-adenylate the 3' ends of the rRNAs and mRNAs (Attardi 1987).

The two H strand transcription initiation sites show different sensitivities to low temperature, [Ca^{2+}] and [Mg^{2+}], and have different ATP requirements when studied using isolated mitochondria (Gaines and Attardi 1984a, 1984b, 1987), suggesting differential control of expression. Since these latter transcripts are synthesised at a
much higher rate (Montoya et al 1983) this probably accounts for the 15 to 60 fold higher synthesis of the rRNA species compared to the mRNAs (Gelfand and Attardi 1981).

The regulation of the rRNAs is not only dependant on the differing activities of the two H strand initiation sites. A DNA binding protein has been isolated in mitochondrial lysate which attaches to a 28 nucleotide region (MTERM), just downstream of the 3' end of the 16S rRNA. Stimulation of the upstream H strand initiation site which generates the two rRNAs, as well as the tRNA$^{\text{Val}}$ and tRNA$^{\text{Phe}}$, also appears to promote termination of this transcript via this DNA binding protein (Kruse et al 1989).

Although the tRNA set is generated from three separate transcription units synthesising at widely different rates, their levels are broadly comparable (Attardi 1987). This situation does not result from differential stability of the mature tRNAs since their half lives are in fact similar (Attardi 1987). Given the half life of the tRNAs, which is more than 24 hours, the rate of synthesis of the whole H strand transcript is barely sufficient to generate the required tRNAs (Attardi et al 1989). The uniformity of tRNA levels must therefore result either from the loss of L strand and short H strand transcripts or of their tRNAs prior to entry into the mature tRNA pool. The high turn over rate of the L strand transcripts (Aloni and Attardi 1971, Cantatore and Attardi 1980) suggests that most of these transcripts are degraded before generating any tRNAs. Another, more speculative, mechanism may apply, particularly for the short H strand, involving limiting levels of specific stabilising factors for the tRNAs,
especially for the otherwise over-produced tRNA\textsuperscript{Phe} and tRNA\textsuperscript{Val}. A possible candidate for such specific factors might be the tRNA synthetases found to play a similar role in \textit{E. Coli} (Neidhardt \textit{et al} 1977).

Unlike the tRNAs, the mature mRNAs have shorter and more variable half lives. Those mRNAs most abundantly transcribed have the shorter half lives, in the order of 7 minutes for the L strand encoded mRNA for ND6 (Gelfand and Attardi 1981), while for the slower transcribed H strand encoded mRNAs this value ranges from 25 to 90 minutes. Furthermore, for the whole H strand transcript the mRNA levels are very variable and unrelated to their relative position compared to the promoter. Thus it seems that for mRNAs, differential stability of the mature mRNAs is the determining factor in their overall lower level, compared with the tRNAs, as well as the differing amounts of individual mRNAs (Attardi \textit{et al} 1989).

4. Control Of Translation

Compared with the emerging information on transcriptional control of mtDNA, very little is known about the regulation at the translational level. Pulse labelling of the mitochondrially encoded polypeptides in HeLa cell cultures with \textsuperscript{35}S methionine gives a value for the relative rates of synthesis of the subunits, after correcting for the differing methionine contents, as tabulated in Table 2.3 (Attardi 1987);

It can be seen that there is a greater than 10 fold variation in the rates of synthesis of some of the subunits but that subunits of the same complex have similar
Table 2.3 Relative rates of mitochondrial polypeptide synthesis
(Attardi 1987)

<table>
<thead>
<tr>
<th>Complex</th>
<th>Relative rates of synthesis</th>
<th>Relative efficiency of mRNA translation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ND1</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>ND2</td>
<td>2.0</td>
<td>1.8</td>
</tr>
<tr>
<td>ND3</td>
<td>2.8</td>
<td>8.0</td>
</tr>
<tr>
<td>ND4</td>
<td>1.7</td>
<td>1.1</td>
</tr>
<tr>
<td>ND4L</td>
<td>1.8</td>
<td>1.2</td>
</tr>
<tr>
<td>ND5</td>
<td>0.9</td>
<td>3.6</td>
</tr>
<tr>
<td>ND6</td>
<td>0.4</td>
<td>&gt;5.8</td>
</tr>
<tr>
<td>Complex II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyto. b</td>
<td>4.6</td>
<td>5.3</td>
</tr>
<tr>
<td>Complex IV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COX I</td>
<td>5.9</td>
<td>4.0</td>
</tr>
<tr>
<td>COX II</td>
<td>7.0</td>
<td>3.8</td>
</tr>
<tr>
<td>COX III</td>
<td>5.2</td>
<td>3.4</td>
</tr>
<tr>
<td>Complex V</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATPase 6</td>
<td>4.7</td>
<td>3.9</td>
</tr>
<tr>
<td>ATPase 8</td>
<td>6.7</td>
<td>5.6</td>
</tr>
</tbody>
</table>

rates of synthesis. Comparing this variation in the rates of synthesis with the abundance of the corresponding mRNAs the conclusion is that the mRNAs differ in the efficiency of their translation as shown above. The basis for this variation is conjectural. There is no preponderance of rarely used codons in those mRNAs which have a lower rate of translation (Attardi 1987). Variations in the accessibility of the initiation codons, in the secondary structure of the mRNAs, or their affinity to the rRNAs have been suggested.
as possible mechanisms. The availability of cytoplasmically synthesised subunits for a given complex may influence the synthesis of mitochondrially encoded subunits for that complex. The nuclear genome may exert a more direct influence via specific translation regulating factors such as have been reported in yeast for the cytochrome b, COX III and possibly the COX II mRNA (Fox 1986, Attardi and Schatz 1988).

5. The Role Of The Nuclear Genome

Nuclear genes play a major role in the replication and expression of mtDNA. Most of the mitochondrial proteins are of nuclear origin and their transport into the relevant mitochondrial compartment is controlled by the products of nuclear genes. This is also true of the majority of the subunits comprising the complexes of the oxidative phosphorylation apparatus, the assembly of which requires close cooperation between many nuclear genes and the mitochondrial genome. All these interactions necessitate complex signalling arrangements to and from the nuclear and mitochondrial genes as well as between numerous nuclear genes. The elucidation of these information loops is at a very early stage and what examples can be demonstrated are mainly in yeast mitochondrial systems which differ in significant aspects from mammalian systems.

Some defects in nuclear genes obviously affect mitochondrial biosynthesis but may not directly affect mitochondrial gene expression. Thus respiratory deficient nuclear petite (pet) yeast mutants have been reported with defects in the genes encoding enzymes of intermediary metabolism such as haem synthesis, cytochrome c, or the
carrier systems, such as the ADP/ATP translocase. Impairment of the protein import machinery has also been found with defects in the matrix localised proteases which cleave targeting presequences from imported precursor proteins (Attardi and Schatz 1988).

Another category of nuclear defects would be those affecting components of the mitochondrial genetic system such as RNA polymerase, ribosomal proteins or aminoacyl-tRNA synthetases which would clearly affect mtDNA expression more directly. Again there is precedence for such nuclear gene mutations seen in pet yeast mutants (Attardi and Schatz 1988).

A further group of yeast mutants have been characterised which lack a nuclear encoded protein which normally binds to the untranslated 5' leader sequence of the mitochondria mRNA for one of the COX subunits. They are therefore unable to translate the relevant polypeptide. In fact for COX III, four such binding proteins seem to be required. Multiple nuclear gene products are also required for the translation of the mitochondrial mRNA for the cytochrome b subunit (Fox 1986). Similar examples in eukaryotic cells are rare (Hinnebusch 1984).

The polycistronic transcripts of mtDNA need to be carefully spliced to remove the intervening tRNAs and generate the individual mRNAs, thus requiring a whole family of nuclear encoded splicers (see page 29). Splicing mutants are described in yeast but the relevance of these to mammalian mitochondria is more speculative since these apply to the removal of introns which are not a feature of mammalian mtDNA.
There has been little direct evidence for any influence of the mitochondrial genome on nuclear genes. Some yeast nuclear transcripts have been found to vary in amount according to the integrity of the mtDNA (Parikh et al 1987). Murine cells express a maternally inherited cell surface antigen which suggests that it is mtDNA influenced (Smith et al 1983). This supposition was strengthened by the fact that a selective mitochondrial inhibitor, rhodamine 6G, blocked expression of this antigen (Fischer Lindahl 1986). More recently this antigen has been shown to be analogous to the mitochondrial subunit ND1 (Loveland et al 1990). Early attempts to show evidence for mitochondrial export of polypeptides or RNA were negative (Schatz and Mason 1974) but this possibility is not excluded.

6. Physiological Control Of Mitochondrial DNA Expression

Mention has already been made of the changes in mitochondrial mass that accompany developmental or environmental challenges. These responses would entail the coordinated control of the two separate genomes involved. Little is known about the details of the bigenomic interactions that must occur, or of the regulation of the mitochondrial genome in these situations.

In HeLa cell cultures the ratio of mitochondrial rRNA genes to mitochondrial rRNA molecules is three orders of magnitude greater than seen in the cytoplasmic equivalents. This implies either, a considerably slower rate of transcription of these genes than seen in their nuclear equivalents, or that only a fraction of the mtDNA genomes are transcribing their rRNAs (Attardi and Schatz 1988). In HeLa cell
mitochondria, the protein coding genes are transcribed even less frequently, perhaps only once or twice per cell generation (Attardi et al 1987). However despite this, transcription of mtDNA is not rate limiting for protein synthesis and in fact is much in excess of that required. Extensive inhibition of mitochondrial RNA synthesis by up to 85% fails to reduce mitochondrial protein production for at least 48 hours. Recalling the much shorter half lives of the mitochondrial mRNAs and rRNAs, this implies that the residual 10 to 15% of mtDNA transcription is sufficient to maintain a normal rate of translation (Lansman and Clayton 1975). Given both the excess mtDNA copy number and the great transcriptional reserve it might seem reasonable to expect that these would provide a mechanism whereby mitochondria could respond rapidly to changing respiratory demand. In fact in such circumstances the mtDNA content increases with no suggestion that the rate of transcription has increased. In rabbit striated muscle of different fibre type composition, the mtDNA content, the level of mtDNA transcripts, and the oxidative phosphorylation capacity rise in parallel. Increasing the respiratory capacity of such muscle by electrical stimulation also induces parallel changes in mitochondrial gene copy number and mitochondrial transcripts (Williams 1986a, 1986b).

With developmental changes it is a similar pattern with an increases in mitochondrial rRNA shadowing the rise in mtDNA content, as seen in rat hepatocytes after birth (Cantatore et al 1986). Conversely, the decline in the synthesis of rat muscle mitochondrial proteins, which is rapid shortly after birth and slower over the next two years, is not accompanied by a decline in the mRNA levels. The need for the excess mtDNA is unknown. In contrast to these findings is the observation that, in the
senescent rat, a 50 to 70% reduction in 12S rRNA and mRNA for COX I is seen in the senescent heart and brain tissue compared with the adult equivalents. This decline was not seen in senescent liver tissue. Pre treatment of the senescent rats with acetyl-L-carnitine restored the reduced transcript levels to that seen in adults (Gadaleta et al 1990).

As well as changes in the overall expression of mitochondrially encoded proteins there is also evidence for differential tissue expression of a particular protein coding gene. The expression of the ND5 subunit appears to be much less in rat muscle and rat brain synaptosome mitochondria than in exponentially growing rat fibroblast cultures (Attardi et al 1989). There is no difference however in the mRNA level for ND5 in these situations, again pointing to control at the translational level. The mechanism for such differential expression is unknown but a specific translation factor, if involved, could be tissue specific.

7. Maternal Inheritance Of Mitochondrial DNA

Another of the distinctive features of mitochondrial DNA is that it is wholly maternally transmitted. Analysis of mtDNA nucleotide sequence polymorphisms between individuals in several families shows that, in cases where the parents have different restriction endonuclease cleavage patterns, the offspring showed the maternal pattern. This finding has been shown to hold true for up to three generations (Case and Wallace 1980, Giles et al 1980). Similar findings have been shown for a variety of other species, both primate and non-primate. Such experiments might not detect
paternal mtDNA molecules in progeny, at levels less than 5%. However an analysis of 45 and 91 generation progeny resulting from repeated paternal backcrossed matings in lepidopteran insects failed to show any paternal mtDNA and set an upper limit to paternal leakage at 1 molecule per 25,000 per generation (Lansman et al 1983).

One explanation for this maternal inheritance may be that sperm contain very few mitochondrial genomes compared with the greater than $1 \times 10^6$ contained in oocytes. Furthermore the spermatozoon mtDNA is located in the middle section which does not penetrate the ovum.

8. Mitochondrial DNA Mutation Rate

Although the organisation of the mtDNA genes is conserved in mammalian mtDNA there is considerable sequence variation among mammalian mtDNAs as evidenced by restriction endonuclease polymorphisms. Such variations have been exploited for anthropological studies (Brown 1980, Denaro et al 1981, Johnson et al 1983). Analysis of the changes in restriction endonuclease sites suggests that mtDNA sequences are changing 5 to 10 times faster than single copy nuclear sequences (Brown et al 1979).

This rapid rate of mtDNA mutation may be the result of several factors applicable to mitochondrial, but not nuclear, DNA. These include a faster turnover rate, its location in a hostile environment containing free radicals and other mutagens, and a lack of mtDNA repair mechanisms (Brown 1981, Richter et al 1988).
It has been hypothesised that the accumulation of mtDNA mutations in life may contribute both to the effects of ageing, and to several human degenerative diseases (Linnane et al 1989).
1. A Historical Review

The concept of a primary mitochondrial myopathy first arose with the publication of a case report describing a 35 year old woman who had hypermetabolism of nonthyroid origin associated with longstanding fatiguable weakness (Luft et al 1962). On electron microscopy her muscle biopsy showed peripheral and intermyofibrillar accumulations of mostly enlarged mitochondria showing abnormalities of cristae and sometimes osmiophilic paracrystalline inclusions. Biochemical studies of her mitochondria showed them capable of generating ATP from exogenous ADP and inorganic phosphate but the mitochondria were loosely coupled, continuing to consume oxygen at an almost maximal rate even in the absence of phosphate acceptor and thereby generating a grossly raised basal metabolic rate.

This abnormality of mitochondrial function is rare with only one other case being reported (Afifi et al 1972, DiMauro et al 1976) however similar ultrastructural abnormalities of muscle mitochondria began to be recognised as the major morphological finding in a variety of myopathies. A modification of the Gomori trichrome stain enabled light microscopic detection of this mitochondrial proliferation (Engel and Cunningham 1963) which produced deep red staining of the affected muscle fibres creating the so called "ragged red fibre" appearance. Many of these cases presented with fixed or slowly progressive proximal muscle weakness with fatiguability,
particularly in children (Shy and Gonatus 1964, Shy et al 1966). Ragged red fibres were also found to be a frequent finding in the syndrome of progressive external ophthalmoplegia (Olsen et al 1972). Later similar histological features were recognised to occur in patients with syndromes predominately or exclusively affecting the central nervous system. In children this could include psychomotor retardation, episodic headache, somnolence and vomiting, movement disorders, seizures, short stature, ataxia and visual impairment. In adults, dementia, deafness, ataxia, seizures, peripheral neuropathy and a wide range of other symptoms could occur (Spiro et al 1970a, 1970b, Shapira et al 1975, Fukuhara et al 1980, Morgan-Hughes et al 1979, 1982, Hayes et al 1984). Other systems apart from muscle and the central nervous system could be affected including haemopoietic tissue, endocrine organs, the kidneys, liver, heart, and eye (Petty et al 1986).

Early biochemical studies in these cases seemed to show defective coupling of the mitochondria with various substrates but without a raised basal metabolic rate which was difficult to interpret. With refinement of biochemical techniques a clearer picture emerged with reports of a respiratory chain defects with deficiencies of cytochrome b (Spiro et al 1970b), cytochrome c oxidase (French et al 1972), succinate-ubiquinone reductase (Goldfischer et al 1973) and NADH-ubiquinone reductase (Morgan-Hughes et al 1979), as well as defects in other enzyme systems such as in the pyruvate decarboxylase complex (Blass et al 1970) and deficiencies of muscle carnitine (Engel and Angelini 1973) and the carnitine palmitoyl transferase system (DiMauro and DiMauro 1973). Gradually these reports were substantiated by additional cases and the list of defects extended with defects of the respiratory chain being the most numerous.
particularly those of Complexes I, III and IV. Ragged red fibres were not an invariate association with all mitochondrial biochemical defects but seemed to be particularly associated with defects in the respiratory chain or the oxidative phosphorylation coupling.

The term "mitochondrial encephalomyopathy" was coined to describe cases of complex multisystem disease associated with "structurally and/or functionally abnormal mitochondria in the brain and/or muscle" (Shapira et al 1977). However the older term "mitochondrial myopathy" is still in use since many of the syndromes do involve muscle and this remains the main source of material for morphological and biochemical study (Morgan-Hughes et al 1982). Others prefer the term "mitochondrial cytopathy" reflecting the protean manifestations of these disorders.

In 1981 the sequence of the mitochondrial genome was published (Anderson et al 1981) and in the next few years its thirteen protein coding genes were assigned to components of complexes I, III and IV of the respiratory chain as well as of complex V (ATP synthetase). This gave rise to speculation that at least some of the mitochondrial myopathies could be the result of defects in the mitochondrial DNA particularly as the commonest biochemical abnormalities were also of complexes I, III, and IV. Although most cases of mitochondrial myopathy are sporadic, familial cases where they occurred, showed a preponderance of maternal inheritance as would be expected for a mtDNA defect (Egger and Wilson 1983, Harding et al 1988). Direct evidence for such mitochondrial DNA defects came with the finding of large, up to 7kb, deletions in a proportion of the mtDNA in the muscle but not the blood of some
patients with mitochondrial myopathies all of whom had ophthalmoplegia as part of their clinical picture (Holt et al 1988). This finding was rapidly confirmed by others (Zeviani et al 1988, Moraes et al 1989, Goto et al 1990).

These findings stimulated the search for other human mitochondrial genome defects particularly concentrating on diseases showing maternal inheritance. One such disease was Leber's hereditary optic neuropathy (LHON) (see chapter 3, section 2diii). A point mutation in the gene coding for the ND4 subunit of Complex I was found to be associated with this disease (Wallace et al 1988a). Additional point mutations of both protein coding and tRNA genes have been reported for these, and other mitochondrial diseases.

The list of mtDNA defects is increasing all the time (Wallace et al 1991), and encompassing diseases which may not have demonstrable morphological abnormalities of the mitochondria ie. ragged red fibres. In some the biochemical defect of mitochondrial function arising from these genetic defects has yet to be determined.

It therefore seems reasonable to expand the original definition of the diseases to include those having "morphological, biochemical or genetic defects affecting mitochondrial energy metabolism".
2. Clinical Features Of The Mitochondrial Myopathies

A wide range of clinical features have been described with ragged red fibres and although the brunt of the disease is neurological, any system may be affected. As always when faced with such a diversity of symptoms and signs there is a drive to lump frequently co-existing features into more readily remembered, and therefore recognised, syndromes. Such classification is difficult to do on purely clinical grounds since there may be considerable overlap between proposed syndromes. Even within a given family a variety of phenotypes may be seen (Bercovic et al 1989). In some cases such clinically derived syndromes turn out to have an aetiological rationale, but advances in genetics may also stretch the boundaries of, and blur the distinction between, syndromes. "Splitters" favour the recognition of syndromes such as Kearns Sayre Syndrome (KSS), Myoclonus Epilepsy with Ragged Red Fibres (MERRF), Mitochondrial Encephalopathy with Lactic Acidaemia and Stroke-like episodes (MELAS) (Rowland et al 1991). Others, "lumpers", prefer a more loose framework of clinical features viz; ophthalmoplegia with or without myopathy, myopathy only, and predominantly CNS disease (Petty et al 1986). The clinical features of the above mentioned groupings clearly overlap and this is reflected in the description headings that follow.

In paediatric practice a variety of additional CNS syndromes are seen such as Leigh's syndrome and the syndromes of benign and fatal lactic acidaemia.
Table 3.1  Clinical presentations of the mitochondrial myopathies

Myopathy

Ophthalmoplegia ± myopathy
  CPEO
  Kearns Sayre syndrome

Major CNS Involvement
  MERRF
  MELAS
  Leigh syndrome
  Alpers' syndrome
  May White syndrome
  NARP
  MNGIE
  Lethal infantile encephalomyopathy
  Benign infantile lactic acidaemias
  Multiple symmetrical lipomatoses

Other Syndromes
  Cardiomyopathy
  Pearson's syndrome
  Leber's Hereditary Optic Neuropathy

In almost all cases these clinical descriptions bear no correlation with any particular biochemical defect and the severity of the disease does not relate to the extent of the biochemical abnormalities. However some associations can now be made with the known mtDNA defects, described later.
2.a. Myopathy

This may be the main feature of the disease. There are no specific clinical features which distinguish this myopathy from that occurring in a variety of other muscle diseases. The onset of muscle symptoms is usually in childhood or adolescence, but neonatal presentation (Roodhooft et al 1986), and late onset, in the fifth decade (Petty et al 1986), have been described. Fatiguable weakness is a common feature and may be associated with exercise induced cramps without pigmenturia. The distribution of weakness can vary with proximal, limb girdle, and fascioscapulohumeral patterns having been described. Some cases have episodic exacerbations of their muscle weakness, sometimes associated with headaches, nausea and vomiting with severe metabolic acidosis documented in a few. Precipitants for such attacks include intercurrent infection, alcohol and unaccustomed exertion. The myopathy is often associated with external ophthalmoplegia and in some cases pigmentary retinopathy is also present (Petty et al 1986). In some, an initial myopathic presentation leads later to CNS involvement such as dementia and epilepsy. A positive family history is not uncommon. All patients in this group have some degree of lactic acidaemia which increases on exercise.

2.b. Chronic Progressive External Ophthalmoplegia / Kearns Sayre Syndrome

The entity of chronic progressive external ophthalmoplegia (CPEO) has long been recognised, with early discussion centering around whether it was of neurogenic or myogenic origin. Adoption of the term "ophthalmoplegia plus" in the late 1960s was acknowledgement of the frequent association of this entity with a variety of other neorological, cardiac, and retinal manifestations. Within this broad category is said
to be a collection of core features which characterise the Kearns Sayre syndrome as a specific entity (Berenberg et al 1977, Rowland et al 1991), (v.i.).

Frequent associations with CPEO include cerebellar ataxia, short stature, delayed sexual maturation and other endocrine abnormalities, sensori-neural deafness, myopathy, cardiac conduction defects, raised CSF protein and cranial or peripheral neuropathies. Pigmentary retinopathy is a common accompaniment and is more usually of the salt and pepper type, although the more classic bone spicule pattern has also been seen. Visual failure and peripheral field constriction is not usually seen with the salt and pepper type pigmentary retinopathy but may be affected more often with the classical form (Mullie et al 1985). A spongioform encephalopathy has been reported at post mortum in several cases (Daroff et al 1966).

Another distinguishing feature of the CPEO group of mitochondrial myopathies is that most represent sporadic cases although some familial cases have been described. These have included mother and daughter (Ozawa et al 1988), monzygotic twins (Rowland et al 1988), father and son (Jankowitz et al 1977), and two brothers (Schnitzler and Robinson 1979).

Kearns and Sayre drew attention to the frequent co-existence of ophthalmoplegia with retinopathy and cardiac conduction block at or before adolescence (Kearns and Sayre 1958). The Kearns Sayre syndrome has been further delineated as the presence of three obligatory features; onset before 20 years old; ptosis and ophthalmoplegia; and pigmentary retinopathy with one of three other features; heart block, CSF protein
greater than 100mg/dl, or cerebellar ataxia. It is argued by some to be a specific entity within the general umbrella of CPEO (Berenberg et al 1977, Rowland et al 1991).

2.c.Major CNS Involvement

Encephalomyopathy and its associated symptoms usually appear in childhood or early adulthood although presentation at either extreme of life have been described (Roodhooft et al 1986, Morgan-Hughes et al 1985). A major feature of this group is the occurrence of episodic headaches and vomiting, stroke-like events and seizures with dementia. Others have combinations of myoclonic epilepsy, ataxia, dementia and sensorineural deafness. These two presentations have been highlighted as specific syndromes in adults namely MERRF and MELAS. Myopathy is usually mild and there may be neurophysiological evidence of muscle involvement in cases without clinically detectable weakness or fatiguibility. Extra-pyramidal features of dystonia and choreoathetoid movements have been described (Truong et al 1990). Widespread multiple necrotic foci, haemorrhage and spongiform degeneration of the brain is seen at post mortum. The ophthalmoplegia, retinopathy and cardiac conduction block characteristic of KSS are not generally seen in this group.

2.c.i.MERRF

The acronym MERRF for myoclonic epilepsy with ragged red fibres was proposed by Fukuhara (1980). As well as myoclonic epilepsy common clinical associations include; cerebellar ataxia, dementia, sensorineural deafness while less common were optic atrophy and a sensory peripheral axonopathy (Berkovic et al 1989). A noticeable attribute of this syndrome is the occurrence of familial cases. A
particularly large pedigree in which maternal inheritance could be demonstrated has been reported (Rosing et al 1985) while many other smaller pedigrees exist in which maternal inheritance is a possibility, but cannot be confirmed. Such inheritance is of course consistent with a mtDNA defect and a proportion of cases have been shown to have a point mutation of a mitochondrial tRNA (see section 7.f.).

2.c.ii.MELAS

The syndrome of mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes, or MELAS, was advanced by Pavalikis et al (1984). The hallmark of this syndrome; the stroke-like events, are so named because, despite the sudden onset of neurological deficit, and the CT appearances of low attenuation lesions, as seen in cerebro-vascular disease, the underlying pathophysiology is of abnormal metabolism rather than vascular in nature (Seyama et al 1989). The stroke-like events are often preceeded by migraine-like headache, nausea and vomiting. The syndrome is said to be further distinguished from MERRF by the lack of myoclonus, optic atrophy and peripheral neuropathy but this separation may not be generally applicable (Petty et al 1986).

2.c.iii.Leigh syndrome

This represents the commonest manifestation of cytochrome oxidase deficiency but can result from Complex I, pyruvate dehydrogenase complex deficiency and pyruvate decarboxylase deficiency. In many cases no biochemical defect can be detected. It was initially defined as a pathological entity characterised by a spongy necrosis of the CNS, with vascular proliferation and glial reaction (Leigh 1951).
Affecting mainly infants and children, there are a variety of features with combinations of delayed milestones, dementia, seizures, cerebellar ataxia, nystagmus, optic atrophy, ptosis, ophthalmoplegia, deafness spasticity, hypotonic weakness, and anorexia with vomiting. Cardiac and renal complications of hypertrophic cardiomyopathy and Fanconi syndrome have been described. In cases with cytochrome oxidase deficiency the defect is widespread in all tissues and this together with known autosomal recessive inheritance suggested a nuclear gene defect. However recently, two cases have been shown to have a tRNA point mutation (Hammans et al 1991).

2.c.iv.Alpers' syndrome

This syndrome is characterised by the development of intractable seizures and liver failure following a period of developmental delay and failure to thrive in infancy. Very rarely cases may present in early adulthood (Harding 1990).

2.c.v.May White syndrome

May and White described a combination of familial cerebellar ataxia, myoclonus and deafness which probably is synonymous with MERRF (May and White 1968).

2.c.vi.NARP

This syndrome is maternally inherited and includes retinitis pigmentosa, ataxia, dementia, seizures, proximal muscle weakness and sensory neuropathy, in variable combination (abbreviated to NARP; neurogenic weakness, ataxia and retinitis pigmentosia) (Holt et al 1990).
2.c.vii. MNGIE

Myo-Neuro-Gastro-Intestinal Encephalopathy includes ophthalmoplegia, gastric and intestinal dysfunction and peripheral and autonomic neuropathy (Bardosi et al 1987, Blake et al 1990).

2.c.viii. Infantile lactic acidaemias/encephalopathies

These are characterised by a severe neonatal lactic acidaemia with respiratory embarrassment, feeding difficulties, failure to thrive, severe hypotonic weakness, mental impairment, seizures with renal, hepatic and cardiac involvement. They may result from a variety of defects of the respiratory chain and are usually fatal. However a benign form is recognised with cytochrome oxidase deficiency, the initial presentation of which is clinically identical but subsequently shows a gradual gain in muscle strength to virtual normality by age 2 or 3 years old, with an increase in the proportion of muscle fibres with normal cytochrome oxidase activity (DiMauro et al 1983, Zeviani et al 1987). The genetic basis of this is discussed later (see section 5).

2.c.ix. Multiple symmetrical lipomatoses

A combination of multiple symmetrical lipomatoses and peripheral neuropathy with variable manifestations of deafness, myopathy, cerebellar and pyramidal features and seizures has been shown to have evidence of mitochondrial dysfunction in skeletal muscle (Berkovic et al 1991).
2.d. Other Syndromes

2.d.i. Cardiomyopathy

A four week old girl with an isolated fatal histiocytoid cardiomyopathy was shown to have a marked deficiency of cytochrome b (Papadimitriou et al 1984). A one year old boy with fatal infantile cardiomyopathy associated with mitochondrial encephalopathy, lactic acidosis and stroke like episodes has been shown to have a mitochondrial tRNA point mutation (Tanaka et al 1990) (see section 7.f).

2.d.ii. Pearson’s syndrome

This consists of a usually fatal, neonatal pancreatic and hepatic dysfunction, pancytopenia and lactic acidosis (Rotig et al 1989).

2.d.iii. Leber’s Hereditary Optic Neuropathy

Leber’s hereditary optic neuropathy (LHON) is a syndrome of progressive optic nerve degeneration leading to bilateral sequential blindness without ragged red fibres. Although maternally inherited it shows a marked male preponderance perhaps pointing to an interaction between the mtDNA mutation and an X chromosome defect (see sections 5 and 6). In some cases of LHON, more widespread CNS involvement occurs with dystonia (Novotny et al 1986), multiple sclerosis-like symptoms (Harding et al 1992), or encephalopathy (Howell et al 1991).
3. Clinical Evaluation Of The Mitochondrial Myopathies

3.a. Initial Investigations

Routine haematological and biochemical investigations are usually normal with the exception of the sideroblastic anaemia or pancytopenia seen in Pearson’s syndrome. Perhaps the most useful biochemical screen is for serum lactate and pyruvate concentrations. Resting, fasting levels of lactate may be raised, or carefully graded aerobic exercise may show an exaggerated rise and slow recovery of the lactate levels. Occasionally the pyruvate to lactate ratio may be abnormal while the absolute values of each are normal. In cases confined to the CNS, the abnormal lactate, or pyruvate to lactate ratio, may only be seen in the cerebrospinal fluid (CSF). CSF protein may be raised and this is one of the criteria for Kearns Sayre syndrome. An ECG may show cardiac conduction defects.

3.b. Neurophysiology

Electromyography may show myopathic features even in cases with apparently exclusive CNS involvement, but these features are not specific for mitochondrial disease. Similarly EEG may show non-specific evidence of brain dysfunction as well as evidence of myoclonic and, or, generalised epileptiform activity. Nerve conduction studies may show evidence of a sub-clinical peripheral axonopathy which is said to be more often seen in MERRF (Peyronnard et al 1980, Mizusawa et al 1991, Eymard et al 1991). Subclinical hearing loss or retinal pathology may be disclosed by audiometry, and electro-retinograms respectively.
3.c. Imaging Studies

Both computerised tomography (CT) and magnetic resonance imaging (MRI) may be abnormal in cases with CNS disease. Multiple low density CT, or high signal MRI lesions may be seen anywhere in the cerebral hemispheres or posterior fossa. There is a predilection, particularly in Leigh's disease, for the basal ganglia, where there may also be calcification. Sub-cortical white matter lesions may be seen. Wedge shaped lesions resembling vascular infarction are seen especially in the MELAS syndrome. Less specific findings of cerebral and, or, cerebellar atrophy may occur.

3.d. Functional Studies

$^{31}$P Phosphorus nuclear magnetic resonance ($^{31}$P NMR) spectroscopy performed on exercising forearm muscle can show patterns of abnormality which are specific for respiratory chain impairment as opposed to other defects of muscle energy production. There is a low resting phosphocreatine (PCR) to inorganic phosphate (Pi) ratio which falls more rapidly on exercise, with a small drop in intracellular pH. On cessation of exercise, the low pH appears to recover quickly, while recovery of PCR is delayed (Radda et al 1982, Griffiths and Edwards 1987). $^{31}$P NMR spectroscopy of the brain has also revealed a defect of oxidative phosphorylation in one patient with mitochondrial encephalopathy (Hayes et al 1985).

Positron emission tomography (PET) has shown, in three cases with known respiratory chain dysfunction, evidence for reduced cerebral oxygen consumption with glucose metabolism continuing, presumably anaerobically. This is consistent with impairment of oxidative phosphorylation (Frackowiak et al 1988).
3.e. Histocytochemistry

Histological studies can be performed on frozen cross sections of a small muscle biopsy obtained under local anaesthetic from usually, the triceps, quadriceps or vastas lateralis muscle. The modified Gomori trichrome stain (Engel and Cunningham 1963) stains phospholipid red and thus highlights the proliferation of mitochondria characteristic of these diseases. The red granular staining may be confined to the periphery of the fibres but may also be seen within the intermyofibrilar network with disruption of the fibres giving rise to the ragged red fibre appearance (Olson et al 1972). Cytochemistry for the intra-mitochondrial enzyme, succinate dehydrogenase, (part of Complex II) may show a similar pattern of intense staining and may be more sensitive. There may also be excessive deposits of glycogen or lipid occurring as a secondary phenomenon and shown by the periodic acid Schiff (PAS), or sudan black stains respectively. Stains for cytochrome oxidase activity can show negative staining fibres particularly in the CPEO group where they may be more numerous than ragged red fibres.

There is no correlation between the severity of the disease and the frequency of ragged red fibres. In some cases mitochondrial disease may exist without ragged red fibres as in the family described by Holt et al (1990) or the case reported by Turnbull et al (1985) in which only cytochrome oxidase negative fibres were apparent histologically. Conversely the presence of a few ragged red fibres does not necessarily imply a mitochondrial disease, particularly in the elderly.
3.f. Electron-microscopy

As well as being increased in numbers, mitochondria may show a variety of structural abnormalities which can be observed on electron-microscopy (Morgan-Hughes 1983). The mitochondria can be greatly enlarged, have unusual arrangements of cristae, and contain abnormal inclusions. Cristae can be branched, concentric, peripherally located, or transverse within elongated mitochondria. Mitochondria may appear empty, vacuolated, or show granules which are similar to that seen in normal mitochondria, but larger and more prominent. Much bigger osmiophilic bodies may be present. Large regular rectangular structures may be seen either within the cristae or the intermembranous space. These paracrystalline inclusions may occupy part or the whole width of the mitochondria or may be present in small groups of four parallel stacked lines within a common outer membrane; the so-called parking lot inclusions. All these morphological abnormalities appear to be non-specific in that they do not indicate any precise biochemical defect (Morgan-Hughes 1983).

3.g. Biochemistry

Skeletal muscle represents the commonest source of mitochondria for biochemical evaluation of respiratory chain disorders. Mitochondria are extracted by a combination of mechanical and enzymatic disruption of the tissue, followed by differential centrifugation, with a low speed spin pelleting the nuclear elements and unbroken cells, and a high speed spin of the supernatant producing a mitochondrial pellet.
Freshly isolated intact mitochondria are used for oxygen electrode studies. Mitochondrial fractions can be incubated with various substrates, in the presence of limiting amounts of ADP and the consumption of oxygen monitored polarographically. The different substrates feed electrons into the respiratory chain at different points, and this knowledge allows deduction of the site(s) of the defect of the respiratory chain.

![Diagram](image)

**Figure 3.1** Substrates feeding into the respiratory chain

Such measurements test the function of the respiratory chain complexes as a whole as well as the function of the many enzyme processes supporting it. As such this method can be argued to represent a close approximation of the in vivo situation. The results can be classified as shown in Table 3.2 (Morgan-Hughes et al 1979).
Table 3.2 Classification of polarographic results

<table>
<thead>
<tr>
<th>Oxygen utilisation with</th>
<th>NAD-linked substrates</th>
<th>Succinate</th>
<th>Ascorbate + TMPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>No defect</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Complex I deficiency</td>
<td>Decreased</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Complex I-III deficiency</td>
<td>Decreased</td>
<td>Decreased</td>
<td>Normal</td>
</tr>
<tr>
<td>Complex I-IV deficiency</td>
<td>Decreased</td>
<td>Decreased</td>
<td>Decreased</td>
</tr>
</tbody>
</table>

Individual enzyme measurements can be made spectrophotometrically after permeabilisation of the mitochondria, with sonication or freeze thawing, to allow access by substrates. The cytochromes can be assayed using room temperature or low temperature spectroscopy.

3.3. Molecular Biology

Detection of deletions of mtDNA is most readily accomplished by digesting the mtDNA with an endonuclease which cuts the circular genome at a single site thereby linearising it. Where a deletion is present Southern blotting will then reveal two bands, the higher molecular weight wild type mtDNA, and a smaller molecular weight mutant mtDNA. Such analysis is generally done on total DNA extracted from muscle with radiolabelled mtDNA as the probe, without the need to separate out mtDNA. Deletion detection can also be achieved by selective amplification across the break point using polymerase chain reaction (PCR) (Zeviani et al 1990a, Johns et al 1989).
Point mutations of mtDNA may result in the loss or gain of a restriction site (v.i.). PCR can be used to amplify the region of interest, and the PCR product digested with an appropriate endonuclease. Restriction site gain or loss can be detected following agarose gel electrophoresis visualisation of the digest under UV light following ethidium bromide staining. In some cases such as the tRNA$^{ lys}$ point mutation a suitable restriction site is created by minor modification of the PCR technique in which one primer creates a PCR product containing a Bg/I restriction site in the mutant but not the wild type mtDNA (Zeviani et al 1991).

4. The Aetiology Of The Mitochondrial Myopathies

From the foregoing account of mitochondrial respiratory chain function and biogenesis one can propose several mechanisms by which abnormal mitochondrial respiratory chain function may arise. These may be classified as follows;

Table 3.3 Possible aetiologies for the mitochondrial myopathies

<table>
<thead>
<tr>
<th>Possible aetiologies for the mitochondrial myopathies</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Defects of the nuclear genome;</td>
</tr>
<tr>
<td>a) affecting nuclear encoded mitochondrial protein subunits.</td>
</tr>
<tr>
<td>b) regulating mitochondrial genome function.</td>
</tr>
<tr>
<td>c) regulating transport of cytoplasmic proteins into the mitochondria.</td>
</tr>
<tr>
<td>2) Defects of the mitochondrial genome affecting mitochondrial protein synthesis.</td>
</tr>
<tr>
<td>3) Defects of both genomes (bigenomic defects)</td>
</tr>
<tr>
<td>4) Acquired defects of mitochondrial function.</td>
</tr>
</tbody>
</table>
5. Defects Of The Nuclear Genome

To date there has been no direct demonstration of a nuclear genetic defect in any of the mitochondrial myopathies but there has been indirect evidence both clinically and biochemically for such a defect.

Several patients have been reported with deficiencies in components of the respiratory chain known not to have a mitochondrial genomic contribution. An isolated deficiency of Complex II has been reported in at least three cases; a case of a 25 year old woman with Kearns Sayre syndrome (Rivner et al 1989), a 22 year old man with exercise intolerance and myoglobinuria (Haller et al 1990), and a 14 year old girl with myopathy (Garavaglia et al 1990). A CoQ\textsubscript{10} deficiency was found in a 14 year old girl and her 12 year old sister with a familial MELAS syndrome (Ogasahara et al 1989).

Immunoblotting of mitochondrial protein with Complex I specific antibodies in cases with Complex I deficiency has revealed specific deficiencies in the nuclear encoded subunits in 6 out of 18 cases, which were of the 24kDa iron sulphur protein in 3, the 13kDa iron sulphur protein in 1, and both of these subunits in 2 (Schapira et al 1988, Morgan-Hughes et al 1988, 1990). However two of these patients with specific deficiency of nuclear encoded subunits have since been demonstrated to have the tRNA\textsuperscript{Leu(UUR)} point mutation (Hammans et al 1991).

In children, Complex IV deficiency can result in a lactic acidaemia which can be either fatal or benign. Both have initially severe symptoms associated with
cytochrome oxidase negative muscle fibres. However the benign form shows a gradual
gain in muscle strength to virtual normality by age 2 or 3 years old, with an increase
in the proportion of muscle fibres with normal cytochrome oxidase activity, while the
fatal form shows no such improvement and no change in the cytochrome oxidase
activity. This difference in outcome could be attributed to a defect in a nuclear encoded
cytochrome oxidase subunit(s) which is developmentally regulated with a defect of a
foetal isoform which gradually becomes replaced by the normal mature isoform. This
hypothesis has been recently strengthened by immunoblotting studies showing that in
the early stages of both forms of myopathy there is an absence of the nuclearly encoded
COX subunits VIIa,b which subsequently ameliorates in the benign form. However this
study also showed deficiency of the COX II subunit in the benign form which also
improved (Trischler et al 1991).

6. Bigenomic Defects

Several defects of mtDNA have been described which, because of their
autosomal pattern of inheritance, are believed to result from a primary nuclear gene
defect perhaps controlling mtDNA replication. These include autosomal dominant and
recessive cases of multiple mtDNA deletions, and autosomal recessive cases of mtDNA
depletion (see sections 7.b and 7.d).

The male preponderance seen in Leber's hereditary optic neuropathy may result
from an interaction between the mtDNA point mutations seen in these diseases and an
X chromosome gene. Linkage of susceptibility to visual loss was said to occur with an
X chromosome gene closely linked to DXS7, but this has been disputed (Vilkki et al 1991, Sweeney et al 1992a).

7. Defects Of The Mitochondrial Genome

Various types of mtDNA defect have been described since the original description of single deletions in the mitochondrial DNA (Holt et al 1988).

<table>
<thead>
<tr>
<th>Table 3.4 Mitochondrial DNA defects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single deletions</td>
</tr>
<tr>
<td>Multiple deletions</td>
</tr>
<tr>
<td>Duplications of mtDNA</td>
</tr>
<tr>
<td>Depletions of mtDNA</td>
</tr>
<tr>
<td>Point mutations</td>
</tr>
<tr>
<td>of tRNA genes</td>
</tr>
<tr>
<td>of protein coding genes</td>
</tr>
</tbody>
</table>

7.a. Single deletions of mtDNA

The deletions seem to result in two main phenotypes. They are seen in up to 40% of the adult population with a diagnosis of mitochondrial myopathy, all of whom have ophthalmoplegia as a constant feature with limb weakness a frequent accompaniment. Some fulfilled the classic criteria for the Kearns Sayre syndrome but apart from these cases none had major central nervous system involvement. While not all cases with ophthalmoplegia had a mtDNA deletion, the addition of retinopathy or

Deletions have also been described in the lymphocytes of neonates with Pearson’s syndrome (see section 2.d.ii)(Rotig et al 1989, 1990, 1991).

**Figure 3.2** Linearised map of the mitochondrial genome indicating the extent of the deletions in 30 cases.

Within a given individual, the size and site of the deletion is usually constant (see Figure 3.2) but variations in both these parameters are seen in different patients. The size of the deletions in mitochondrial myopathies can range from 1.3 to 7.6kb (Holt et al 1988, 1989b, Zeviani et al 1988, Moraes et al 1989, Goto et al 1990b). Although the site of the deletion can vary there is a predilection for the 7000 to 15 000 bp region which avoids the origins of replication of the heavy and light strands (O_H and O_L), but encompasses genes for subunits ND3, 4L, 4 and 5 of Complex I, COX I, II and III.
subunits, both ATPase subunits and several tRNA's; the so-called "common deletion". Flanking this region is a probable deletion "hot spot" with a 13 nucleotide direct repeat giving rise to the theoretical possibility of replication slippage resulting in the "common deletion" (Zeviani et al 1988, Shoffner et al 1989, Holt et al 1989a), (see Figures 3.3 and 3.4).

![Figure 3.3](image)

Such a slip replication mechanism depends upon the presence of large stretches of complementary single stranded DNA and has been demonstrated in deletions of the human β-globulin genes (Efstratiadis et al 1980). Although similar large single strands of complementary DNA are not a feature of mtDNA replication, many deletions of mtDNA have been found to occur within direct repeat sequences. One survey of deletion break points in 28 patients found that a majority (71%) were flanked by perfect direct repeats of between 5 to 13 base pairs located, in normal mtDNA, at the edges of the deletion. In the remainder of cases such direct repeats were not found or were not so closely related to the break point region (Mita et al 1990).
In contrast to the large numbers of reported deletions located in the large arc of mtDNA between O_L and O_H, only four cases (eg case 2 illustrated in Figure 3.2) are recorded of deletions affecting the smaller arc between O_H and O_L (Holt et al 1989b, Moraes et al 1991b, Johns and Comblath 1991). This may be because of the relative paucity of base pair repeats in this region, or to differences in the secondary structure of the replicating strands (Johns and Comblath 1991).
A small deletion of 0.4 kb involving the ND5 gene was apparently found in all the mtDNA of liver and muscle in a girl with myoclonic epilepsy, ataxia dysarthria and sensorineural deafness (Noer et al 1988). However this claim was later shown to be incorrect and the patient was subsequently found to have a tRNA* point mutation (Seibel et al 1991).

7.b. Multiple deletions of mtDNA

Multiple mtDNA deletions of variable length within an individual, have been described in the muscle of four individual members of a large family who manifested progressive external ophthalmoplegia, limb weakness, cataract and premature death. All deletions had a common origin, being within a twelve nucleotide region of the D-loop, extending over 1000 bp with different break points for each individual (Zeviani et al 1989). The autosomal inheritance of this defect was felt to be indicative of an intergenomic defect (see sections 5 and 6). A second pedigree with similar features has since been reported (Zeviani et al 1990b).

Multiple deletions of mtDNA were found in two brothers of a consanguineous marriage who had CPEO, optic atrophy and an axonal neuropathy. At least twelve different deletions were seen, without a common origin, most of which were flanked by direct repeats (Yuzaki et al 1989).

Multiple mtDNA deletions have also been reported in two brothers with recurrent myoglobinuria (Ohno et al 1991), and in a case of idiopathic cardiomyopathy (Ozawa et al 1990).
7.c. Duplications of mtDNA

Two patients with Kearns Sayre syndrome and insulin dependant diabetes have been found to have tandem direct duplications of their mtDNA (Poulton et al 1989a,b). This abnormality was not seen in the maternal relatives, was heteroplasmic (see section 8.a) and was present in the blood as well as in many other tissues. The duplications were about 8kb long and were similar to the undeleted regions of mtDNA seen in the cases with single deletions. In both cases the COX I gene was interrupted.

![Figure 3.5 Duplication of mitochondrial DNA](image)

7.d. Depletion of mtDNA

Generalised depletion of mtDNA has been demonstrated in the brain kidneys and liver in a case of infantile lactic acidosis with a suspected autosomal recessive inheritance (Arnaudo et al 1991). Four cases with fatal infantile cytochrome oxidase deficiency variously manifesting with myopathy, CPEO, liver and renal tubular failure,
also had severe depletion of mtDNA (Moraes et al 1991a). A failure of mtDNA replication has been suggested as the basis for the low copy number of mtDNA.

7.e. Point mutations of mtDNA protein coding genes

7.e.i. Associated with LHON

Several point mutations have now been found in association with Leber's hereditary optic neuropathy (LHON), (see Table 3.5). The commonest, seen in two thirds of cases, is the G to A base change at position 11,778 in the ND4 gene which changes a highly conserved arginine at position 340 to histidine (Wallace et al 1988a, Vilkki et al 1989, Holt et al 1989c). Johns and Berman (1990) found multiple, sometimes simultaneous, point mutations, including the 11778 mutation described above, but also what was felt to be pathologically significant 4917, 4261 and 13708 mutations affecting the ND2, ND1 and ND5 genes respectively. Also described is a G to A base substitution at position 3460 changing an alanine to threonine in the ND1 subunit (Huoponen et al 1991). A large Queensland pedigree with LHON which is particularly aggressive, and associated with neurological abnormalities such as severe infantile encephalopathy, ataxia, tremor, dysarthria, spasticity and posterior column signs, has been found to have a T to C base change at position 4160. This changes a highly conserved leucine at position 285 of the ND1 gene for a proline and is predicted to disrupt a small $\alpha$ helix in a hydrophilic loop. In addition, a branch of this pedigree, with less severe ophthalmological, and lacking the neurological, features had an additional point mutation at position 4136. Here an A to G transition results in a substitution of a cysteine for tyrosine at position 277 of the ND1 subunit. This second
mutation is suggested to function as an intragenic suppressor mutation which ameliorates the clinical features (Howell et al 1991).

Table 3.5 Point mutations of mtDNA

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Position</th>
<th>Substitution</th>
<th>Gene affected</th>
<th>Method of Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>MERRF</td>
<td>8344</td>
<td>A to G</td>
<td>tRNA$^{ly}$</td>
<td>Bgl I gain</td>
</tr>
<tr>
<td>MELAS</td>
<td>3243</td>
<td>A to G</td>
<td>tRNA$^{lu}$</td>
<td>Apa I gain</td>
</tr>
<tr>
<td></td>
<td>3271</td>
<td>T to C</td>
<td>tRNA$^{lu}$</td>
<td>Afl II gain</td>
</tr>
<tr>
<td></td>
<td>3260</td>
<td>A to G</td>
<td>tRNA$^{lu}$</td>
<td>Xmn I gain</td>
</tr>
<tr>
<td></td>
<td>3250</td>
<td>T to C</td>
<td>tRNA$^{lu}$</td>
<td>Nae I gain</td>
</tr>
<tr>
<td></td>
<td>3252</td>
<td>A to G</td>
<td>tRNA$^{lu}$</td>
<td>-</td>
</tr>
<tr>
<td>Infantile cardiomyopathy</td>
<td>4317</td>
<td>A to G</td>
<td>tRNA$^{le}$</td>
<td>Afl II gain</td>
</tr>
<tr>
<td>NARP / Leigh disease</td>
<td>8993</td>
<td>T to G</td>
<td>ATPase 6</td>
<td>Ava I gain</td>
</tr>
<tr>
<td>LHON</td>
<td>11778</td>
<td>G to A</td>
<td>ND4</td>
<td>SfaNI loss</td>
</tr>
<tr>
<td></td>
<td>4160</td>
<td>A to G</td>
<td>ND1</td>
<td>Mae III / Bst XI gain</td>
</tr>
<tr>
<td></td>
<td>3460</td>
<td>G to A</td>
<td>ND1</td>
<td>Acy I loss</td>
</tr>
</tbody>
</table>

7.e.ii. Associated with NARP or Leigh Syndrome

A point mutation, substituting a T for a G at position 8993 in the gene for subunit 6 of ATPase was found in the family with NARP. This base substitution results in the conversion of a highly conserved hydrophobic leucine for a hydrophilic arginine at position 156 of the ATP 6 subunit. (Holt et al 1990). Recently the same mutation has been reported in another family, one member of which had similar features to that described by Holt et al (1990), while three maternally related members died in infancy, with Leigh disease pathologically proven in one (Tatuch et al 1992).
7.f. Point mutations of mt tRNA genes

Point mutations of three transfer RNA genes are now recognised.

7.f.i. tRNA\textsuperscript{by}

The A to G transition at base pair 8344 affecting the pseudouridine (Tc) loop of the tRNA\textsuperscript{by} was described in association with MERRF (Shoffner et al 1990). Although it is not invariably present in all cases of MERRF, the presence of this mutation seems to be consistent with the core features of this syndrome (Zeviani et al 1991, Seibel et al 1990, Hammans et al 1991).

![Diagram of tRNA\textsuperscript{by} mutation](image)

Figure 3.6 Point mutation in the tRNA\textsuperscript{by}

7.f.ii. tRNA\textsuperscript{leu(UUR)}

The A to G transition at base pair 3,243 affecting the dihydrouridine loop of the tRNA\textsuperscript{leu(UUR)} gene has been found in cases of MELAS (Goto et al 1990, Kobayashi et
al, 1990, Ino et al, 1991) but a subsequent survey screening for this mutation found that the clinical features were more variable with only half having the MELAS phenotype. The rest had combinations of myopathy, ataxia and deafness, progressive external ophthalmoplegia or myopathy alone (Hammans et al, 1991). Four additional point mutations within this gene have also been reported in association with major CNS disease (see Table 3.5) (Wallace et al, 1991, Morten et al, 1992).

![Figure 3.7 Point mutation in the tRNA^{ile(URI)}](image)

7. f. iii. tRNA^{ile}

A point mutation of A to G at position 4,317 in the tRNA^{ile} has been described in a one year old boy with fatal infantile cardiomyopathy, mitochondrial encephalopathy, lactic acidosis and stroke like episodes (Tanaka et al, 1990).
8. General Properties Of Mitochondrial DNA Defects

8.a. Heteroplasmy

In the initial reports of the 11788 point mutation associated with Lebers hereditary optic neuropathy, all the mtDNA genomes were affected (Wallace et al 1988). The two mutations described in the Queensland pedigree are present in all the mtDNA (Howell et al 1991).

More usually however mtDNA mutations do not affect all the mitochondrial genomes thus resulting in a mixed population of wild type and mutant mtDNA; a situation termed heteroplasmy. The degree of heteroplasmy may vary in the different tissues of any one individual.
The original detection of the deletions was by Southern blotting and the defect was only detectable in the muscle mtDNA and not the leucocyte mtDNA (Holt et al 1989). Several autopsy studies (Moraes et al 1989, Shanske et al 1990, Zeviani et al 1990) have demonstrated variable proportions of the deletion containing mtDNA in different tissues with percentage mutant mtDNA ranging from 4% in smooth muscle, 14% in liver, 40% in heart and kidney, and 50% in skeletal muscle. The proportion of abnormal mtDNA was however constant in different samples obtained from the same tissue. By contrast different areas of the brain did show variation, with 15% in the cerebellum compared with 44% in the frontal lobe, reflecting the different phylogenetic origin of these two areas of the brain.

One post-mortem study of a patient with MELAS and the tRNA^{leu(UUR)} mutation showed heteroplasmy, but in five tissues the percentage of mutant genomes was similar ranging from 79 to 88% (Ciafaloni et al 1991).

Another post-mortem study of a T to G mutation within the ATPase 6 gene at 8993 in an infant who died of Leigh's disease showed greater than 95% mutant mtDNA in brain, kidney, and liver (Tatuch et al 1992).

8.b. Inheritance Of Defects

The originally described deletions appear to be sporadic rather than showing the maternal inheritance predicted for a mtDNA disorder. Only a few reports of familial ophthalmoplegia associated with a single deletion have been published (Holt et al 1989b). In the case of a mother and daughter with ophthalmoplegia reported by Ozawa
et al (1988) the deletion was different in the two individuals. Subsequent mtDNA mutations, in particular the point mutations whether of protein or tRNA genes, have shown maternal transmission, while multiple deletions and depletions of mtDNA have shown autosomal inheritance.

8.c. Association With Ragged Red Fibres

Not all the diseases associated with mtDNA defects are associated with ragged red fibres on muscle biopsy and so would not be classically labelled as "mitochondrial myopathies". Mutations involving tRNAs, whether deletions or point mutations, are commonly associated with ragged red fibres. Exceptions include one case who had the tRNA_{ly} mutation, and two with the tRNA_{leu(UUR)} point mutation who did not have ragged red fibres (Hammans et al 1991). By contrast mutations affecting only protein coding genes as seen in Leber's hereditary optic atrophy, the cases of NARP (Holt et al 1990) and the case of Leigh's disease (Tatuch et al 1992) did not show ragged red fibres.

9. Defects Of Mitochondrial Protein Import

Defects of protein import into mitochondria have been implicated in human mitochondrial disease.

Fibroblasts cultured from a patient with methylmalonic aciduria had a defect in the import of the non-respiratory chain matrix enzyme methylmalonyl co-enzyme A mutase (Fenton et al 1987). This has been found to be due to a nonsense point
mutation in the DNA coding for the leader aminoacid sequence required for the correct targeting and translocation of this enzyme (Ledley et al 1990).

Schapira et al (1990c) reported a case of a 14 year old girl with congenital myopathy whose muscle showed no ragged red fibres, had absent staining for succinate dehydrogenase activity, and had cytochrome oxidase negative fibres. Immunoblotting studies showed that the 22.5kD Rieske protein of Complex III was present in the muscle homogenate and cytosol, in a precursor form with an apparent molecular mass of 26kD, but not in the mitochondrial fraction suggesting a defect in the transport of this protein into mitochondria.

10. Acquired Defects Of Mitochondria

Several acquired defects of mitochondrial function have been postulated to be of relevance to human disease. Mitochondrial function can also be disturbed by toxins or drugs with symptomatic effect.

10.a. Parkinson’s Disease

A specific deficiency in Complex I has been reported in the substantia nigra of patients with Parkinson’s disease. This defect seemed to be disease specific as it was not seen in the control group, nor in patients with Parkinson’s-like disease of Multiple Systems Atrophy (Schapira et al 1989, 1990a). Reduced Complex I activity was also seen in the platelets of patients with Parkinson’s disease (Parker et al 1989). A syndrome identical to Parkinsons disease also results from exposure to the neurotoxin
1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP is converted to the active toxin 1-methyl-4-phenyl-pyridinium (MPP⁺) by the mitochondrial enzyme monoamine oxidase B and then selectively concentrated in the mitochondria of dopaminergic neurones where it specifically inhibits Complex I activity leading to neuronal cell loss (Ramsay et al 1989, Langston et al 1983). These two observations raise the intriguing possibility that the idiopathic form of Parkinson’s disease may be the result of a endogenous or exogenous neurotoxin which either inhibits Complex I directly, or specifically affects mtDNA synthesis of ND subunits. There is evidence for ongoing toxic exposure to free radicals in Parkinson’s disease with increased mitochondrial superoxide dismutase activity (Saggu et al 1989), and increased basal lipid peroxidation (Dexter et al 1989), and this may be damaging to mtDNA.

10.b. Zidovudine Induced Myopathy

Zidovudine (AZT) is a dideoxy nucleoside triphosphate used in the long term treatment of human immunodeficiency virus (HIV) infection. A proportion of patients so treated develop an inflammatory myopathy characterised histologically by the proliferation of abnormal mitochondria with ragged red fibres (Dalakas et al 1989). Such patients have also been shown, by Southern blotting, to have an up to 78% reduction in mtDNA content (Arnaudo et al 1991). This may be the result of AZT’s action on the DNA polymerase γ responsible for mtDNA replication since it can act as a substrate for this enzyme.

10.c. Autoimmunity

Schapira et al (1990b) described the case of a 15 year old boy with
encephalomyopathy characterised by focal myoclonus, generalised seizures, dementia, cerebellar ataxia deafness and recurrent stroke-like episodes. Biochemical analysis showed a Complex I deficiency and low cytochrome aa3, while immunoblotting revealed a deficiency of the nuclear encoded 24 kD FeS protein of Complex I and of the mitochondrially encoded subunit II of complex IV. In addition there was a circulating antibody to an unidentified, nuclear encoded 41 kD mitochondrial matrix polypeptide which was not a component of the respiratory chain. This protein appears to have an important role since it was remarkably conserved, being present not only in several tissues, but in several species, including human, bovine, rabbit and rat. Whether this antibody is relevant to the pathogenesis of the disease in this patient is unclear. The mechanism of such an aetiology is therefore speculative, but might involve a role for the 41 kD protein in the processing of both nuclear and mitochondrial encoded protein subunits.

A clinically similar case described by Skoglund (1979) had an unidentified antimitochondrial antibody and was markedly steroid responsive. This clinical improvement may have been the result of immunosupression of the antibody. Steroid responsiveness has been noted in other cases (Shapira et al 1975). There has also been an association of Kearns Sayre syndrome with other autoimmune diseases such as hypoparathyroidism (Pellock et al 1978, Horwitz and Roessmann 1978).

There are strong clinical grounds for suggesting that autoimmunity underlies the pathogenesis of LHON (Harding et al 1992). The identification of the murine maternally transmitted histocompatibility antigen as a component of ND1 (Loveland et
al 1990) may provide a mechanism by which mtDNA defects may alter immune systems.

10.d. Viral

A viral aetiology for Kearns Sayre syndrome has been proposed (Berenberg et al 1977).

11. The Expression Of Mitochondrial DNA Defects

Contrasting with the expanding list of mtDNA defects being recognised in mitochondrial disease is the paucity of information on how these various genetic defects give rise to the widely variable phenotypes seen, both clinically, and biochemically. The precise role of the various mtDNA defects in the aetiology of these diseases is unclear.

11.a. Relationship between mtDNA defects and clinical features

Mention has been made of the clinical correlations with some of the mtDNA defects. For the point mutations the association may be good as in the case of the mutations seen in LHON, or the tRNA^lys mutation and the core features of the MERRF syndrome. The clinical correlates are less firm for the tRNA^lea point mutations; only 10 out of 27 index cases with this mutation had MELAS syndrome with the rest having myopathy alone, CPEO, other encephalomyopathies, or gastrointestinal renal or endocrine manifestations (Sweeney et al 1992b). The deletions have all had ophthalmoplegia as a common characteristic but there may be a variable number of other clinical features with some fulfilling the classical criteria for the Kearns Sayre
syndrome. But cases of ophthalmoplegia including cases of classic KSS exist for whom a mtDNA deletion is not found (Holt et al 1988, 1989b). Recently point mutations of either the tRNA$^{\text{lys}}$ or tRNA$^{\text{leu(UUR)}}$ genes have been reported in cases of CPEO, though not with the classic Kearns Sayre phenotype (Hammans et al 1991). Thus in the majority of cases there is little relationship between the precise clinical presentation and the genetic abnormality. There is variation in the range of tissues affected, the severity of the tissue involvement and the age of onset of the disease.

For the nuclear gene defects, the variability in the range and severity of tissue involvement seen clinically, can be explained on the basis of tissue specific isoenzymes. Probes for the 24kD FeS protein of Complex I have revealed the possibility of more than one expressed gene for this product (Pilkington and Walker 1989). Two separate genes are known to encode the subunits of mitochondrial ADP/ATP translocase (Houldsworth and Attardi 1988) and tissue specific variations of this enzyme are known to be expressed (Powell et al 1989). There is also good evidence for tissue specific isoforms of Complex I (Clay and Ragan 1988) and IV (Kuhn-Nentwig and Kadenbach 1985) subunits. The compact but limited genetic information contained in the mitochondrial genome does not code for tissue specific polypeptide iso-forms. However post-transcriptional processes can account for variation in the tissue expression of ND5 (Attardi et al 1989).

For mtDNA defects, additional variation in tissue expression may be the result of differing degrees of heteroplasmy for the mutant genomes. Mitochondrial DNA single deletions may be more widespread in the full Kearns Sayre syndrome phenotype
as compared with incomplete cases (Zeviani et al 1988). The two phenotypes of Kearns Sayre and Pearson's syndromes seen with single deletions of mtDNA may be the result of the latter having a higher proportion of mutant versus wild type mtDNA in a greater variety of tissues; a theory supported by the fact that in one case of Pearson's syndrome mutant mtDNA made up 80-90% of the total blood mtDNA compared with consistently less than 10% seen in the blood samples from cases of mitochondrial myopathy. The report of a case of Pearson's syndrome which survived to develop a Kearns Sayre syndrome suggests a change of phenotype occurring with decline of the proportion of mutant mtDNA in dividing tissues (McShane et al 1990). The supposition in this case was that the mutant mtDNA was selected out in the rapidly dividing cell lines such as the haemopoetic stem cells, leading to amelioration of symptoms in those tissues.

Variations in the clinical phenotype in family members with tRNA<sup>lyt</sup> point mutations and the T to G mutation at 8993 have been correlated with the percentage of mutant mtDNA (Shoffner et al 1990, Tatuch et al 1992).

However, although the percentage of mtDNA with a deletion, in the muscle of different patients varied from 20 to 80% there was no correlation with the severity of the biochemical defect (Holt et al 1989b). It might therefore be the absolute number of normal mitochondrial genomes that determines the extent of tissue involvement, particularly if mutant mtDNA is non-functional.

For both nuclear and mitochondrial genome defects, further variability of disease expression may occur depending on the energy requirements of the different tissues and
their reliance on oxidative phosphorylation. This balance may not remain constant throughout life since mitochondrial function declines with age (Trounce et al 1989) and this may result in worsening of pre-existing abnormalities, or the emergence of new symptoms and signs.

11. b. Relationship between mtDNA defects and the biochemical abnormalities.

The relationship between the biochemical deficiencies and either the clinical or mtDNA defect is tenuous. The benign form of infantile myopathy seems to be consistently related to cytochrome oxidase deficiency, but this is likely to result from a nuclear DNA mutation. Usually it is impossible to predict the biochemical impairment from either the clinical features or the mtDNA abnormality.

One of the confounding variables is the common occurrence of combined respiratory chain deficiencies in the mitochondrial myopathies. Considering the multiplicity of polypeptides, from separate genomes, that comprise the functional entity that is the respiratory chain, one must appreciate that a complex system of intergenomic and intragenomic regulation must underlie such organisation. It should not therefore be surprising that defects of one element may cause perturbations elsewhere in the system. Several examples of a possible defect of intergenomic regulation have already been mentioned (v.s.). The occurrence of multiple biochemical abnormalities does make correlation with any specific mtDNA defect difficult since it may be hard to establish which of the biochemical abnormalities is the primary defect rather than merely a secondary phenomenon. Combined deficiencies of Complexes I and IV are
common. Cases with severe Complex I deficiency and a selective defect of the nuclear encoded 24kD and, or 13kD FeS subunits, frequently had also, a selective deficiency of subunit II of cytochrome oxidase (Schapira et al 1988). Complex I deficiency with a mtDNA deletion confined to protein coding genes for ND subunits and not involving COX subunit genes, could nevertheless be associated with reduced cytochrome oxidase activity and low levels of the Complex IV associated cytochrome aa₃ (Morgan-Hughes et al 1990). However these cases appear to have normal Complex IV activity in vivo, as judged by the normal utilisation of succinate or ascorbate plus TMPD measured polarographically, suggesting that the latter defects are functionally less important and may be secondary to the Complex I deficiency.

For mtDNA deletions some biochemical correlation has been made in those cases with an isolated defect of Complex I activity, measured polarographically. In 5 out of 6 such cases the mtDNA deletion involved protein coding genes for Complex I subunits only, but also deleted the intervening tRNAs, with the deletion extending to involve the two ribosomal RNAs in 1 case. In more generalised respiratory chain deficiencies, the deletion was more extensive, encompassing 4 ND subunits, 1 or 2 COX subunits and both ATPase subunits as well as intervening tRNAs. However similar extensive deletions were associated with normal polarography in 7 cases, as well as accounting for the 1 out of 6 cases with Complex I deficiency only.

For point mutations, measurements of mitochondrial enzyme activities in platelets have shown a deficiency of Complex I in some cases of LHON consistent with the defect in mtDNA ND gene(s) (Parker et al 1989).
11.c. The role of "complementation"

The fact that a deletion confined to ND subunit reading frames does correlate with a pure Complex I defect, despite loss of intervening tRNAs, suggests that the missing tRNAs are available from normal genomes within the same organelle, thus facilitating the expression of the intact reading frames of the mutant mtDNA. This cooperation between mutant and normal mtDNA genomes, is termed "complementation". The frequent preservation of the levels of cytochrome b, whose reading frame is not affected by any of the deletions, even in cases with as high a proportion of deleted genomes as 80%, also argues the case for complementation. If complementation did not occur, even in heteroplasmic organelles, all deletions regardless of size or site, would express the same biochemical phenotype since even intact reading frames of mutant mtDNA could not be translated.

The degree of complementation may be a variable phenomenon, thus accounting for the differences in biochemical expression of identical deletions. This in turn may depend on the relative proportions of mutant and wild type mtDNA within mitochondria. At one extreme, would be homoplasmic organelles, as illustrated in Figure 3.9, where the segregation of mutant mtDNA from normal mtDNA, prevents complementation for the missing tRNAs and therefore the expression of any of the intact reading frames of the mutant mtDNA. At the other end of the spectrum would be heteroplasmic organelles, as illustrated in Figure 3.10, which might be able to compensate for the missing tRNAs of mutant mtDNA.
Figure 3.9 Mitochondria heteroplasmic for a mtDNA deletion

Figure 3.10 Mitochondria homoplasmic for a mtDNA deletion
An additional variable affecting the degree of complementation might be the length of the deletion and consequently the number of tRNAs that have been deleted. Patients with more extensive deletions tended to have more comprehensive respiratory chain defects polarographically (Hammans et al. 1991).

11.d. Expression of mtDNA deletions

In situ hybridisation studies in a single case, have shown that the segmental abnormalities of COX staining and "ragged red" changes are associated with aggregations of mutant mtDNA with reduced levels of wild type genomes. Abundant mutant transcripts were detected in these regions but no translation products could be found (Mita et al. 1989). Transcription, but not translation, of mutant mtDNA was also demonstrated in a cloned fibroblast cell line containing 60% mutant mtDNA with a deletion (Nakase et al. 1990). Northern blot analysis of total cellular RNA from skeletal muscle and EBV transformed cell lines from two patients with the tandem duplication has confirmed the presence of mutant RNA conforming to the predicted transcript (Poulton et al. 1989c). It is not known whether this transcript is translated. Failure of translation has been attributed to the lack of tRNAs (Mita et al. 1989), suggesting that complementation does not occur, in contrast to the conclusion from the biochemical studies cited above. In situ studies in two cases with mtDNA deletion, by Shoubridge et al. (1990) found normal levels of wild type mtDNA transcripts in ragged red fibres. They therefore proposed an alternative hypothesis, that such mutant mtDNA were functionally dominant and disrupted the expression of adjacent normal mtDNA.

Histochemical analysis of muscle biopsies from 23 patients with mitochondrial
myopathy showed that those patients with deletions not affecting COX subunits had significantly less focal cytochrome oxidase deficiency relative to the number of ragged red fibres. Polarographic measurements on this sub-group showed isolated Complex I deficiency rather than the more widespread abnormalities seen in the rest (Hammons et al 1992). Thus not all deletions are identical in either their histochemical or biochemical effects suggesting that complementation does occur to some extent in some of these patients.

Deletions of the smaller arc of mtDNA between \( O_h \) and \( O_L \) may extend close to or partly into the mitochondrial transcription factor 1 binding site regulating the light strand promoter region (Topper and Clayton 1989). In theory this could affect expression of the genes on that strand, i.e. ND6, and 8 of the tRNAs. Small arc deletions may also affect the heavy strand promotor (Holt et al 1989b, Moraes et al 1991b).

11.e. The presence and effect of "fusion" proteins

The common deletion of 4977bp flanked by a 13bp direct repeat, results in the merging of nucleotide position 8483 in the ATPase 8 gene to position 13460 in the ND5 gene. The resultant frame shift in the fusion gene generates a premature stop codon 12 nucleotides downstream of the break point (Holt et al 1989a). The tandem duplication results in a truncated COX I gene (Poulton et al 1989c). These fusion genes, if transcribed and translated would give rise to fusion proteins. The existence of such proteins would therefore be conclusive proof of complementation in the case of the deletions. Abnormal fusion proteins might interfere with the assembly of functional
respiratory chain complexes by completing with normal products.

11.f. Mechanisms of disease expression applicable to point mutations of tRNAs

Single base substitutions affecting protein coding genes are well recognised to result in human disease. Complex I deficiency has been reported in the platelets of some patients with LHON where there is such a mutation affecting the ND1 subunit. Mechanisms applicable to the point mutations of mitochondrial tRNAs are less well understood.

The tRNA\textsuperscript{lys} point mutation occurs in the Tc loop (see Figure 3.6) and since this is believed to interact with the ribosome, this may affect the incorporation of lysine residues into mitochondrial polypeptides. This would be consistent with the finding of multiple respiratory chain defects in MERRF (Wallace et al 1988b, Shoffner et al 1990).

Difficulties with leucine incorporation may occur in the tRNA\textsuperscript{leu(UUR)} mutation but since the H strand transcription termination site is located at the boundary of the 16SrRNA and the tRNA\textsuperscript{leu(UUR)} genes an additional mechanism may apply. The mutation has been found to be embedded in a tridecamer sequence responsible for the formation of the 3\textsuperscript{1} ends of 16SrRNA and has been shown to result in severe impairment of the 16SrRNA transcription termination correlating with reduced binding of the termination (mTERM) protein (Hess et al 1991). This might lead to a defect in the proportion of rRNA transcribed relative to other RNA species.
Since tRNAs often serve as "punctuation marks" between intervening protein coding genes (see page 28) changes in their secondary structure might interfere with the precise cleavage of the polycistronic transcript.

For all the tRNA defects the deleterious effects may be modified by complementation as may occur for the mtDNA deletions.

11.g. Intragenic suppressor gene mutations

Not all mutations may be deleterious. The defects found in LHON detailed above suggest that there may be "sets" of point mutations contributing to the disease phenotype, with at least one acting as an intragenic suppressor gene. In this respect it is interesting to note that the abnormal platelet Complex I activity reported by Parker et al (1989) was seen in the more severely affected members of the Queensland pedigree who lacked this 4136 point mutation (see page 68).
CHAPTER 4: IN VITRO MITOCHONDRIAL PROTEIN SYNTHESIS

1. Introduction

The original experiments on mitochondrial protein synthesis were performed in the late 1950's at a time when the synthetic capability of mitochondria was in doubt. Although in 1950 Ephrussi had suggested the existence of extra-chromosomal genetic factors in yeast mitochondria it was more than 10 years before mitochondrial DNA was first visualised and then isolated (see page 15).

In vivo experiments involving the injection of radiolabelled amino acid into rats or guinea pigs, and following the label's incorporation into the various sub cellular compartments seemed to show that the labelled amino acids were rapidly incorporated into the microsomal fraction, and only very slowly into the mitochondrial fraction (Siekevitz 1952, Zamecnik and Keller 1954). It was felt that this slow rate of incorporation reflected migration of synthesised proteins from the microsomal fraction or contamination of the mitochondria with microsomes. This misconception may have been the result of the preferential use of liver tissue which has a particularly rich and synthetically active microsomal fraction. The presence of bile salts also made the extraction of a mitochondrial fraction free from microsomal contamination difficult (Kroon 1963). In retrospect it also seems likely that bacterial contamination of the mitochondrial preparations was an additional factor obscuring the true magnitude of mitochondrial protein synthesis (see sections 4 and 5), (Beattie et al 1967). When these
in vivo studies were applied to muscle it became apparent that the rate of incorporation of label into mitochondria was comparable with that seen with the microsomal fraction (Simpson et al 1957).

Subsequently successful incorporation of labelled aminoacid into isolated mitochondria from muscle (McLean et al 1958) and liver (Reis et al 1959, Bates et al 1958) was reported. Unfortunately most of the results have been expressed in cpm radiolabelled amino acid incorporated per mg mitochondrial protein making direct comparison between different workers impossible.

2. Mitochondrial Preparations

The early experiments used large amounts of mitochondria and large reaction volumes. Reis et al (1959) used mitochondria prepared from 3g rat liver in 5.5ml of incubation medium. Roodyn et al (1961) used reaction volumes of 3 to 6ml containing 10 to 20mg of mitochondria and found that the optimum concentration of mitochondria for their system was 4mg/ml if incubation was continued for 2 hours, or 2mg/ml for a 1 hour incubation. Beattie et al (1967) employed an incubation volume of 2ml containing 2-3 mg/ml of mitochondrial protein. Towers et al (1972) reduced the volume of medium to 0.5ml with 0.5-1 mg of mitochondria being added. For studies using human mitochondria, 200 to 600 μg were required in 0.5ml of medium (Marzuki et al 1988).

The vast majority, up to 90%, of the radioactive incorporation was into the inner
membrane fraction of the mitochondria (Beattie et al 1967). Inner membrane particles prepared by the digitonin method were also able incorporate radiolabel in a rate and manner comparable with intact mitochondria, but the response to the succinate energy system was lost. Bhat et al (1982) found an enhancement of the rate of radiolabel incorporation using digitonin at concentrations of up to 50\mu g/mg protein, with a substantial fall off in incorporation at higher concentrations. This enhancement may be due to the removal of lysosomes and hydrolytic enzymes by the digitonin or due to swelling of the mitochondrial particles consequent on the removal of the outer membrane (Bhat et al 1982). Inner membrane fractions obtained with sonication, Ficoll or phospholipase methods did not incorporate labelled amino-acid (Beattie et al 1967, Towers et al 1972). However Kroon (1973) found that sonicated mitochondrial particles had a higher level of amino acid incorporation than intact mitochondria, and an altered response to energy systems.

Treatment of the mitochondria with freezing, or with hypotonic sucrose during extraction or fractionation procedures decreased the amino acid incorporation in parallel with the reduction in respiratory control and ADP:O ratios seen with oxidative phosphorylation studies. This suggested that a degree of structural integrity of the inner membrane was required for successful in vitro protein synthesis (Beattie et al 1967, Towers et al 1972).
3. Energy Systems

It was soon recognised that mitochondrial protein synthesis was ATP dependant (McLean et al 1958). Reis et al (1959) established that incorporation of amino acids into isolated rat liver mitochondria had similar requirements as for oxidative phosphorylation. The rate of decline in both processes was similar when inhibited by lack of oxygen, or the presence of dinitrophenol (DNP) or cyanide (Reis et al 1959, Roodyn et al 1961).

ATP on its own was able to support in vitro protein synthesis but not as effectively as an external ATP generating system (McLean et al 1958). McLean et al utilised two different ATP generating systems; one depending on creatine phosphate and the other depending on d-3-phosphoglycerate. For both there was a relative requirement for added ADP due to the presence of endogenous ADP. Liver soluble fraction was also present and was a source of the enzymes appropriate for the energy substrate used viz. phosphoglycero-mutase, enolase and pyruvate kinase. Creatine kinase was added where creatine phosphate was used.

Lederman and Attardi (1970), using an external ATP generating system consisting of 2mM ATP together with sucrose, GTP, creatine phosphate and creatine phosphokinase, showed that the incorporation of tritiated leucine was indeed sensitive to inhibitors of the oxidative phosphorylation system such as dinitrophenol or oligomycin. The inhibition produced by DNP was not absolute, the insensitive
proportion possibly reflecting ATP production by substrate level phosphorylation (Lederman and Attardi 1970).

Another external energy system was used by Towers et al (1972) consisting of 2.5mM ATP, 12.5mM phosphoenol pyruvate, 5eU pyruvate kinase and 10μ oligomycin /ml, and was claimed to be independant, i.e. exogenous of the respiratory chain.

Kroon (1973) found that amino acid incorporation using submitochondrial particulate fractions prepared by sonication depended on ATP generated by oxidative phosphorylation and that there was no benefit in adding an oxidisable substrate.

Beattie et al (1979) describe a unique energy system consisting of glutamate and atracyloside, a specific inhibitor of adenine translocase. This gave a ten fold increase in the rate of amino acid incorporation seen with external ATP generating systems and five times that seen with glutamate and ADP.

Marzuki et al (1988) found that human placental mitochondria could incorporate 35S methionine in the absence of exogenous ATP (see section 10).

4. Other requirements

Tricine or bicine buffer systems were superior to tris (Good et al 1966, Beattie et al 1967). The oxidative phosphorylation capacity of isolated mitochondria is unchanged by the substitution of bicine for tris (Beattie et al 1967), but it is possible
that the spontaneous hydrolysis of tRNA
 is accelerated by tris (Heredia and Halvorson 1966). K⁺ ions are preferred to Na⁺ or NH₄⁺ ions for maximum incorporation but concentrations varying from 30mM to 120mM have been used. The presence of EDTA and phosphate is advantageous, while there is an absolute requirement for Mg²⁺ the optimum concentration of which may be species specific (Beattie 1979, Towers et al 1972, Marzuki et al 1988).

Initial work suggested a dependence of in vitro mitochondrial protein synthesis on the presence of cell sap, (Reis et al 1959). Boiling followed by discarding of the coagulated protein, or pre-treatment with ribonuclease did not alter the beneficial effect of cell sap, suggesting that the responsible factors were of low molecular weight, probably amino acids (Roodyn et al 1961). Cell sap has now been effectively substituted by amino acid mixtures (Reis et al 1959, Roodyn et al 1961). Incorporation rates were maximised by the presence of a full set of amino acids and the omission of any of the amino acids did reduce the rate of protein synthesis. This reduction was small for most of the amino acids but more substantial when either serine, proline or methionine were omitted (Beattie et al 1967).

Also present in cell sap were adenine nucleotides. The supplementation of additional adenine nucleotides to boiled cell sap, was able to prolong the in vitro oxidative phosphorylation of the mitochondria by up to 2 hours at 30°C but the in vitro amino acid incorporation was falling off after 1 hour (Reis et al 1959). The further supplementation of the system with hexokinase and glucose, acting as an acceptor for energy rich phosphate bonds, enabled amino acid incorporation to continue linearly for
up to 2 hours (Reis et al 1959). It was also suggested that the presence of hexokinase and glucose lowered the concentration of extra-mitochondrial ATP hence minimising the effect of microsomal contamination (Roodyn et al 1961).

5. Bacterial Contamination

As mentioned above, the original in vitro mitochondrial protein synthesis experiments were probably confounded by the presence of bacterial contamination. Due to their similar size any bacteria present would tend to be concentrated in the mitochondrial fraction. The use of sterile solutions and aseptic technique reduces such contamination. Everett et al (1980) quote levels ranging from 70 to 1400 bacteria per ml, as not affecting the results of mitochondrial protein synthesis. Towers et al (1972) routinely recorded less than 100 bacteria per incubation and rejected experiments recording more than 1000 bacteria per incubation.

6. Effects Of Inhibitors

Amino acid incorporation by isolated mitochondria was not inhibited by even high concentrations of cycloheximide in concentrations up to 500μg/ml in contrast to the inhibition seen with microsomal amino acid incorporation at concentrations as low as 0.25μg/ml (Ashwell and Work 1968). This loss of inhibition could not be explained on the basis of impermeability of the mitochondrial membrane to cycloheximide since it persisted even following permeabilisation procedures (Beattie et al 1967), and uptake of cycloheximide into the mitochondria could be demonstrated (Ashwell and Work
Chloramphenicol was found to markedly inhibit mitochondrial amino acid incorporation (Kroon 1963, Wheeldon and Lehninger 1966, Beattie et al 1967). Protein synthesis by intact rat liver and in yeast mitochondria was also strongly inhibited by mikamycin, carbomycin, and spiromycin but the mammalian mitochondria differed from yeast mitochondria in that its protein synthesis was insensitive to erythromycin, lincomycin, and paromycin. The insensitivity to the latter antibiotics was not altered by osmotic damage to the mitochondria sufficient to destroy any permeability barrier (Towers et al 1972).

These differential effects of various inhibitors seen with yeast and mammalian mitochondria are thought to arise from phylogenetic differences in the mitochondrial ribosomes. Mammalian mitochondrial ribosomes are considerably smaller than their yeast counterparts. Similarly the differences between nuclear and mitochondrial ribosomes may underlie the differential effects of inhibitors on these two protein synthesising systems.

7. Nuclear Influences

The protein synthesising capacity of isolated yeast mitochondria was extended beyond the usual 30 minutes by the addition of an S100 fraction obtained from
homogenised yeast, E coli or rat liver (Everett et al 1980). This increased rate of radiolabelled amino acid incorporation into protein was due to mitochondrial protein synthesis since it was inhibited by the addition of chloramphenicol just prior to the introduction of the S100 fraction. The stimulatory effect was not the result of the non-specific introduction of protein since it was not seen when bovine serum albumin was added instead of the S100 fraction. The stimulatory effect of addition of S100 fraction was greatest if the fraction was present at the onset of incubation but also occurred when it was added to depleted mitochondria. The electrophoretic profile of the synthesised proteins showed that synthesis of COX III, cytochrome b and VAR-3 was most enhanced by the S100 fraction. The stimulatory activity of the E Coli and the rat liver S100 fraction was non-dialysable, ribonuclease A resistant but sensitive to trypsin suggesting that the enhancing factor(s) were proteins.

In vitro protein synthesis may therefore be limited by the relative lack of nuclear encoded "partner" proteins, or proteins which directly regulate mitochondrial protein synthesis (Everett et al 1980).

8. The Identity Of Synthesised Proteins

In early experiments electrophoretic separation of the labelled proteins revealed four major and some minor bands (Haldar et al 1966, Beattie et al 1967). The radioactivity was found to have been incorporated into insoluble inner membrane proteins especially the cytochrome fraction known, at the time, to include cytochromes a, b and c. Succinate dehydrogenase and cytochrome a were found not to be labelled
Later workers found about eight polypeptides in the size range of $6 \times 10^3$ to $5.5 \times 10^4$ daltons and these showed a similar polypeptide pattern to that seen in cell cultures labelled in the presence of cycloheximide (Schatz and Mason 1974, Coote and Work 1971, Lederman and Attardi 1973). The advent of higher resolution electrophoresis revealed up to 24 labelled polypeptide bands up to a molecular weight of $1 \times 10^5$ (Bhat et al 1982).

The variable numbers of polypeptide bands reported may have a variety of explanations. The hydrophobic mitochondrially synthesised proteins are known to have a propensity for aggregation during electrophoresis (Schatz and Mason 1974, Downer et al 1976). Various methods have been used to avoid this have been used including pre-treatment with alkali (Tzagoloff 1972), or heating to 90°C, or electrophoresis in the presence of urea, 2-mercaptoethanol, or high concentrations of sodium dodecylsulphate (Bhat et al 1982). It was originally proposed that the presence, on electrophoresis, of more than the 13 proteins known to be encoded by mtDNA might be due to proteins coded for by imported cytoplasmic mRNAs but there is as yet, no evidence that this occurs. It was speculated that uncombined mitochondrially synthesised proteins may be more susceptible to proteases (Beattie et al 1967). However pulse chase analysis of the radiolabelled proteins, and the effects of protease inhibitors on the electrophoretic profiles did not suggest any significant proteolytic degradation during incubation (Bhatt et al 1982).

The definitive assignment of the polypeptides comes from the immunoprecipitation studies using antibodies to purified protein subunits such as those
for cytochrome oxidase (Attardi and Ching 1979, Ching and Attardi 1982,) or those raised against chemically synthesised peptides predicted from the mtDNA sequence in the case of the Complex I subunits (Mariottini et al 1983, 1986; Chomyn et al 1983, 1986). Identification of proteins separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) is usually on the basis of migration distance. This assumes that proteins fully solubilised by SDS are uniformly attracted by the electrical field and are therefore effectively sieved through the polyacrylamide gel purely on the basis of size so that the migration distance is proportional to the logarithm of the molecular weight. However this assumption depends crucially on the complete reduction of the protein by the SDS (Pitt-Rivers and Impiombato 1968, Reynolds and Tanford 1970) and also assumes that the different detergented coated proteins have similar hydrodynamics ie shape. Molecular weights below 15kD are less reliable because of hydrodynamic differences. Glycoproteins (Grefarth and Reynolds 1974) and hydrophobic proteins show incomplete binding of SDS. Thus the hydrophobic mitochondrialy synthesised proteins show anomalous mobilities in gels (Sierra and Tzagoloff 1973), with migration distances greater than expected for their molecular weight. The apparent molecular weight of mitochondrialy synthesised proteins is thus less than that predicted from the mtDNA sequence and differs with variations in sample preparation and with different gel systems. Relative mobilities may also vary with different gel systems eg those of COX II and ATP8 (Wallace et al 1991), (see Table 8.11 ). Mariottini et al (1986) showed that the migration distance of mitochondrialy synthesised proteins in a SDS urea PAGE system was proportional to the logarithm of the predicted molecular weight with the exception of COX II and URF A6L (now designated ATP8). This was attributed to the greater water solubility of
those subunits.

9. Species, Tissue, and Age Specific Differences

The rate of mitochondrial protein synthesis varied in mitochondrial from different tissue sources. McLean et al (1958) noted that rat liver mitochondria had a higher rate of labelled amino acid uptake than rat muscle mitochondria. The synthesis rate was highest in mitochondria from Lettre Ehrlich ascites cells and from beef heart, was less in rat liver, rat brain and mouse liver mitochondria, and reduced still further in kidney and spleen mitochondria (Bhat et al 1982). Human placental mitochondria had a two fold higher rate of protein synthesis than human muscle mitochondria (Marzuki et al 1988).

The mitochondrial protein synthesis rate in rat muscle showed a sharp decline in the first weeks of life and a slower decline in the next 2 years (Attardi et al 1989). Rat brain synaptosome mitochondrial protein synthesis showed a postnatal burst of synthesis at 10-13 days with a sharp decline in the third week, thererafter remaining constant for the next two years also (Polosa and Attardi 1991).

The electrophoretic profile of the mitochondrially synthesised proteins was said to show marked species specific variations which were both quantitative and qualitative (Bhat et al 1982). Yatscoff et al (1978) found a "common band" pattern of twelve mitochondrially synthesised proteins in 24 out of 27 human cell culture lines, while in a further three cell lines derived from HeLa cell lines, a different band pattern was
seen, lacking the 14kD band, but having an additional 13kD band. Oliver et al (1983) identified two forms of ND3 in HeLa cells with estimated molecular weights of 15kD and 14kD. This was confirmed in subsequent immunoprecipitation studies (Attardi et al 1987). The same polymorphic variant was also found in two out of 31 individuals tested, with another three polymorphisms for polypeptides of apparent molecular weights 28kD, 23.75kD and 8.1kD, amongst 92 unrelated individuals (Spinner and King 1986). A variety of protein polymorphisms were reported in in vitro human mitochondrial synthesis studies (see section 10).

The labelling of the ND5 subunit was found to be much less in rat muscle and rat brain synaptosome mitochondria than in rat fibroblast mitochondria. Since comparable levels of ND5 mRNA were found in all three cell lines this suggested post translational control of the synthesis of ND5 (Attardi et al 1989, Polosa and Attardi 1991).

10. Human Mitochondrial Protein Synthesis

There have been only two publications reporting in vitro mitochondrial protein synthesis in isolated human mitochondria, both from the same group in Australia, (Marzuki et al 1988, Byrne et al 1987).

In their system mitochondria were incubated for 1 hour at 37°C, at a final concentration of 0.4 to 1.2mg/ml protein, in a bicine KOH buffer containing sucrose, KCl, MgCl and EDTA. The energy system was a exogenous one comprising of ATP,
phosphoenol pyruvate, pyruvate kinase and oligomycin. However succinate, a Complex II dependant substrate was also present, as was GTP. Three protease inhibitors and cycloheximide were added routinely. Synthesised proteins were labelled with $^{35}$S methionine.

Initial experiments were performed using human placental mitochondria (Marzuki et al 1988). The optimum Mg$^{2+}$ concentration was determined to be 2.5mM as compared with 5mM quoted for rat skeletal muscle mitochondria (Towers et al 1972). The omission of GTP, or ATP did not make any significant difference to the rate of mitochondrial protein synthesis.

Using human placental mitochondria a linear incorporation of $^{35}$S methionine into mitochondrial synthesised proteins of $40 \times 10^4$ dpm/mg protein in 1 hour was achieved. This was equivalent to the incorporation of 0.4pmol $^{35}$S methionine/ mg protein/ min. Human skeletal muscle mitochondria had a lower rate of incorporation of label with a mean and standard deviation in five cases of $0.20 \pm 0.06$ pmol $^{35}$S methionine/ mg protein/ min. (Marzuki et al 1988). Four additional control values for human skeletal mitochondrial $^{35}$S methionine incorporation were quoted in the second paper, with the method having been optimised for human skeletal muscle mitochondria, and these gave a mean and standard deviation of $277\pm 79 \times 10^3$ dpm/ mg protein/ hr. (Byrne et al 1987). This same paper studied two patients with Kearnes Sayre syndrome and found a five to ten fold increase in mitochondrial protein synthesis as compared to controls, with $^{35}$S methionine incorporation rates of 938 and $1,625 \times 10^3$ dpm/ mg protein/ hr.
Using this system with human skeletal mitochondria from control patients, thirteen mitochondrial translation products were observed with apparent molecular weights ranging from 5.5 to 75 kD. In a total of eight controls reported in the two papers, two cases showed an absence a 10.5 kD product, three showed an electrophoretic mobility polymorphism for a 20kD product, and in two a 45kD translation product had an increased electrophoretic mobility of 47kD. Two cases of Kearns Sayre syndrome with normal respiratory chain activity were studied (Byrne et al 1987). In one there was apparent oversynthesis of a 10.5kD product and in both the 5kD product was undetectable. The 5kD product was thought to be a product of either, the URF4L (ND4L) gene, or the URF6L (ATPase 8).
CHAPTER 5: CELL CULTURES IN MITOCHONDRIAL DISEASES

1. Muscle Cell Cultures

Myogenic cell cultures were first established in 1963 (Konigsberg 1963) initially in non human species, and using embryonic or new born muscle (Hauschka 1972). Subsequently primary cultures of adult human muscle became possible (Bateson 1972, Askanas and Engel 1975, Yasin et al 1977), and this led to considerable interest in their use for studying muscle disease. For reviews see (John and Jones 1985, Witkowski 1986).

The starting point for primary muscle cell culture is the myosatellite cell. These are small flattened spindle shaped cells probably representing remnants of the myogenic stem cells (Mauro 1961). These cells are the source of additional nuclei for muscle growth and hypertrophy, and for muscle regeneration in response to injury or disease. The numbers of stem cells declines with age from 10% of the total cell population in the young adult to 2 to 3% in mature adults. The myosatellite cell numbers increase in denervated muscle, and in muscle diseases which stimulate muscle regeneration, such as polymyositis (Landon 1992). The myosatellite cells can be released from muscle by a combination of mechanical and enzymatic dissociation (Yasin et al 1977), or allowed to migrate out of muscle explants (Askanas and Engel 1975).

In a process which recapitulates normal muscle development (Landon 1992), the
myogenic stem cells mature to form spindle shaped myoblasts. Influenced by a variety of factors including cell density, the presence of a collagen substratum and media conditions, the dividing myoblasts line up along their long axis and fuse to form multinucleated myotubules. These syncytial myotubes are terminally differentiated and do not have the ability to divide. The myotubules accumulate muscle specific proteins and isoenzymes and with maturity shown cross striations. In parallel with these changes is an increasing excitability of the cell membrane which can be manifest as spontaneous contractions of the myotubules. Both the development of cross striations and spontaneous contractions show species variation being less readily seen in human myotubule cultures than in cultures from other species such as rodents. Myotubes can be co-cultured with neuronal elements resulting in innervated muscle cell cultures and these cultures show a greater degree of differentiation morphologically and enzymatically, with a propensity to spontaneous contractions (Kobayashi et al 1987, Ecob-Prince and Brown 1988, Martinuzzi et al 1987).

Muscle cell cultures can be transformed by transfection with origin defective simian virus 40 (SV40). Such cultures show enhanced growth rate and unlimited proliferation, while retaining the characteristics of differentiated myoblasts, providing a stable supply of cultured human myoblast cells. The characteristics of these transformed cell lines also facilitates the production of clonal myogenic cell lines (Hurko et al 1986, Nakamigawa et al 1988).
2. Human Muscle Disease In Culture

Muscle cultures have been used to study a variety of diseases including dystrophies and metabolic myopathies (John and Jones 1985, Askanas and Engel Miranda et al 1990). However the emergence of foetal isoenzymes in culture can lead to the disappearance of the defect of interest as was first shown for myophosphorylase deficiency (DiMauro et al 1978). This phenomenon is true for most defects of enzymes which have tissue specific isoforms and are likely to have foetal isoforms. Often the brain specific isoform of an enzyme is also the foetal isoform (Miranda et al 1990, John and Jones 1985). Tissue specific variation in respiratory chain complexes subunits has been demonstrated for Complex I and IV (see Chapter 3, section 11). This highlights the necessity for establishing that cultures express the defect of interest before they can be used to study the mechanisms of expression of a given disease.

Similar strictures apply to fibroblast and leukocyte cultures. Although they are easy to set up from patients, requiring only a small skin biopsy or blood sample, they may not express the mitochondrial defect.

In cases where the enzyme defect resides in a mature isoform it may be possible to induce expression of the defect in a suitably differentiated cell line (Matinuzzi et al 1987). Unlike leukocyte and fibroblast cell lines, muscle cell lines have the potential for such differentiation, which may be further extended by co-culture with neuronal elements (v.s.).
3. Respiratory Chain Deficiencies In Culture

Respiratory chain deficiencies have been studied in animal cell lines and in human fibroblast and muscle cell cultures.

3.a. Animal Cell Lines

A cell line of Chinese hamster lung cell mutants unable to grow on galactose instead of glucose was found to have a combined Complex I and IV defect with a significant reduction in rotenone sensitive NADH oxidase activity and in cytochrome oxidase activity. This was thought to be due to an undisclosed single gene defect (Whitfield et al 1981, Malczewski and Whitfield 1984).

3.b. Fibroblast Cell Lines

Respiratory chain defects were demonstrated in fibroblasts cultured from neonates presenting in the first few days of life with lactic acidosis, usually fatal. They were shown to have reduced ATP and oxygen consumption with NAD-linked substrates suggesting a Complex I deficiency (Robinson et al 1985, Robinson et al 1989). Both of these reports predate the first documentation of human mtDNA deletions and so do not specify whether there was a mtDNA defect. However the cases cited above did not have ophthalmoplegia as a clinical feature as has been seen in all cases with all the cases with mtDNA length deletions hitherto described. Other mtDNA defects may have been present.

There are several reports of fibroblast cultures from patients with Leigh’s
syndrome demonstrating cytochrome oxidase deficiency (De Vivo et al 1986, DiMauro et al 1987, Miranda et al 1989). Miranda and colleagues (1989) used one such cytochrome oxidase deficient cell line to demonstrate evidence for a nuclear encoded mutation. Fibroblast culture was used to examine mitochondrial protein synthesis in a case of MERRF with a tRNA<sup>6*</sup> point mutation at nucleotide pair 8344. Fibroblasts from the patient were found to have 31% mutant mtDNA while fibroblasts from the mother had 48% mutant mtDNA (Seibel et al 1991).

3.c. Muscle Cell Lines

Askanas and colleagues (1978) reported on the morphology of muscle cultures from two cases of mitochondrial myopathy who had failure to thrive, stroke like episodes, epilepsy and cerebellar features. Their cultures showed cytochrome oxidase negative fibres and large "abnormal" mitochondria on electronmicroscopy. No mention was made of the succinate dehydrogenase reaction or whether the Gomori trichrome stain showed typical ragged red fibres. However paracrystalline inclusions as observed under electron microscopy of the muscle biopsy were not seen in the mitochondria obtained from cultures of this muscle.

Muscle cultures from three patients with Kearns-Sayre syndrome exposed to the uncoupling agent 2,4, dinitrophenol (2,4, DNP) were found to develop cytoplasmic granules visible with phase contrast microscopy and a 40 to 20% decrement in all mitochondrial enzyme activity not seen with control cultures (Meola et al 1987).

Muscle cell cultures from a patient with the MERRF phenotype and a partial
deficiency of cytochrome oxidase showed markedly reduced or absent staining for
cytochrome oxidase in 30% of the cells with the rest showing normal activity. This was
adduced as evidence for the presence of a dual population of mitochondria (Miranda
et al 1989).

3.d. Clonal Cell Cultures

In primary myoblast cultures obtained from patients with Kearns Sayre syndrome
and a mtDNA deletion, the relative number of deleted genomes was found to decline
and eventually disappear from the cultures within a limited number of population
doublings (Moraes et al 1990). By contrast the same group found that mtDNA
deletions were maintained in clonal cultures of either fibroblasts or myoblasts.
Different clones showed varying degrees of heteroplasmy for the deleted genomes
which remained stable despite up to 38 cell doublings. Such clones showed reduced
histochemical staining for cytochrome oxidase but normal succinate dehydrogenase
staining. The growth rate of the heteroplasmic clones was found to be slower than the
homoplasmic normal clones. It was hypothesised that in uncloned cultures the loss of
deleted genome was the result of this differential rate of cell division with the normal
cells overgrowing the heteroplasmic cells (Moraes et al 1990).

Shimoizumi and colleagues (1989) reported on a cytochrome oxidase negative
transformed muscle cell line which showed reduced synthesis of the mitochondrially
encoded subunits I, II and III of cytochrome oxidase, and subunit 6 of ATPase. The
patient from whom the cultures were derived did not have ophthalmoplegia and probing
of the total DNA from the cultures (digested with one restriction endonuclease) with a
labelled HeLa cell mtDNA fragment containing the coding regions for the above subunits did not show a reduction in mtDNA coding for those subunits. It was suggested that the apparent reduced synthesis of the above subunits may represent a consequence of a nuclear DNA defect resulting in faulty post transcriptional processing of the mitochondrial messenger RNA.

4. Mitochondrial Protein Synthesis In Culture

With the presence of inhibitors of cytoplasmic protein synthesis such as cycloheximide or emetine, it is possible to differentially label mitochondrially synthesised proteins in culture.

Attardi's group have made extensive use of HeLa cell cultures in this way and the mitochondria isolated from these cultures have formed the basis of immunoprecipitation experiments, using synthetic antibodies, to identify several of the hitherto unidentified reading frames of the mitochondrial genome (see page 27).

The combined deficiency of Complex I and IV activity seen in the Chinese hamster cell line (v.s), was found to have reduced synthesis of five subunits of Complex I and COX subunits I, II, and III (Malczewski and Whitfield 1984).

Mitochondrial translation products were studied in leukocyte cultures obtained from a four month baby with a lethal infantile mitochondrial disease characterised by cardiomyopathy, hypotonia and failure to thrive. Biochemical studies showed a
combined Complex I and IV deficiency with no specific mtDNA abnormality and normal mitochondrial translation products. On this basis it was concluded that the defects arose from a nuclear mutation (Zheng et al. 1989). However it was not established that the leukocyte cultures expressed the biochemical defect.

Mitochondrial protein synthesis in fibroblasts obtained from a case of MERRF with a tRNA\textsuperscript{Lys} point mutation showed reduced incorporation of \textsuperscript{35}S methionine into those polypeptides with a high lysine content (Seibel et al. 1991).
METHODS
CHAPTER 6: METHODS

1. Materials

The chemicals and materials used and their source is listed in Appendix 2.

1.a. Animals

The rats used were six week old males of the Wistar strain which had unrestricted access to water and a standard laboratory diet.

1.b. Patients

Fresh human muscle biopsies were obtained with the full and informed consent of patients of the National Hospital For Neurology and Neurosurgery, Queen Square, London. Small muscle biopsies under local anaesthetic from either vastus lateralis or triceps muscle were performed as part of the diagnostic work up for a variety of neuromuscular problems. Those with no clinical or histochemical evidence of mitochondrial disease were deemed to be controls. Some had other muscle pathology, detailed, while others had normal histochemical appearances. Such material was sufficient for the establishment of muscle cell cultures but not for the isolation of mitochondria suitable for oxygen electrode assays or the in vitro labelling of newly synthesised proteins.

A proportion of patients with clinical and histochemical evidence of a mitochondrial myopathy on a small muscle biopsy, underwent a large muscle biopsy
with the excision of approximately 6g of muscle from vastus lateralis under general anaesthetic. This provided sufficient material for the isolation of mitochondria.

2. Preparation Of Muscle Mitochondria

2.a. General Principles

Mitochondrial preparations were performed in collaboration with Dr. J.M. Cooper (Department Of Biochemistry, St. Bartholomew's Hospital Medical School, London). All mitochondrial preparations were isolated from freshly obtained muscle taken immediately in the case of rats and within 10 minutes of biopsy in the human cases. The extraction procedures were carried out at 4°C throughout and mitochondria were isolated within 90 minutes from the time of muscle isolation. Sterile precautions were taken throughout the procedure. All instruments and centrifuge tubes were sterilised by autoclaving. The Ultra Turrax shaft was alcohol sterilised and rinsed in sterile distilled water. Glassware was autoclaved and pre-packed, gamma irradiated, single use plastic ware was used where appropriate. Solutions were autoclaved or sterile filtered (as detailed in the tables) through a 2.2μm filter using an Acrodisc for small volumes, or a bottle top filter for larger quantities.

2.b. Preparation Of Rat Muscle Mitochondria

The rats were killed by cervical dislocation. The skin over the gastrocnemius muscle was sterilised with absolute ethanol prior to the removal of approximately 6g of this muscle. The muscle was repeatedly washed in ice cold high EDTA isolation medium while being finely minced with scissors thus removing blood and fat. The
muscle was then incubated for 30 minutes with trypsin (activity 40U/mg) at a concentration of 0.5mg per g of muscle. Digestion was then halted by the addition of a 3 fold excess of soya bean trypsin inhibitor which was then diluted out. The digested muscle was made up to a volume of 60 to 80ml and then homogenised using a Ultra Turrax fitted with a 18N shaft at full speed for two five second bursts.

The homogenate was then centrifuged for 5 minutes at 1 500g resulting in a nuclear pellet. The resultant supernatant was strained through muslin and then centrifuged for 10 minutes at 7 000g. Gentle shaking removed any fluffy layer which was then discarded along with the supernatant. The pellet was suspended in low EDTA isolation medium (see Table 6.1) and hand homogenised using a loose, glass-teflon, Potter type homogeniser. The resulting crude mitochondrial fraction was made up to 50ml and any remaining contaminants were removed by centrifugation at 1 500g for 5 minutes. The supernatant was removed and centrifuged at 7 000g for 10 minutes. This final purified mitochondrial pellet was resuspended in low EDTA isolation medium to

<table>
<thead>
<tr>
<th>Table 6.1 EDTA isolation medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Mannitol</td>
</tr>
<tr>
<td>Sucrose</td>
</tr>
<tr>
<td>EDTA low</td>
</tr>
<tr>
<td>high</td>
</tr>
<tr>
<td>Tris pH 7.4</td>
</tr>
</tbody>
</table>

Adjust to pH 7.2. Store at 4°C for up to 3 days
a final protein concentration of about 10mg/ml.

2.c. Preparation Of Human Muscle Mitochondria

Human muscle was placed in ice cold low EDTA isolation medium for transfer to the lab where isolation of the mitochondrial proceeded as for rat muscle, detailed above.

Typical yields of mitochondria ranged from 1.5 to 3.0 mg per g wet weight of muscle from rats, and 0.5 to 2mg per g wet weight of muscle from human cases. This represents a yield of roughly 10-20% of the total muscle mitochondria with around 80% being retained in the nuclear pellet. The nuclear pellet has been used as a source of mitochondrial DNA (see section 9a).

3. Human Adult Muscle Cell Cultures

3.a. Culture Materials

The source for the cell culture materials is given in Appendix 2.

3.b. Batch Testing Of Foetal Calf Serum

The growth medium contained 20% foetal calf serum from reserved batches of serum which had been previously tested for optimum myoblast growth and fusion using hamster muscle cell cultures. Professor F. Walshe (Dept Of Neurochemistry, Institute Of Neurology, London) kindly allowed the use of their batch tested serum.
3.c. Detoxification Of Chick Embryo Extract

The growth medium also contained 2% detoxified chick embryo extract. Detoxification of the chick embryo extract was carried out in 80ml batches. It was first heated to 65°C in a water bath for 10 minutes with gentle intermittent agitation and then cooled quickly in cold water. The resulting precipitate was centrifuged down in a 8 x 50 rotor at 15,000rpm for 30 minutes at 4°C using a Beckman MSE 18 Centrifuge. The decanted supernatant was filtered through an Amicon filter holder lined with a 62mm PM 10 Diaflo membrane. The volume was reduced to a third under 40 psi pressure using O₂ free nitrogen and then made up to its original volume with phosphate buffered saline (PBS). Three such cycles were performed. The final product was then sterile filtered through a 2.2μm Acrodisc filter and stored in 10ml aliquots at -20°C.

3.d. Collagen Coating Of Culture Dishes

Disposable sterile plastic culture dishes were used and these were pre-coated with collagen. Using the laminar flow hood with full sterile precautions, the dishes were flooded with a 1 in 70 solution of Vitrogen in sterile phosphate buffered saline (PBS). These were then left, either overnight at room temperature, or for 4 hours at 37°C, before the solution was aspirated off. Two rinses with PBS followed and the treated dishes then stored, in their original wrapping, at -4°C.

3.e. The Establishment Of Human Adult Muscle Cell Cultures.

The methods used were based on those of Yasin et al (1977). The muscle biopsy was placed into a sterile 40ml beaker and 10mls of dissociation solution was added.
Table 6.2 Dissociation solution for muscle

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dulbecco’s Modified Eagle’s Medium</td>
<td>Make up to 40ml</td>
</tr>
<tr>
<td>Bovine Serum Albumin</td>
<td>0.1% w/v</td>
</tr>
<tr>
<td>Collagenase Type II</td>
<td>0.15% w/v</td>
</tr>
<tr>
<td>Trypsin</td>
<td>0.15-0.2% w/v</td>
</tr>
</tbody>
</table>

The muscle fibres were teased apart using sterile needles and the beaker then covered with sterile aluminium foil prior to incubation in a slowly shaking water bath at 37°C for 15 minutes. 10mls of growth medium was added to neutralise the enzymes and the muscle fragments triturated through a 25ml wide bore pipette. After allowing the muscle fragments to settle the supernatant was pipetted off and passed through a filter consisting of two layers of 50μm and one bottom layer of 22μm nylon mesh supported by a 4cm plastic funnel, all previously alcohol sterilised and rinsed with sterile Dulbecco’s Modified Eagle’s Medium (DMEM). Another 10mls of dissociation solution was then added to the beaker and the above cycle repeated three more times. Following the 2nd enzyme digest the muscle fragments were thereafter trituratable through a 10ml pipette. The resulting four filtered supernatants were centrifuged at 1000rpm for 10 minutes in Sterilin universal containers to sediment the cells. The supernatants were then aspirated off and the cell pellets resuspended, combined in 1.5ml of growth medium and placed in a 35mm tissue culture dish. This was incubated overnight at 37°C in 9% carbon dioxide. The following day the medium was aspirated off and the plate washed twice with DMEM to remove the red blood cells and fresh medium was added.
3.f. Establishment Of Cloned Muscle Cell Cultures.

Dissociated muscle cells were resuspended in a large volume of growth medium and 1ml of this cell suspension was pipetted into each well of a 24 well tissue culture plate. The remaining cell suspension was diluted 1 in 5 with additional growth medium and a further 24 well tissue culture plate was seeded. Further 1 in 5 dilutions of the cell suspension were made and the procedure repeated, resulting in several 24 well culture plates containing cell suspensions of decreasing density. The next day the growth medium was removed and the wells washed with DMEM to remove red blood cells, before fresh medium was added. Each well was then inspected daily and those showing a single cell were marked. Plates showing a high proportion of such wells were retained while the others were discarded. When the marked wells were 70 to 80% confluent they were passaged into 35mm culture dishes and from thence, into dishes of increasing size as detailed below.

3.g. Maintenance Of Muscle Cell Cultures.

Cultures were incubated at 37°C in a 9% CO₂ atmosphere. The 20% foetal calf serum growth medium (see Table 6.3) was changed every third or fourth day.

<table>
<thead>
<tr>
<th>Table 6.3 Growth medium for muscle cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dulbeco's Modified Eagle's Medium (DMEM)</td>
</tr>
<tr>
<td>Foetal Calf Serum</td>
</tr>
<tr>
<td>Detoxified Chick Embryo Extract</td>
</tr>
<tr>
<td>Glutamine</td>
</tr>
<tr>
<td>Penicillin and Streptomycin</td>
</tr>
</tbody>
</table>

119
Cultures were passaged when approximately 70% confluent. In the case of newly established cultures, the passaging was into a sequence of enlarging culture plate sizes from 35mm to 60mm and thereafter 100mm diameter. Passaging was achieved by aspirating off the growth medium, washing the culture in serum free DMEM and then incubating with trypsin solution (see Table 6.4) until the cells detached; aided occasionally by sharp taps of the side of the dish.

<table>
<thead>
<tr>
<th>Table 6.4 Trypsin solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Versine 1:5000</td>
</tr>
<tr>
<td>Trypsin 1:250</td>
</tr>
</tbody>
</table>

The addition of growth medium containing serum, neutralised the trypsin, and the cells were then pelleted down at 1000rpm for 10 minutes, before resuspended in the appropriate volume of growth medium and placed in the new culture dish.

Cultures designed for experiments were allowed to grow beyond 70% confluency at which point fusion of the myoblasts, forming myotubes would occur. During this latter phase the growth medium was changed to one containing 10% horse serum; differentiation medium, (see Table 6.5).
Table 6.5 Differentiation medium for muscle cultures

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dulbeco's Modified Eagle's Medium (DMEM)</td>
<td>500ml</td>
</tr>
<tr>
<td>Horse Serum</td>
<td>50ml</td>
</tr>
<tr>
<td>Glutamine</td>
<td>0.145g</td>
</tr>
<tr>
<td>Penicillin and Streptomycin</td>
<td>5ml</td>
</tr>
</tbody>
</table>

3.h. Harvesting Of Cell Cultures.

Confluent myotubule cultures were harvested for biochemical assay, for mtDNA analysis and for protein electrophoresis, following radiolabelling of the mitochondrial proteins. The medium was aspirated off and the cultures washed twice in ice cold PBS. They were then scraped off the dish into 1ml of cold PBS using a teflon cell scraper and taken up into an Eppendorf tube. A short high speed spin in a microfuge pelleted the cells allowing aspiration of the PBS and the solid pellet was then stored at -70°C until used.

3.i. Freezing, Storage And Thawing Of Cell Lines.

Cell culture lines destined for storage were trypsinised when 70% confluent in a 100mm dish as detailed above. The cell pellet was resuspended in a small volume of growth medium containing 10% dimethyl sulfoxide (DMSO); typically 0.3ml for cell pellet obtained from a 100mm dish. The cell suspension was placed in a 2ml screw cap cryotube and frozen down with the minimum of delay. Freezing was carried out as a two step procedure using a Jencons freezing tray. The tubes were placed at the top level (-70°C) for 20 minutes and then wound down to bottom level (-120°C) for 15 minutes, before being transferred to liquid nitrogen.
When required, frozen cell lines were rapidly thawed out with hand heat, before the contents were diluted in the appropriate volume of growth medium for plating out into a dish, usually one size smaller than the dish from which the cells had originated.

4. In Vitro Labelling Of Mitochondrially Synthesised Proteins

4.a. General Method

Sterile precautions, detailed above also applied to the equipment and solutions used for the labelling of the mitochondria. Materials used are listed in Appendix 2.

Isolated mitochondria were incubated in sterile 2ml cryotubes with screw caps containing a mixture of buffer and salts (see Table 6.6). Amino acid mixture deficient in methionine was added. Several energy systems were evaluated (see Table 6.7) with the optimum system being glutamate and atractyloside (see results). In later experiments 1mM ATP was included in the energy system. Specific inhibitors appropriate to the experiment were added (see Table 6.8). Sterile double distilled water was added to bring the final reaction volume to 100ul containing mitochondria at 1.8mg/ml. After the addition of the mitochondria and initial incubation at 30°C for three minutes, 50uCi $^{35}$S methionine (specific activity >1000 Ci/mM) was added at time zero. The final composition of the incubation mixture is summarised in Table 6.9. Incubation was continued in a shaking waterbath at 30°C for 60 minutes. The reaction was then stopped on ice, transferred to an Ependorf tube and centrifuged at high speed in a microfuge. The supernatant was discarded and the labelled mitochondrial pellets stored at -70°C.
### Table 6.6 Stock buffer and salt solution

<table>
<thead>
<tr>
<th></th>
<th>For volume of 50ml</th>
<th>Stock conc.</th>
<th>Final conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>0.670g</td>
<td>180mM</td>
<td>90mM</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>0.102g</td>
<td>10mM</td>
<td>5mM</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.136g</td>
<td>20mM</td>
<td>10mM</td>
</tr>
<tr>
<td>Bicine</td>
<td>0.815g</td>
<td>100mM</td>
<td>50mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.292g</td>
<td>2mM</td>
<td>1mM</td>
</tr>
</tbody>
</table>

Adjusted to pH 7.6 with KOH, sterile filtered and stored at 4°C for up to two weeks.

### Table 6.7 Energy systems used (volumes stated are for five incubations ie. final vol. 500μl)

<table>
<thead>
<tr>
<th></th>
<th>Stock concentration</th>
<th>Volume added</th>
<th>Final conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamate</td>
<td>250mM</td>
<td>10μl</td>
<td>25mM</td>
</tr>
<tr>
<td>Atractyloside</td>
<td>50mM</td>
<td>5μl</td>
<td>50μM</td>
</tr>
<tr>
<td>ATP</td>
<td>10mM</td>
<td>50μl</td>
<td>1mM</td>
</tr>
<tr>
<td>Phospho-enol pyruvate (PEP)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>1mg/ml</td>
<td>5μl</td>
<td>10μg/ml</td>
</tr>
<tr>
<td>Phosphoenol pyruvate</td>
<td>250mM</td>
<td>10μl</td>
<td>5mM</td>
</tr>
<tr>
<td>ATP</td>
<td>250mM</td>
<td>10μl</td>
<td>5mM</td>
</tr>
<tr>
<td>Succinate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>100mM</td>
<td>50μl</td>
<td>10mM</td>
</tr>
<tr>
<td>ADP</td>
<td>20mM</td>
<td>50μl</td>
<td>2mM</td>
</tr>
</tbody>
</table>
Table 6.8 Inhibitors used

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Stock concentration</th>
<th>Volume added</th>
<th>Final conc. (per incubation fv 100μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycloheximide</td>
<td>4mg/ml</td>
<td>5μl</td>
<td>200μg/ml</td>
</tr>
<tr>
<td>Emetine</td>
<td>4mg/ml</td>
<td></td>
<td>Used for cell culture labelling only</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>4mg/ml</td>
<td>5μl</td>
<td>200μg/ml</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>4mg/ml</td>
<td>5μl</td>
<td>200μg/ml</td>
</tr>
<tr>
<td>Rotenone</td>
<td></td>
<td></td>
<td>Variable concentration. See text</td>
</tr>
</tbody>
</table>

Table 6.9 Composition of incubation mixture per single incubation (fv 100μl)

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Volume added</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer and salts</td>
<td>Stock</td>
<td>50μl</td>
</tr>
<tr>
<td>Amino acid mixture</td>
<td>1mM</td>
<td>10μl</td>
</tr>
<tr>
<td>Energy system</td>
<td></td>
<td>See Table 6.7</td>
</tr>
<tr>
<td>Inhibitors</td>
<td></td>
<td>See Table 6.8</td>
</tr>
<tr>
<td>Sterile water</td>
<td></td>
<td>To final volume 100μl</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>15-20mg/ml</td>
<td>15-20μl</td>
</tr>
<tr>
<td>35S Methionine</td>
<td>500mCi/33μl</td>
<td>3.3μl</td>
</tr>
</tbody>
</table>

4.b. Quantitation Of The Mitochondrial Protein Synthesis

The time course of the radiolabel incorporation into mitochondrial protein was followed by the removal of triplicate 5μl aliquots from the reaction mixture, at time zero and again after 60 minutes, and these were then spotted onto Whatman GF/C 2cm glass fibre discs. The discs were plunged into cold 10% trichloroacetic acid containing 0.5% w/v unlabelled methionine for 1 hour to stop the reaction. They were then boiled
in fresh 10% and then 5% trichloroacetic acid for 20 minutes each before being rinsed in ethanol and air dried. The dried discs were then placed in scintillation vials containing 5ml of Aquasol scintillation fluid for counting.

4.c.Radiolabel Counting

Counting was performed using either a Beckman or Packard 2500 TR liquid scintillation analyser. Counting was done for 1 minute. For each incubation the mean baseline count was subtracted from the mean final count, corrected for the half life decay, and expressed as disintegrations per minute (dpm) $^{35}$S methionine incorporated per mg mitochondrial protein (dpm/mg mito.protein).

5.Labeling The Cell Culture Synthesised Mitochondrial Proteins.

Confluent myotube cultures in 100mm dishes were incubated overnight in 5ml of growth medium containing 200$\mu$g/ml chloramphenicol. This selective inhibition of mitochondrial protein synthesis has been shown to increase the subsequent labelling of these proteins when the inhibition is discontinued. This medium was then aspirated off and the cultures washed twice in medium free of chloramphenicol to remove traces of this inhibitor. The cultures were then incubated in 2mls of Minimum Eagles Medium, lacking methionine, containing 5% dialysed foetal calf serum and 200$\mu$g/ml emetine. After an initial 30 minute incubation, 50uCi of $^{35}$S methionine was added and incubation continued for a further 4 hours. At the end of this period the medium was removed and after two washes with DMEM was replaced with normal growth medium containing unlabelled methionine and no inhibitor. A "chase" with this medium
continued for 30 minutes following which the cells were harvested as detailed above.

6. Histochemical And Cytochemical Staining Of Muscle Sections

These were carried out by Mrs M. Gilbert (The National Hospitals For Neurology and Neurosurgery, Queen Square, London). The following stains and methods were utilised:

Table 6.10 Histochemical stains used

<table>
<thead>
<tr>
<th>Stain</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemotoxin and Eosin</td>
<td>Dubowitz and Brooke (1973)</td>
</tr>
<tr>
<td>Modified Gomori Trichrome</td>
<td>Engel and Cunningham (1963)</td>
</tr>
<tr>
<td>ATPase reactions (at pH's 9.4, 4.3 and 4.5)</td>
<td>Drews and Engel (1966)</td>
</tr>
<tr>
<td>NADH-tetrazolium reductase</td>
<td>Dubowitz and Brooke (1973)</td>
</tr>
<tr>
<td>Periodic Acid Schiff</td>
<td>Dubowitz and Brooke (1973)</td>
</tr>
<tr>
<td>Sudan Black</td>
<td>Dubowitz and Brooke (1973)</td>
</tr>
<tr>
<td>Succinate Dehydrogenase</td>
<td>Dubowitz and Brooke (1973)</td>
</tr>
<tr>
<td>Cytochrome Oxidase</td>
<td>Seligman et al (1968)</td>
</tr>
</tbody>
</table>

7. Biochemical Assays On Mitochondria And Muscle Cell Cultures

Biochemical assays were performed in collaboration with Dr. J.M. Cooper (Department Of Biochemistry, St. Bartholomew's Hospital Medical School. London).

Oxygen utilisation was measured using a Clark electrode (Yellow Springs Instrument Co., Ohio, USA.) which was fitted into the top of a incubation chamber of 0.5 to 1.5ml capacity, which contained a magnetically stirred flea and was thermostatically regulated to 25°C. The following individual substrates and inhibitors were used:

Table 6.11 Substrates and inhibiters used for polarography (all had maleate added)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Final conc.</th>
<th>Inhibiter</th>
<th>Final conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex I linked pyruvate</td>
<td>5mM</td>
<td>rotenone</td>
<td>10μM</td>
</tr>
<tr>
<td>(with maleate 2.5μM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-hydroxybutyrate</td>
<td>10mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>glutamate</td>
<td>10mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complex II linked succinate</td>
<td>10mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complex IV linked ascorbate +</td>
<td>2mM</td>
<td>antimycin</td>
<td>10μM</td>
</tr>
<tr>
<td>TMPD</td>
<td>50μM</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

State 3 respiration was induced by the addition of 250nM ADP, and the respiratory control ratio (RCR) was calculated from the ratio of state 3 (with ADP): state 4 (without ADP) respiration rates. Uncoupled rates were determined by the addition if CCCP (fc 1μM)

The results were expressed as nmoles O utilised per minute per mg of mitochondrial protein, (nmoles O/min/mg mito.protein) for each substrate. Using these absolute measurements, each patient was assigned to one of four categories based on these results as defined by Morgan-Hughes et al (1979):
### Table 6.12 Classification of polarographic defects

<table>
<thead>
<tr>
<th>Oxygen utilisation with</th>
<th>NAD-linked substrates</th>
<th>Succinate</th>
<th>Ascorbate + TMPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>No defect</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Complex I deficiency</td>
<td>Decreased</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Complex I-III deficiency</td>
<td>Decreased</td>
<td>Decreased</td>
<td>Normal</td>
</tr>
<tr>
<td>Complex I-IV deficiency</td>
<td>Decreased</td>
<td>Decreased</td>
<td>Decreased</td>
</tr>
</tbody>
</table>


Enzyme assays were all carried out at 25°C in a final volume of 1ml using a variety of spectrophotometers and expressed, either as units (U) where 1 unit represented 1μmole of substrate utilised per minute per g wet weight of tissue, or as nmoles per minute per mg mitochondrial protein. Where appropriate, intramitochondrial enzymes were liberated by the addition of 0.1% v/v (fc) Triton X-100, and membranes were rendered permeable by three freeze thaw cycles to achieve maximal enzyme activities. The enzyme activities measured and the methods employed are shown in Table 6.13.
Table 6.13 Measurement of enzyme activities

<table>
<thead>
<tr>
<th>Enzyme activities</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATPase</td>
<td>Vasilyeva et al 1982</td>
</tr>
<tr>
<td>Citrate synthetase</td>
<td>Coore et al 1971</td>
</tr>
<tr>
<td>Cytochrome oxidase</td>
<td>Wharton and Tzagoloff 1967</td>
</tr>
<tr>
<td>NADH CoQ₁ reductase</td>
<td>Hatefi et al 1962</td>
</tr>
<tr>
<td>NADH ferricyanide reductase</td>
<td>King and Howard 1967</td>
</tr>
<tr>
<td>NADH cytochrome c reductase</td>
<td>Hatefi and Rieske 1967</td>
</tr>
<tr>
<td>Succinate cytochrome c reductase</td>
<td>King 1967</td>
</tr>
</tbody>
</table>

7.c. Measurement of Cytochromes

Mitochondrial cytochrome spectra were generated by the methods of Wilson and Epel (1968), and measured at 77K on an Aminco Chance DW2 recording spectrophotometer in the split beam mode with the low temperature attachment. Cytochromes b, c₁, c and aa₃ were measured and expressed as nmoles per mg mitochondrial protein.

8. Urea-Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

(Urea SDS PAGE).

8.a. Materials

Materials used for electrophoresis are listed in Appendix 2.

Electrophoretic separation of the proteins was performed using a discontinuous
buffer system (*Laemeli 1971*) with a 6M urea SDS polyacrylamide gel (*Kadenbach et al 1983*), using LKB vertical electrophoresis units run with LKB 2197 power supply. Gel dimensions were 14cm x 18cm x 0.7mm.

8.b. Solubilisation Of Samples

Mitochondrial or cell culture samples were solubilised in SDS sample buffer, containing 5mM aminobenzamidine as a protease inhibitor. Cell culture samples were sonicated on ice for three five second bursts.

<table>
<thead>
<tr>
<th>Table 6.14 Sample buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>For 50ml</strong></td>
</tr>
<tr>
<td>Tris base</td>
</tr>
<tr>
<td>SDS</td>
</tr>
<tr>
<td>Glycerol</td>
</tr>
<tr>
<td>Aminobenzamidine</td>
</tr>
<tr>
<td>Bromophenol blue</td>
</tr>
</tbody>
</table>

Adjust to pH 6.8.

Samples were not boiled but were left at room temperature for at least 2 hours. Just prior to loading onto the gel, mercaptoethanol was added to the sample to a final concentration of 1%. The sample was spun at 10 000g for 5 minutes and the supernatant used for electrophoretic separation. Samples for loading were adjusted to equal volume, usually 50ul, with the addition of sample buffer as required.
8.c. Polymerisation, Loading And Running Of Gels

Separation and stacking gel were made up from stock solutions (see Tables 6.15 and 6.16) and adjusted to the correct pH just prior to use. TEMED (5ul) was added to each solution which was then poured sequentially with a 10 well sample comb in situ, so that polymerisation of the two occurred together.

<table>
<thead>
<tr>
<th>Table 6.15 Composition of gels</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stacking Gel (4.83ml)</strong></td>
</tr>
<tr>
<td>Acrylamide Solution</td>
</tr>
<tr>
<td>Stacking gel buffer</td>
</tr>
<tr>
<td>Ammonium persulphate (25mg/300ul)</td>
</tr>
<tr>
<td>TEMED</td>
</tr>
<tr>
<td>Adjust to pH 6.8.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Separation Gel (20ml 18.75% acrylamide)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide solution</td>
</tr>
<tr>
<td>Separation gel buffer</td>
</tr>
<tr>
<td>Urea (fc. 6M)</td>
</tr>
<tr>
<td>Ammonium persulphate (25mg/300ul)</td>
</tr>
<tr>
<td>TEMED</td>
</tr>
<tr>
<td>Adjust to pH 8.8</td>
</tr>
</tbody>
</table>
Table 6.16 Stock solutions for electrophoresis

<table>
<thead>
<tr>
<th>Stacking gel buffer</th>
<th>For 50ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>0.125M</td>
</tr>
<tr>
<td>SDS</td>
<td>0.125%</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Separation gel buffer</th>
<th>For 50ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>0.75M</td>
</tr>
<tr>
<td>SDS</td>
<td>0.2%</td>
</tr>
<tr>
<td>Urea</td>
<td>7.2M</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Acrylamide solution</th>
<th>For 50ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>48% w/v</td>
</tr>
<tr>
<td>Bis acrylamide</td>
<td>1.5% w/v</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

When polymerisation was complete the sample comb was removed and the samples loaded using a Unimetrics microsyringe. \(^{14}\)C labeled "Rainbow" molecular weight markers, covering the range 2.3 to 46 kD, were added for each gel. The samples were overlaid with electrode buffer.

Table 6.17 Electrode buffer

<table>
<thead>
<tr>
<th>Glycine</th>
<th>190mM</th>
<th>300g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>25mM</td>
<td>60g</td>
</tr>
<tr>
<td>SDS</td>
<td>0.1%</td>
<td>20g</td>
</tr>
</tbody>
</table>

|                      | Adjust to pH 8.45 just prior to use. |
Gels were run at 80V constant at least until the samples had entered the separation gel, and usually overnight at this voltage. For faster runs the voltage was increased to a maximum of 180V constant once the samples were in the separation gel. No cooling circuit was employed. Separation was continued until the 2.3 kD "Rainbow" marker had reached the end of the gel.

8.d. Fixing And Staining Of The Gels

After running the stacking gel was cut off and the separation gel fixed and stained on a shaker overnight in 50% methanol, 10% acetic acid containing 0.2% Coomassie Blue. Background staining was removed by soaking in several changes of destain containing 50% methanol and 10% acetic acid.

8.e. Fluorography Of Gels

Stained gels were dehydrated in two changes of scintillation grade dimethyl sulphoxide (Scintran DMSO) for 30 minutes each, on a shaker. A soak in 20% PPO in DMSO on a shaker for 60 minutes followed. The fluor was then precipitated in the gel by transferring it to water for 60 minutes on a shaker.

8.f. Drying Of Gels

Following fluorography gels were equilibrated in 40% methanol, 10% acetic acid and 10% glycerol for at least 60 minutes before being dried onto Whatman 3M paper for 1 hour, at 60°C, under vacuum.
8.g. Autoradiography Of Gels

After staining, fluorography and drying, gels were placed in opposition to Fuji FX film between sheets of glass which were placed in light-tight bags and the "sandwich" was clamped together using bulldog clips on all four sides. Autoradiography took place at room temperature. For each gel, several sequential autoradiographs were exposed for variable periods of time. For gels containing in vitro labelled mitochondrial proteins, exposures ranged from 4 hours to several days, while for cell culture labelled proteins, longer exposures of up to three weeks were required.

9. Mitochondrial DNA Studies

All DNA extraction and mitochondrial DNA (mtDNA) analyses were performed in collaboration with Dr Ian Holt and Ms Mary Sweeney (Dept Of Clinical Neurology, Institute Of Neurology).

9.a. DNA Extraction

Approximately 1mg of cells was digested with 2mg proteinase K in a total volume of 200ul 75mM NaCl, 50mM EDTA buffer (at pH 8.0), with SDS added to a final concentration of 0.5%. Samples were incubated overnight at 37°C and then phenol chloroform extracted using standard techniques.

A similar procedure was used for the extraction of DNA from mitochondrial preparations using 500 to 1000ug of mitochondria in a total volume of 100ul. DNA was also extracted from the nuclear pellet produced in the isolation of mitochondria (see
section 2c) and this was mixed with equal volume of NaCl/EDTA buffer with SDS and proteinase K added, and processed as above.

9.b. Mitochondrial DNA Analysis

Restriction endonuclease digestion was performed on 3ul (1ug/ml) muscle mtDNA in a total volume of 30ul containing, usually, 10 units of enzyme per digest. Reaction conditions were as recommended by the manufacturer with the addition of bovine serum albumen to 100ug/ml and spermidine to 30mM.

Digested DNA samples were separated by horizontal agarose (0.8% (w/v)) gel electrophoresis in TAE buffer at 40-50V overnight.

<table>
<thead>
<tr>
<th>Table 6.18 TAE buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
</tr>
<tr>
<td>EDTA</td>
</tr>
<tr>
<td>Acetic acid</td>
</tr>
</tbody>
</table>

The inclusion of 0.5ug/ml ethidium bromide in the gel allowed the visualisation and photography, under UV light, of the DNA fragments.

After denaturing and neutralisation of the gels the DNA was transferred to nylon membrane (Hybond-N) by Southern blotting.
Whole human placental mtDNA labelled with $^{32}$P using a random primer kit (Amersham) was used to probe for the mtDNA fragments present in the membrane. Filters were hybridised at 65°C with the labelled probe; washed in 2 x SSC buffer and autoradiography carried out at -70°C for 2 hours to 7 days.

Table 6.19 SSC buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>0.3M</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>30mM</td>
</tr>
</tbody>
</table>

Adjust to pH 7.2
RESULTS
1. Rate Of Incorporation

The rate of incorporation of the $^{35}$S methionine label in vitro was linear for the 60 minute incubation period (figure 7.1), and in the examples shown was at a rate of $1.06 \times 10^5 \text{ dpm label incorporated per mg. mitochondrial protein per minute}$. 

![Graph showing the rate of methionine incorporation into mitochondrial proteins using isolated rat muscle mitochondria. Two independent experiments are shown.](image)
There was a range of total incorporation from $44.80 \text{ to } 134.88 \times 10^5 \text{ dpm/mg.}$ mitochondrial protein in 60 minutes with a mean of $77.37 \times 10^5 \text{ dpm/mg.}$ mito. protein, and a standard deviation of 28.40. The profile of the labelled proteins was similar for incubations performed at ten minute increments up to 60 minutes, (figure 7.2).

This suggests that the synthesis of the individual polypeptides was also linear during this period. This suggests that the synthesis of the individual polypeptides was also linear during this period. The assignment of the bands is discussed in Chapter 8 (Section 8, 3).

Figure 7.2 Autoradiograph showing the profile of mitochondrial proteins synthesised after varying periods of incubation.
Despite storage of the isolated mitochondria on ice, there was a progressive decline in the rate of incorporation of $^{35}$S methionine the longer the delay between the end of the mitochondrial isolation and the commencement of the labelling experiment (figure 7.3).

**Figure 7.3** The effect of delay between the end of mitochondrial extraction and the commencement of in vitro incubation on mitochondrial protein synthesis.
2. Sensitivity Of Incorporation To Inhibitors

The incorporation of the $^{35}$S methionine was consistently shown to be into mitochondrially synthesised proteins. The incorporation of $^{35}$S methionine was not significantly affected by the inclusion of cycloheximide (200μg/ml) but was reduced by 94.3% by the addition of chloramphenicol (200μg/ml), as would be expected for mitochondrial protein synthesis. There was relative resistance to erythromycin (200μg/ml), an inhibitor of mycoplasmal protein synthesis (figures 7.4 and 7.5).

Figure 7.4 The effect of cycloheximide (CHX), chloramphenicol (CAP), and erythromycin (ERYTH), compared with no inhibitor (None), on the level of $^{35}$S methionine incorporation.
Figure 7.5 Autoradiograph of the in vitro translation products of rat muscle mitochondria without inhibitor (None) and in presence of cycloheximide (CHX), and chloramphenicol (CAP).

The addition of sterile precautions in the preparation of the mitochondria resulted in a 100 to 1000 fold reduction in the bacterial count obtained from cultures of aliquots from the incubation mixture taken at the end of the incubation period and at this level would not have contributed to the protein synthesis being observed (Everet et al 1980).
The optimum concentration of magnesium in this system was between 7.5 and 10 mM, (figure 7.6). This result is reflected in the autoradiograph of the same experiment (figure 7.7).

![Figure 7.6](image-url)
Figure 7.7 Autoradiograph of translation products of rat muscle mitochondria illustrating the effects of Mg$^{2+}$ concentration.

4. Assessment Of Different Energy Systems

Without any added energy system or ATP, mitochondrial protein synthesis proceeded at a low level, presumably utilising endogenous substrates and ATP stores. The rate of protein synthesis increased linearly as ATP was added, but remained at a low rate, (figure 7.8). This result is reflected in the autoradiograph (figure 7.9) which also shows the result obtained with the optimal added energy system for comparison.
The rate of $^{35}$S methionine incorporation increases as the concentration of ATP is increased, but remains at a low level.

![Figure 7.8](image)

*Figure 7.8* The rate of $^{35}$S methionine incorporation increases as the concentration of ATP is increased, but remains at a low level.

<table>
<thead>
<tr>
<th>ATP Alone</th>
<th>Glutamate</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP conc (mM/l)</td>
<td>0</td>
</tr>
</tbody>
</table>

![Figure 7.9](image)

*Figure 7.9* Mitochondrially synthesised proteins can just be discerned when ATP is the sole added energy source, as compared with when the added energy system is optimal (see text for details).
Three external energy systems were evaluated as detailed in the methods including a glutamate based, a phosphoenol pyruvate (PEP) based, and a succinate based system. The glutamate based energy system achieved the best incorporation which was at least twice that seen with the PEP based energy system, and four times that seen with the succinate based energy system, (figure 7.10). These results are reflected in the autoradiograph (figure 7.11).

![Diagram](image-url)

**Figure 7.10** Showing the efficacy of the three different energy systems used, on the rate of in vitro mitochondrial protein synthesis and the effect of the addition of rotenone (5μM) in each case.
Figure 7.11 Autoradiograph of the mitochondrial translation products obtained with the different energy systems and the effect of complex I inhibition by 5μM rotenone.

Also shown in figures 7.10 and 7.11 are the effects of the addition of 5μM rotenone, sufficient to completely inhibit Complex I activity, on each of the energy systems. Mitochondrial protein synthesis was almost completely inhibited, by 98.6%, with the glutamate based system, and by 91.4% with the PEP based system. The succinate based system was also affected by the addition of rotenone but to a lesser degree (46.2%).

The inhibition of the glutamate based energy system by rotenone occurred in a dose dependant manner, (figure 7.12). The profile of the labelled polypeptides obtained
under these conditions does not suggest that this inhibition differentially affects the synthesis of some polypeptides more than others, (figure 7.13).

Figure 7.12 The dose response curve obtained with increasing concentrations of rotenone using the glutamate based energy system.
Figure 7.13 The translation products obtained with increasing inhibition of the glutamate based energy system by rotenone.

5. Variations To The Glutamate Energy System

The glutamate based energy system originally comprised glutamate and atracyloside alone (see page 93). The effect of variations to the glutamate and atracyloside energy system was investigated (figures 7.14 and 7.15). The absence of either the glutamate or the atracyloside reduced the efficacy of this energy system. The addition of 1mM ATP to incubations containing glutamate alone or containing glutamate and atracyloside enhanced the rate of protein synthesis particularly in the latter case. Using 5mM ATP was not advantageous and indeed reduced the rate of protein synthesis compared with when only 1mM ATP was used. The combination of glutamate, atracyloside and 1mM ATP was therefore adopted as the energy system of choice.
Figure 7.14 The effect of variations to the glutamate based energy system on the rate of mitochondrial protein synthesis in isolated rat muscle mitochondria.

Figure 7.15 Autoradiograph illustrating the translation products obtained with variations to the glutamate based energy system. The numbers above each lane refer to the concentration (in mM/l) of ATP added.
CHAPTER 8: PROTEIN SYNTHESIS IN ISOLATED HUMAN MUSCLE MITOCHONDRIA

1. Patients

1.a. Control patients

Eleven control patients were studied comprising 5 males and 6 females whose ages ranged from 20 to 73 years old with a mean age of 50.6 ± 18.38 years. The clinical details of these subjects is listed in Table 8.1, with their biochemical data in Tables 8.2, 8.3 and 8.4.

Six of the patients had a clinical diagnosis of idiopathic Parkinson’s disease. It has been suggested that this disease may be due to an acquired defect of mitochondrial function, specifically, a deficiency of Complex I activity (see Chapter 3 section 10b). However the mtDNA and biochemical studies on these patients has not shown any abnormality of mitochondrial function and it was therefore felt reasonable to consider them as control patients. These patients did however tend to be older with ages ranging from 49 to 73 years old. The normal ranges for all the biochemical data quoted in the tables represents overall means and standard deviations of the control data collected over the years by Dr J.M. Cooper and co-workers but it is now apparent that there is an age related decline in mitochondrial function as measured polarographically and enzymatically.
Table 8.1 Clinical features of the control patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Hosp.No.</th>
<th>Sex</th>
<th>Age</th>
<th>Clinical details</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB</td>
<td>B64456</td>
<td>M</td>
<td>20</td>
<td>Exercise induced fatigue and myoglobinuria</td>
</tr>
<tr>
<td>SC</td>
<td>B84028</td>
<td>F</td>
<td>49</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>HC</td>
<td>B83148</td>
<td>F</td>
<td>65</td>
<td>Multiple system atrophy: ptosis, myopathy, ataxia, extra-pyramidal</td>
</tr>
<tr>
<td>MD</td>
<td>PP</td>
<td>F</td>
<td>42</td>
<td>Possible carnitine deficiency</td>
</tr>
<tr>
<td>EE</td>
<td>B84531</td>
<td>F</td>
<td>67</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>JG</td>
<td>B82709</td>
<td>F</td>
<td>58</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>AH</td>
<td>B87446</td>
<td>M</td>
<td>27</td>
<td>Akinetic-rigid syndrome of unknown aetiology</td>
</tr>
<tr>
<td>EK</td>
<td>B82794</td>
<td>M</td>
<td>63</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PM</td>
<td>B81823</td>
<td>M</td>
<td>73</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>RS</td>
<td>B82527</td>
<td>M</td>
<td>68</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>JS</td>
<td>B70686</td>
<td>F</td>
<td>25</td>
<td>Exercise induced fatigue</td>
</tr>
</tbody>
</table>
Table 8.2 Polarographic results in the control patients

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Pyruvate</th>
<th>Glutamate</th>
<th>Succinate</th>
<th>Ascorbate + TMPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control mean ± STD</td>
<td>106±17</td>
<td>107±25</td>
<td>147±49</td>
<td>305±109</td>
</tr>
<tr>
<td>Control range</td>
<td>77-129</td>
<td>72-148</td>
<td>97-230</td>
<td>167-485</td>
</tr>
<tr>
<td>LB</td>
<td>102</td>
<td>148</td>
<td>158</td>
<td>349</td>
</tr>
<tr>
<td>SC</td>
<td>96</td>
<td>60</td>
<td>104</td>
<td>203</td>
</tr>
<tr>
<td>HC</td>
<td>72</td>
<td>43</td>
<td>105</td>
<td>199</td>
</tr>
<tr>
<td>MD</td>
<td>119</td>
<td>127</td>
<td>183</td>
<td>298</td>
</tr>
<tr>
<td>EE</td>
<td>72</td>
<td>62</td>
<td>94</td>
<td>460</td>
</tr>
<tr>
<td>JG</td>
<td>105</td>
<td>101</td>
<td>83</td>
<td>257</td>
</tr>
<tr>
<td>AH</td>
<td>149</td>
<td>154</td>
<td>208</td>
<td>440</td>
</tr>
<tr>
<td>EK</td>
<td>89</td>
<td>109</td>
<td>142</td>
<td>195</td>
</tr>
<tr>
<td>PM</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RS</td>
<td>71</td>
<td>48</td>
<td>99</td>
<td>430</td>
</tr>
<tr>
<td>JS</td>
<td>112</td>
<td>102</td>
<td>139</td>
<td>255</td>
</tr>
</tbody>
</table>
Table 8.3 Enzyme activities in the control patients

<table>
<thead>
<tr>
<th></th>
<th>NADH fericyanide reductase</th>
<th>NADH CoQ&lt;sup&gt;a&lt;/sup&gt; reductase</th>
<th>Succinate&lt;sup&gt;b&lt;/sup&gt; cytochrome c oxidase</th>
<th>ATPase&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control mean±STD</strong></td>
<td>3854±1152</td>
<td>182±64</td>
<td>224±134</td>
<td>44.4±14.6</td>
</tr>
<tr>
<td><strong>Control range</strong></td>
<td>2403-5409</td>
<td>117-284</td>
<td>113-430</td>
<td>28.4-65.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Patient</th>
<th>Value</th>
<th>Value</th>
<th>Value</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB</td>
<td>3781</td>
<td>117</td>
<td>113</td>
<td>65.1</td>
</tr>
<tr>
<td>SC</td>
<td>6138</td>
<td>103</td>
<td>216</td>
<td>55.2</td>
</tr>
<tr>
<td>HC</td>
<td>-</td>
<td>73</td>
<td>322</td>
<td>23.9</td>
</tr>
<tr>
<td>MD</td>
<td>2283</td>
<td>124</td>
<td>121</td>
<td>24.1</td>
</tr>
<tr>
<td>EE</td>
<td>3528</td>
<td>80</td>
<td>186</td>
<td>27.1</td>
</tr>
<tr>
<td>JG</td>
<td>4472</td>
<td>128</td>
<td>161</td>
<td>44.7</td>
</tr>
<tr>
<td>AH</td>
<td>-</td>
<td>226</td>
<td>351</td>
<td>40.2</td>
</tr>
<tr>
<td>EK</td>
<td>3616</td>
<td>84</td>
<td>298</td>
<td>45.4</td>
</tr>
<tr>
<td>PM</td>
<td>3253</td>
<td>69</td>
<td>505</td>
<td>20.3</td>
</tr>
<tr>
<td>RS</td>
<td>3498</td>
<td>79</td>
<td>180</td>
<td>26.2</td>
</tr>
<tr>
<td>JS</td>
<td>4902</td>
<td>164</td>
<td>224</td>
<td>53.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> rotenone sensitive,  <sup>b</sup> antimycin A sensitive,  <sup>c</sup> 1st order rate constant (/min/mg),  <sup>d</sup> oligomycin sensitive
Table 8.4  Cytochrome levels in the control patients

<table>
<thead>
<tr>
<th></th>
<th>nmoles/mg mt.protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>b</td>
</tr>
<tr>
<td>Control mean±STD</td>
<td>0.41±0.09</td>
</tr>
<tr>
<td>Control range</td>
<td>0.29-0.51</td>
</tr>
<tr>
<td>LB</td>
<td>0.31</td>
</tr>
<tr>
<td>SC</td>
<td>0.36</td>
</tr>
<tr>
<td>HC</td>
<td>0.29</td>
</tr>
<tr>
<td>MD</td>
<td>-</td>
</tr>
<tr>
<td>EE</td>
<td>0.29</td>
</tr>
<tr>
<td>JG</td>
<td>0.43</td>
</tr>
<tr>
<td>AH</td>
<td>0.59</td>
</tr>
<tr>
<td>EK</td>
<td>0.44</td>
</tr>
<tr>
<td>PM</td>
<td>-</td>
</tr>
<tr>
<td>RS</td>
<td>0.40</td>
</tr>
<tr>
<td>JS</td>
<td>0.29</td>
</tr>
</tbody>
</table>
Patient HC was aged 65 and had a multi-system neurological degeneration of uncertain cause. Some of the clinical features were suggestive of a mitochondrial disorder, namely the ptosis, myopathy and ataxia. The extra-pyramidal features of akinesia and rigidity were however quite marked and would have been unusual for a mitochondrial disorder. Her biochemical data seemed to suggest that she had a mild Complex I deficiency with glutamate and pyruvate oxidation rates below the normal range, as well as reduced NADH Q₁ reductase activity. However when age related normative data for these results are used she in fact falls within the normal range for these values (Dr J.M. Cooper personal communication). A few ragged red fibres were seen on her muscle biopsy but the numbers involved were considered to be compatible with her age. On balance therefore a mitochondrial disease was considered to be unlikely.

1.b. Mitochondrial Myopathy Patients

Twelve patients with mitochondrial disease were investigated in vitro, comprising eight males and four females whose ages ranged from 13 to 58 years old, with a mean of 30.73 years and a standard deviation of 14.86 years. These cases are grouped according to the absence or presence of a mtDNA defect, and according the nature of the defect, if present, as shown in Table 8.5. Group I contains two patients with a tRNA^leu(UUR) point mutation. Group II contains 5 patients with a single mtDNA deletion, while the remaining 5 cases with no detectable mtDNA defect are in Group III. Case summaries of these patients are given in Appendix 1, while the clinical features are summarised in Table 8.6. The biochemical findings of each patient are listed in Tables 8.7, 8.8 and 8.9.
Table 8.5 Clinical features of the mitochondrial myopathy patients

<table>
<thead>
<tr>
<th>mtDNA Group</th>
<th>Patient</th>
<th>Hosp.No</th>
<th>Sex</th>
<th>Lab No</th>
<th>Age</th>
<th>Clinical category(^1) and details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>VA</td>
<td>B84151</td>
<td>M</td>
<td>113</td>
<td>57</td>
<td>B CPEO, ptosis, pigmented retinopathy, myopathy</td>
</tr>
<tr>
<td>(point mutation)</td>
<td>DL</td>
<td>B68366</td>
<td>F</td>
<td>91</td>
<td>29</td>
<td>D dementia, pigmented retinopathy, myopathy</td>
</tr>
<tr>
<td>Group II</td>
<td>MB</td>
<td>B75728</td>
<td>M</td>
<td>90</td>
<td>34</td>
<td>C CPEO, ptosis, pigmented retinopathy, ataxia, dementia</td>
</tr>
<tr>
<td>(Deletion)</td>
<td>MHO</td>
<td>MVP14944</td>
<td>M</td>
<td>85</td>
<td>48</td>
<td>B CPEO, ptosis, myopathy</td>
</tr>
<tr>
<td></td>
<td>CS</td>
<td>GOS609208</td>
<td>F</td>
<td>112</td>
<td>13</td>
<td>C CPEO, ptosis, pigmented retinopathy, myopathy</td>
</tr>
<tr>
<td></td>
<td>PS</td>
<td>B79277</td>
<td>M</td>
<td>115</td>
<td>58</td>
<td>C CPEO, ptosis, pigmented retinopathy, deafness</td>
</tr>
<tr>
<td></td>
<td>MW</td>
<td>B73071</td>
<td>M</td>
<td>95</td>
<td>22</td>
<td>C CPEO, ptosis, pigmented retinopathy, deafness, dementia, cardiac conduction block, myopathy</td>
</tr>
<tr>
<td></td>
<td>2FB</td>
<td>MV91118</td>
<td>F</td>
<td>16</td>
<td>31</td>
<td>B CPEO</td>
</tr>
<tr>
<td></td>
<td>2YP</td>
<td>B69014</td>
<td>F</td>
<td>83</td>
<td>22</td>
<td>B CPEO</td>
</tr>
<tr>
<td>Group III</td>
<td>SB</td>
<td>B84488</td>
<td>F</td>
<td>21</td>
<td>21</td>
<td>D ataxia, dementia, deafness, short stature, myopathy</td>
</tr>
<tr>
<td>(None detected)</td>
<td>MHE</td>
<td>B72879</td>
<td>M</td>
<td>16</td>
<td>16</td>
<td>A myopathy</td>
</tr>
<tr>
<td></td>
<td>JL</td>
<td>B62495</td>
<td>M</td>
<td>96</td>
<td>23</td>
<td>D myoclonic epilepsy, ataxia, dementia, deafness</td>
</tr>
<tr>
<td></td>
<td>DP</td>
<td>B73500</td>
<td>F</td>
<td>116</td>
<td>29</td>
<td>A myopathy, lactic acidosis</td>
</tr>
<tr>
<td></td>
<td>PW</td>
<td>B74202</td>
<td>M</td>
<td>102</td>
<td>19</td>
<td>C CPEO, ptosis, pigmented retinopathy, ataxia, seizures,</td>
</tr>
</tbody>
</table>

\(^1\)Clinical categories:  
A: limb weakness and exercise intolerance  
B: chronic progressive external ophthalmoplegia (CPEO) ± pigmented retinopathy  
C: chronic progressive external ophthalmoplegia (CPEO) ± central nervous system disease  
D: major central nervous system disease  

\(^2\)Used for muscle culture studies only. See chapter 9.
Table 8.6 Polarographic findings in the mitochondrial myopathy patients

<table>
<thead>
<tr>
<th>Substrate (nMolesO/min/ml)</th>
<th>Control mean±STD</th>
<th>Control range</th>
<th>Polarographic category</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pyruvate</td>
<td>Glutamate</td>
<td>Succinate</td>
</tr>
<tr>
<td></td>
<td>Control mean±STD</td>
<td>106±17</td>
<td>107±25</td>
</tr>
<tr>
<td></td>
<td>Control range</td>
<td>77-129</td>
<td>72-148</td>
</tr>
<tr>
<td>Group I</td>
<td>VA</td>
<td>28 ↓</td>
<td>29 ↓</td>
</tr>
<tr>
<td></td>
<td>DL</td>
<td>51 ↓</td>
<td>44 ↓</td>
</tr>
<tr>
<td>Group II</td>
<td>MB</td>
<td>49 ↓</td>
<td>47 ↓</td>
</tr>
<tr>
<td></td>
<td>MHO</td>
<td>73 ↓</td>
<td>69 ↓</td>
</tr>
<tr>
<td></td>
<td>CS</td>
<td>49 ↓</td>
<td>34 ↓</td>
</tr>
<tr>
<td></td>
<td>PS</td>
<td>74 ↓</td>
<td>64 ↓</td>
</tr>
<tr>
<td></td>
<td>MW</td>
<td>107</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>FB</td>
<td>24 ↓</td>
<td>16 ↓</td>
</tr>
<tr>
<td></td>
<td>YP</td>
<td>30 ↓</td>
<td>12 ↓</td>
</tr>
<tr>
<td>Group III</td>
<td>SB</td>
<td>118</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>MHE</td>
<td>134</td>
<td>129</td>
</tr>
<tr>
<td></td>
<td>JL</td>
<td>33 ↓</td>
<td>35 ↓</td>
</tr>
<tr>
<td></td>
<td>DP</td>
<td>61 ↓</td>
<td>54 ↓</td>
</tr>
<tr>
<td></td>
<td>PW</td>
<td>106</td>
<td>128</td>
</tr>
</tbody>
</table>

1: Used for muscle culture studies only. See chapter 9.
<table>
<thead>
<tr>
<th></th>
<th>NADH fericyanide reductase</th>
<th>NADH CoQ&lt;sub&gt;1&lt;/sub&gt; reductase</th>
<th>Succinate&lt;sup&gt;b&lt;/sup&gt; cytochrome c reductase</th>
<th>Cytochrome c&lt;sup&gt;c&lt;/sup&gt; oxidase</th>
<th>ATPase&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control mean±STD</strong></td>
<td>3854±1152</td>
<td>182±64</td>
<td>242±134</td>
<td>44.4±14.6</td>
<td>812±123</td>
</tr>
<tr>
<td><strong>Control range</strong></td>
<td>2403-5409</td>
<td>117-284</td>
<td>113-430</td>
<td>28.4-65.1</td>
<td>674-910</td>
</tr>
<tr>
<td><strong>Group I</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VA</td>
<td>1744↓</td>
<td>25↓</td>
<td>230</td>
<td>5.8↓</td>
<td>977</td>
</tr>
<tr>
<td>DL</td>
<td>2965</td>
<td>74↓</td>
<td>236</td>
<td>33.7</td>
<td>1100</td>
</tr>
<tr>
<td><strong>Group II</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MB</td>
<td>1999↓</td>
<td>25↓</td>
<td>300</td>
<td>18.7↓</td>
<td>-</td>
</tr>
<tr>
<td>MHO</td>
<td>2106↓</td>
<td>63↓</td>
<td>114</td>
<td>43.2</td>
<td>620↓</td>
</tr>
<tr>
<td>CS</td>
<td>-</td>
<td>45↓</td>
<td>129</td>
<td>21.1↓</td>
<td>-</td>
</tr>
<tr>
<td>PS</td>
<td>-</td>
<td>193</td>
<td>531</td>
<td>34.5</td>
<td>-</td>
</tr>
<tr>
<td>MW</td>
<td>4371</td>
<td>137</td>
<td>234</td>
<td>40.6</td>
<td>260↓</td>
</tr>
<tr>
<td>1FB</td>
<td>-</td>
<td>56↓</td>
<td>338</td>
<td>21.4↓</td>
<td>-</td>
</tr>
<tr>
<td>1YP</td>
<td>-</td>
<td>44↓</td>
<td>117</td>
<td>12.4↓</td>
<td>-</td>
</tr>
<tr>
<td><strong>Group III</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SB</td>
<td>-</td>
<td>226</td>
<td>211</td>
<td>32.5</td>
<td>-</td>
</tr>
<tr>
<td>MHE</td>
<td>2553</td>
<td>159</td>
<td>268</td>
<td>68.8</td>
<td>340↓</td>
</tr>
<tr>
<td>JL</td>
<td>1392↓</td>
<td>15↓</td>
<td>272</td>
<td>4.0↓</td>
<td>-</td>
</tr>
<tr>
<td>DP</td>
<td>-</td>
<td>126</td>
<td>333</td>
<td>7.7↓</td>
<td>-</td>
</tr>
<tr>
<td>PW</td>
<td>1703↓</td>
<td>206</td>
<td>133</td>
<td>34.8</td>
<td>250↓</td>
</tr>
</tbody>
</table>

<sup>1</sup>: Used for muscle culture studies only. See chapter 9.  
<sup>a</sup>: rotenone sensitive,  
<sup>b</sup>: antimycin A sensitive,  
<sup>c</sup>: 1st order rate constant (l/min/mg),  
<sup>d</sup>: oligomycin sensitive
Table 8.8  Cytochrome levels in the mitochondrial myopathy patients

<table>
<thead>
<tr>
<th></th>
<th>b</th>
<th>c</th>
<th>aa&lt;sub&gt;3&lt;/sub&gt;</th>
<th>Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control mean±STD</td>
<td>0.41±0.09</td>
<td>0.54±0.07</td>
<td>0.69±0.10</td>
<td>0.42±0.09</td>
</tr>
<tr>
<td>Control range</td>
<td>0.29-0.51</td>
<td>0.48-0.62</td>
<td>0.56-0.83</td>
<td>0.33-0.59</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group I</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VA</td>
<td>0.28↓</td>
<td>-</td>
<td>0.57</td>
<td>0.12↓</td>
</tr>
<tr>
<td>DL</td>
<td>0.35</td>
<td>0.46↓</td>
<td>0.66</td>
<td>0.22↓</td>
</tr>
<tr>
<td>Group II</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MB</td>
<td>0.30</td>
<td>-</td>
<td>0.73</td>
<td>0.17↓</td>
</tr>
<tr>
<td>MHO</td>
<td>0.43</td>
<td>-</td>
<td>0.79</td>
<td>0.42</td>
</tr>
<tr>
<td>CS</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PS</td>
<td>0.44</td>
<td>0.60</td>
<td>0.72</td>
<td>0.27↓</td>
</tr>
<tr>
<td>MW</td>
<td>0.44</td>
<td>-</td>
<td>0.63</td>
<td>0.28↓</td>
</tr>
<tr>
<td>&lt;sup&gt;1&lt;/sup&gt;FB</td>
<td>0.35</td>
<td>0.41</td>
<td>0.55</td>
<td>0.17↓</td>
</tr>
<tr>
<td>&lt;sup&gt;1&lt;/sup&gt;YP</td>
<td>0.34</td>
<td>0.49</td>
<td>0.65</td>
<td>0.10</td>
</tr>
<tr>
<td>Group III</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SB</td>
<td>0.35</td>
<td>0.44↓</td>
<td>0.55↓</td>
<td>0.16↓</td>
</tr>
<tr>
<td>MHE</td>
<td>0.52</td>
<td>-</td>
<td>0.68</td>
<td>0.47</td>
</tr>
<tr>
<td>JL</td>
<td>0.16↓</td>
<td>-</td>
<td>0.58</td>
<td>undetectable</td>
</tr>
<tr>
<td>DP</td>
<td>0.49</td>
<td>0.49</td>
<td>0.65</td>
<td>0.10↓</td>
</tr>
<tr>
<td>PW</td>
<td>0.49</td>
<td>0.47</td>
<td>0.65</td>
<td>0.47</td>
</tr>
</tbody>
</table>

<sup>1</sup> Used for muscle culture studies only. See chapter 9.
2. Mitochondrial Protein Synthesis: General Quantitative Aspects

2.a. Controls

Of the eleven control patients, two were studied using the glutamate energy system modified by the addition of 1mM ATP thus increasing the rate of incorporation of the radiolabel (see Chapter 7, section 5). The incorporation of $^{35}$S methionine in the nine control patients with the unmodified glutamate energy system ranged from $3.58 \times 10^5$ dpm in 60 minutes, with a mean and standard deviation of $16.80 \pm 7.70 \times 10^5$ dpm in 60 minutes.

Table 8.9 Mitochondrial protein synthesis in human controls

<table>
<thead>
<tr>
<th>Patient</th>
<th>No. Of Results (n =)</th>
<th>Mitochondrial Protein Synthesis (x $10^5$ dpm/mg mt.protein in 60 minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Without ATP</td>
</tr>
<tr>
<td>LB</td>
<td>4</td>
<td>22.62 ± 7.16</td>
</tr>
<tr>
<td>SC</td>
<td>2</td>
<td>14.07, 15.51</td>
</tr>
<tr>
<td>HC</td>
<td>3</td>
<td>6.16 ± 2.98</td>
</tr>
<tr>
<td>MD</td>
<td>3</td>
<td>5.26 ± 1.19</td>
</tr>
<tr>
<td>EE</td>
<td>3</td>
<td>28.25 ± 7.54</td>
</tr>
<tr>
<td>JG</td>
<td>2</td>
<td>15.09, 14.06</td>
</tr>
<tr>
<td>AH</td>
<td>3</td>
<td>23.99 ± 3.06</td>
</tr>
<tr>
<td>EK</td>
<td>3</td>
<td>20.53 ± 4.63</td>
</tr>
<tr>
<td>PM</td>
<td>2</td>
<td>21.83, 16.94</td>
</tr>
<tr>
<td>RS</td>
<td>1</td>
<td>22.28</td>
</tr>
<tr>
<td>JS</td>
<td>3</td>
<td>3.58 ± 0.20</td>
</tr>
</tbody>
</table>

Mean ± STD = 16.80 ± 7.70 30.08 ± 6.08

Combined mean ± STD = 19.22 ± 9.03

Values are mean ± std, except where n < 3 where each value is given.
The total incorporation of label in the two cases using the modified glutamate energy system was 23.99 and $36.16 \times 10^5$ dpm in 60 minutes. Table 8.9 gives the individual results for these patients.

These figures compare with the mean and standard deviation of $77.37 \pm 28.40 \times 10^5$ dpm/mg mitochondrial protein in 60 minutes for rat muscle mitochondria. This significantly lower rate of human mitochondrial protein synthesis entailed longer exposure times for autoradiography.

2.b. Mitochondrial myopathy patients

There was a much wider variation in the total incorporation of radiolabelled methionine in this group as detailed in Table 8.10. In the cases studied with the original glutamate energy system values ranged from $1.07$ to $67.52 \times 10^5$ dpm/mg mitochondrial protein in 60 minutes with a mean of 21.85 and a standard deviation of $20.65 \times 10^5$ dpm/mg mitochondrial protein in 60 minutes. Three cases were studied with the modified glutamate energy system and for these values ranged from $41.72$ to $132.00 \times 10^5$ dpm/mg mitochondrial protein in 60 minutes with a mean and standard deviation of $79.66$ and $38.22 \times 10^5$ dpm/mg mitochondrial protein in 60 minutes respectively. These figures are on the whole higher than for the controls, but with the exception of two cases, DP and PS, still do not approach the levels seen in rat muscle mitochondrial protein synthesis.
Table 8.10 Mitochondrial protein synthesis in mitochondrial myopathies

<table>
<thead>
<tr>
<th>Patient</th>
<th>No. Of Results (n =)</th>
<th>Mitochondrial Protein Synthesis ($\times 10^5$ dpm/mg mt.protein in 60 minutes)</th>
<th>Without ATP</th>
<th>With ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>VA</td>
<td>3</td>
<td>13.61 ± 1.18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DL</td>
<td>2</td>
<td>18.33, 17.80</td>
<td></td>
</tr>
<tr>
<td>Group II</td>
<td>MB</td>
<td>4</td>
<td>7.03 ± 2.60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MHO</td>
<td>3</td>
<td>2.58 ± 0.47</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CS</td>
<td>2</td>
<td>24.03, 18.38</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PS</td>
<td>2</td>
<td>68.95, 61.64</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MW</td>
<td>3</td>
<td>67.57 ± 2.70</td>
<td></td>
</tr>
<tr>
<td>Group III</td>
<td>SB</td>
<td>3</td>
<td>41.72 ± 2.29</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MHE</td>
<td>4</td>
<td>46.98 ± 30.87</td>
<td></td>
</tr>
<tr>
<td></td>
<td>JL</td>
<td>4</td>
<td>18.55 ± 2.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DP</td>
<td>1</td>
<td>132</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PW</td>
<td>2</td>
<td>1.20, 0.94</td>
<td></td>
</tr>
</tbody>
</table>

Mean ± STD = 21.85 ± 20.65 79.66 ± 38.22

Combined mean ± STD = 36.30 ± 36.22

Values are mean ± std, except where n < 3 where each value is given.

2.c. Relationship between mitochondrial protein synthesis and age

Neither the controls or the mitochondrial myopathy cases showed any significant correlation between the rate of mitochondrial protein synthesis and the age of the subject, (figure 8.1).

163
2.d. Relationship between respiratory chain activity and mitochondrial protein synthesis

Since the in vitro energy system used was a glutamate based one which was demonstrated to be sensitive to Complex I inhibition, (see ), the relationship between the rate of mitochondrial protein synthesis and respiratory chain activity as assessed by glutamate oxidation was examined. As shown in figure 8.2 there is no significant correlation between the two.
3. Assignment Of Polypeptide Bands

The pattern of mitochondrially synthesised proteins as revealed by autoradiography was consistent for all the incubations, both with isolated rat and human muscle mitochondria, and in the mitochondrial translation products labelled in culture (see Chapter 9). The urea gel system described by Kadenbach et al (1983) was found
Table 8.11 Molecular weights of protein bands

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tris glycine&lt;sup&gt;a&lt;/sup&gt;</td>
<td>PO&lt;sub&gt;4&lt;/sub&gt;&lt;sup&gt;2-&lt;/sup&gt;-Urea&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ND 5</td>
<td>66.6</td>
<td>43.5</td>
</tr>
<tr>
<td>COX I</td>
<td>57.0</td>
<td>39.5</td>
</tr>
<tr>
<td>ND 4</td>
<td>51.4</td>
<td>36.5</td>
</tr>
<tr>
<td>CYT B</td>
<td>42.7</td>
<td>27.5</td>
</tr>
<tr>
<td>ND 2</td>
<td>38.9</td>
<td>31.5</td>
</tr>
<tr>
<td>ND 1</td>
<td>35.6</td>
<td>29.5</td>
</tr>
<tr>
<td>COX III</td>
<td>30.0</td>
<td>22.5</td>
</tr>
<tr>
<td>COX II</td>
<td>25.5</td>
<td>23.6</td>
</tr>
<tr>
<td>ATP6</td>
<td>24.8</td>
<td>21.6</td>
</tr>
<tr>
<td>ND 6</td>
<td>18.6</td>
<td>16.7</td>
</tr>
<tr>
<td>ND 3</td>
<td>13.2</td>
<td>13.5</td>
</tr>
<tr>
<td>ND 4L</td>
<td>10.7</td>
<td>14.8</td>
</tr>
<tr>
<td>ATP8</td>
<td>7.9</td>
<td>9.8</td>
</tr>
</tbody>
</table>

<sup>a</sup>: tris glycine gel system, <sup>b</sup>: phosphate urea gel system (Wallace et al 1991), <sup>c</sup>: urea gel system described here.

to give the best separation of the subunits. The apparent molecular weights for each of the bands is given in Table 8.11. Shown also in this table for comparison are the predicted molecular weights for the different subunits and the apparent molecular weights for the bands in two other gel systems as quoted in Wallace et al (1991). As highlighted in Chapter 4 (see section 8) the apparent molecular weights of the bands varies according to the precise method of sample preparation, sample solubilisation and gel system used. The bands were assigned according to their relative mobilities as given for the urea gel system (column b).
In figure 8.3 the polypeptide profile of patients EK and JG (both controls) is shown alongside that of a sample (kindly donated by Professor Attardi and Dr Chomyn) of HeLa mitochondrial proteins labelled in culture as described (Chomyn et al 1983). It can be seen that there is good correspondence between the bands in all three lanes.

![Figure 8.3 Autoradiograph comparing the mitochondrial translation products obtained in two in vitro studies with those obtained in HeLa cultures by the method of Chomyn et al 1983.](image)

The COX subunits were often the clearest visualised bands with COX I being the first heavy labelled band visible from the origin, while COX II and III formed a close running doublet sometimes merging into a single band. The ND5 subunit was
only sometimes visible as a faint band above the COX I band. ATP 6 subunit was often the last of the heavily labelled bands with bands below it being less well visualised in some cases. These findings are consistent with previously published studies showing the relative rates of synthesis of the different polypeptides (see Chapter 2 Table 2.3).

4. Mitochondrial Protein Synthesis In The Mitochondrial Myopathies

4.a. Group I (Point mutation)

4.a.i. Patient VA

Patient VA, had the tRNA^{leu(UUR)3243} point mutation presenting with ophthalmoplegia, ptosis, retinopathy and myopathy. Polarography showed a Complex I-III defect, and there was a marginal reduction in cytochrome b, with a more significant reduction in cytochrome aa\textsubscript{3}. The mean rate of mitochondrial protein incorporation was 13.61 x 10\textsuperscript{5} dpm/mg mt.protein/60 mins. compared with the overall mean of 21.85 x 10\textsuperscript{5} dpm/mg mt.protein/60 mins. Figure 8.4 shows the autoradiograph obtained with this case alongside that of four control cases. The profile of the labelled polypeptides is the same in all five cases.
4.a.ii. Patient DL

Patient DL had a tRNA<sup>Leu(UUR)</sup><sup>3252</sup> point mutation (Morten et al 1992) presenting with dementia, myopathy and pigmentary retinopathy, with a Complex I-III defect on polarography and low levels of both cytochrome c<sup>1</sup> and aa<sup>3</sup>. The mitochondrial translation products obtained in this case are illustrated in figure 8.8. Although no control lane was available for this autoradiograph, the profile of the labelled proteins does not seem different to that obtained in normal subjects.

4.b. Group II (Deletions)

Five cases had mtDNA deletions and, as one would expect, these all had
ophthalmoplegia as part of their clinical picture. The mapping details and the affected reading frames for these deletions is listed in Table 8.12 and illustrated in Figure 8.5. Two were the so-called "common" deletion with a 13bp repeat flanking the 4.9kb deletion, another two had a 10bp repeat flanking a smaller deletion of 2.309kb. The final case, MW, had a more extensive deletion of 5.938kb. Also given for the deletions is the percentage of the total mtDNA represented by the mutant DNA.
### Table 8.12 Details of mtDNA deletions studied

<table>
<thead>
<tr>
<th>Patient</th>
<th>Case No.</th>
<th>Proportion</th>
<th>Deletion mapping</th>
<th>Deleted reading frames</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB</td>
<td>90</td>
<td>80%</td>
<td>2.309kb with 10bp repeat (12103-14412)</td>
<td>4,5,6, - - - III 6,8 5</td>
</tr>
<tr>
<td>MHO</td>
<td>85</td>
<td>50%</td>
<td>2.309kb with 10bp repeat (12103-14412)</td>
<td>4,5,6, - - - III 6,8 5</td>
</tr>
<tr>
<td>CS</td>
<td>112</td>
<td>77%</td>
<td>4.9kb with 13 bp repeat (8470-13447)</td>
<td>3,4L,4,5, III 6,8 5</td>
</tr>
<tr>
<td>PS</td>
<td>115</td>
<td>40%</td>
<td>4.9kb with 13 bp repeat (8470-13447)</td>
<td>3,4L,4,5, III 6,8 5</td>
</tr>
<tr>
<td>MW</td>
<td>95</td>
<td>50%</td>
<td>5.938kb (7439-13477)</td>
<td>3,4L,4,5, I,II,III 6,8 8</td>
</tr>
<tr>
<td>FB</td>
<td>16</td>
<td>80%</td>
<td>2.309kb with 10bp repeat (12103-14412)</td>
<td>4,5,6, - - - III 6,8 5</td>
</tr>
<tr>
<td>YP</td>
<td>83</td>
<td>65%</td>
<td>not sequenced</td>
<td>3,4L,4, II 8 6</td>
</tr>
</tbody>
</table>

*^ Used for muscle culture studies only. See chapter 9.*
Figure 8.6 charts the mitochondrial protein synthesis in these cases indicating the biochemical defect and the percentage mutant mtDNA. It would seem from this that there is a lack of correspondence between the percentage of affected mitochondrial genomes and the rate of mitochondrial protein synthesis. This best shown by cases MHO and MW, who both have 50% of the mtDNA affected by a deletion. They have very different rates of mitochondrial protein synthesis being $2.58 \pm 0.47$ and $67.57 \pm 2.70 \times 10^5$ dpm/mg mitochondrial protein in 60 minutes, respectively. The length of the deletion is actually greater in MW and deletes eight, rather than five, tRNAs.
These patients have differing polarographic assessment of the respiratory chain with the case with the lower rate of synthesis, MB, having a Complex I deficiency, while MW is polarographically normal.

Three out of the four cases with defective respiratory chain function polarographically had lower rates of mitochondrial protein synthesis than the single case with normal respiratory chain function. The exception, PS, was studied using the modified glutamate energy system containing 1mM ATP, but also has the lowest proportion of mutant mtDNA (40%).

4.b.i. Patient CS

This 13 year old female patient has a Complex I-III defect on polarography with reduced enzyme activities for NADH CoQ\textsubscript{1} reductase and cytochrome c oxidase. The mtDNA deletion affects 77% of the total mtDNA. Mapping of the deletion reveals the "common" deletion resulting in loss of the reading frames for ND subunits 3, 4L and 4, COX III, ATPase subunits 6 and 8 and for five of the tRNAs. The mtDNA translation products for this patient are shown in Figure 8.7 alongside that of control case JG. Of the ND subunits, ND3 and ND4L appear to be synthesised normally, but ND4 while only faintly visualised in the control is reduced in patient CS. The COX III subunit is clearly reduced in patient CS compared with control, while by contrast the synthesis of COX II, whose reading frame is not within the deletion region is preserved. The intensity of the ATPase 6 band may be reduced while that of the ATPase 8 subunit appears to be unaffected. No additional band is seen that might correspond to a fusion product.
4.b.ii. Patient PS

This 58 year old male patient had a Complex I defect on polarography, with normal enzyme (including NADH CoQ1) activities, but a reduced aa3 level. He has an identical deletion to patient CS, but which affects only 40% of total mtDNA. The mitochondrial translation products obtained in this case are shown in Figure 8.8 together with those obtained from control HC. NDs 3 and 4L are not significantly reduced but ND 4 is undersynthesised. ATPase subunits 6 and 8 are seen at a level comparable with the control. The COX II/III band may be reduced, compatible with reduced synthesis of COX III. Again no candidate fusion protein can be visualised.
4.b.iii. Patient MW

This 22 year old male showed no polarographic defect of mitochondrial function, but ATPase activity was reduced and there were low levels of cytochrome aa₃. The mtDNA deletion was extensive, being 4.9kb long and affecting reading frames for NDs 3, 4L and 4, all three COX subunits, both ATPase subunits and eight tRNAs. Mutant mtDNA comprised half of the total mtDNA. The labelled mitochondrially synthesised proteins are shown in Figure 8.9. Unfortunately no controls were available for this gel run. However there does not appear to be any obvious under synthesis of specific subunits.
Figure 8.9 Mitochondrial translation products in patients DL (Group I), MW (Group II) and MHE (Group III).

4.b.iv. Patient MHO

MHO has a Complex I defect polarographically with normal cytochrome levels, and 50% mutant mtDNA with a deletion which encompasses the ND 4, 5 and 6 reading frames. The level of $^{35}$S methionine incorporation was low at $2.58 \times 10^5$ dpm/mg mitochondrial protein in 60 minutes. ND5 is generally not seen in these
autoradiographs, ND4 appears unaffected allowing for the low level of $^{35}$S methionine labelling, and the ND6 region is poorly resolved on this gel (Figure 8.10).

An autoradiograph of patient MB, who has an identical deletion to MHO affecting 80% of total mtDNA, is not available.
4.c. Group III (No known mtDNA defect)

4.c.i. Patient DP

Patient DP had an exceptionally high level of methionine incorporation with a single value of $132.00 \times 10^5$ dpm/mg mitochondrial protein in 60 minutes, even allowing for the modification of the energy system. She has, as yet, no known mtDNA defect. Clinically she was also unusual in that she had recurrent episodes of myopathy with severe encephalopathy associated with lactic acidosis, but despite this had only

---

**Figure 8.11** Autoradiograph of mitochondrial translation products in patients SB and DP (Group III) compared with those in controls AH, and HC.
mild muscle fatiguability between attacks with no central nervous system abnormality either clinically, or on imaging. Histochemical stains were characterised by a very low level of cytochrome oxidase activity in most of the fibres. Biochemically she was also atypical in that, polarographically she has a Complex I-III defect with a preserved rate of oxygen consumption utilising ascorbate and TMPD, but a markedly low cytochrome oxidase activity. The profile of mitochondrial protein synthesis shown in Figure 8.11, shows no under synthesis of any of the polypeptides compared with that of the controls. In particular synthesis of COX subunits I, II and III seems unimpaired.

4.c.ii. Patient JL

This patient displayed the MERRF phenotype, without any detectable point mutation of the mtDNA. Oxygen uptake rates were below the mean control values for all substrates used with residual rates of 31% and 36% in the case of the NAD-linked substrates pyruvate and glutamate respectively, 48% for succinate, and 39% for ascorbate + TMPD. Enzyme assays show a decrease in NADH ferricyanide reductase (36% of normal), NADH-CoQ1 reductase (8% of normal), and cytochrome c oxidase (9% of normal) activities. Cytochrome measurements show a marked reduction in the levels of cytochromes b and aa3. In two incubations radiolabel was incorporated into mitochondrial protein at rates of 18.48 and 21.84 dpm/mg mt. protein in 60 minutes.

Figure 8.12 shows the autoradiograph of the labelled mitochondrial proteins from JL compared with that from a control case LB. There is relative undersynthesis of the higher molecular weight protein subunits namely ND4, ND2, ND1, COX I, COX
II COX III and cytochrome b. The reduced synthesis of these subunits thus correlates with the biochemical findings of impaired Complex I and IV activity, and the reduced levels of cytochrome b.

4.c.iii. Other Group III Patients

PW had a combination of ophthalmoplegia, ptosis and retinopathy with ataxia and seizures. He had no polarographic defect of the respiratory chain and has normal cytochrome levels. The rate of mitochondrial protein synthesis for his mitochondria was the lowest of all the human cases studied. Allowing for this low level of radiolabel incorporation, the profile of the mitochondrial translation products shown in Figure 8.13 is comparable with that of the control MD.
The mitochondrial translation products for patient MHE are shown in figure 8.7 and that for patient SB in figure 8.11 and neither shows any obvious abnormalities.
1. Patients Studied

The results obtained from four of the mitochondrial myopathy cell lines from cases (CS, MW, MB and JL), also studied in vitro, along with an additional two cases of mitochondrial myopathy (YP and FB) are presented here. The two additional mitochondrial myopathy cases were investigated in detail biochemically and genetically and their details are given in Tables 8.6 to 8.10 of Chapter 8. These six cases were classified as shown in Table 9.1.

<table>
<thead>
<tr>
<th>Group</th>
<th>Point mutation</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group II</td>
<td>Deletion</td>
<td>CS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MW</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MB</td>
</tr>
<tr>
<td></td>
<td></td>
<td>YP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FB</td>
</tr>
<tr>
<td>Group III</td>
<td>No known defect</td>
<td>JL</td>
</tr>
</tbody>
</table>

Six controls cell lines were used for comparison. The sex, age, and muscle histology findings of the additional control cases is given in Table 9.2.
Table 9.2 Details of additional controls used for muscle cultures

<table>
<thead>
<tr>
<th>Patient</th>
<th>Hosp.No</th>
<th>Sex</th>
<th>Age</th>
<th>Muscle Histology</th>
</tr>
</thead>
<tbody>
<tr>
<td>EB</td>
<td>PP</td>
<td>M</td>
<td>33</td>
<td>Low grade polymyositis</td>
</tr>
<tr>
<td>JB</td>
<td>B79850</td>
<td>F</td>
<td>70</td>
<td>Polymyositis</td>
</tr>
<tr>
<td>RC</td>
<td>B80739</td>
<td>M</td>
<td>27</td>
<td>Normal</td>
</tr>
<tr>
<td>BD</td>
<td>B80655</td>
<td>F</td>
<td>59</td>
<td>Type 2 atrophy</td>
</tr>
<tr>
<td>PD</td>
<td>B69011</td>
<td>M</td>
<td>22</td>
<td>Normal</td>
</tr>
<tr>
<td>NT</td>
<td>PP</td>
<td>M</td>
<td>42</td>
<td>Dystrophy</td>
</tr>
</tbody>
</table>

2. The Expression Of Disease In Primary Muscle Cell Culture.

2.a. Growth Characteristics

It was noticeable that the control muscle cultures deriving from cases of polymyositis grew much faster than other controls, consistent with the higher percentage of myosatellite stem cell known to be present in regenerating muscle. With occasional exceptions with very slow growth rates, the growth rate of muscle cultures from cases of mitochondrial myopathy was comparable with that of the controls. The significance of this is uncertain without knowing the numbers of viable mononuclear myosatellite cells dissociated from the original muscle biopsy. Formal culture growth curves were not plotted.

2.b. Morphological Characteristics

Morphological studies of the muscle cultures, using electron microscopy, showed numerous abnormally large mitochondria with unusual arrangement of their cristae.
Figures 9.1 and 9.2.

Figure 9.1 Electronmicrograph showing examples of the abnormal mitochondria seen in culture both in controls and in mitochondrial myopathy.

Figure 9.2 Detail of a single mitochondrion, twice normal size, and with abnormal array of cristae (see text).
These mitochondria resembled those illustrated by Askanas et al (1978) in their cultures obtained from the muscle of two patients with mitochondrial myopathy, (Figure 9.3).

However such abnormalities were seen in the control as well as mitochondrial myopathy cultures and cannot therefore be specific to the disease. Para-crystalline inclusions were not seen.

![Figure 9.3](image)

*Figure 9.3* Electronmicrograph of abnormal mitochondria published by Askanas et al 1978.

With phase contrast microscopy it was possible to see increasing cytoplasmic granularity of the myotubule cultures as they aged. This was an effect seen in most ageing cultures and did not appear to be exclusive to mitochondria myopathy cultures, as suggested by Meola et al (1987).

Preliminary attempts to characterise the cultures in terms of their cytochrome oxidase staining properties were not uniformly reliable.
2.c. Biochemical Characteristics.

Several attempts were made to replicate the methods of Robinson et al (1986, 1989) assaying respiratory function using the oxygen electrode on digitonin treated muscle cultures, both at myoblast and at myotubule stage, without success.

2.d. Mitochondrial DNA Analysis

Southern blot analysis for the length deletions of mtDNA was carried out on the muscle cultures of cases MW, FB, and YP within 5 to 7 passages from the primary cell culture. In each case the proportion of mutant mtDNA present in muscle culture was considerably reduced compared with that present in the mtDNA extracted from the muscle (Figure 9.4).

![Figure 9.4](image)

*Figure 9.4* Southern blot of mtDNA present in the muscle biopsy (M) and muscle cell cultures (C) of three patients with mitochondrial myopathy. Upper band = wild type, lower band = mtDNA with a deletion.
In one of these cases, MW, Southern blot analysis was performed on fibroblast culture obtained from explants of the patients skin, (Figure 9.5). Given the nature of the explant technique for the establishment of the fibroblast cell line, it is likely that many more cell doublings would have occurred prior to harvesting than would have been the case with the muscle cell line. Despite this, and in contrast to the muscle cell line, there was a higher proportion of the total mtDNA containing the deletion in the fibroblast line, although it was still less than seen in the original muscle biopsy.

Figure 9.5 Southern blot of the mtDNA present in the muscle biopsy (M), fibroblast cultures (F) and muscle cultures (C), from patient MW.

187
In a further case, CS, Southern blot analysis was performed on clonal muscle cell cultures (Figure 9.6). Here it can be seen that the relative proportions of mutant versus wild type mtDNA genomes varies in the different clones. Thus clonal lines 4 and 7 show no mutant mtDNA on this blot, while clonal lines 1 and 9 still retain a significant proportion of mtDNA harbouring a deletion.

![Southern blot of the mtDNA present in eleven cloned muscle cell culture lines from patient CS.](image)

**Figure 9.6** Southern blot of the mtDNA present in eleven cloned muscle cell culture lines from patient CS.

3. Mitochondrial Protein Synthesis In Culture

More satisfactory inhibition of nuclear encoded protein synthesis in culture was achieved with emetine than with cycloheximide. Figure 9.7 illustrates the effect of the inhibition of nuclear encoded protein synthesis as seen in lane 1, by emetine. Inhibition of nuclear encoded synthesis is apparent at an emetine concentration of 50μg/ml and remains specific until 400μg/ml where there is a suggestion that the overall mitochondrial protein synthesis is reduced. The prior addition of chloramphenicol, as well as emetine, to the culture results in complete loss of incorporation of $^{35}$S.
methionine as both nuclear and mitochondrial encoded protein synthesis were inhibited.

<table>
<thead>
<tr>
<th>Emetine concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
</tbody>
</table>

Figure 9.7 Protein synthesis in muscle cell cultures incubated with increasing concentration of emetine (0 - 400µg/ml). In the final lane chloramphenicol (CAP) was also present.
The autoradiograph shown in Figure 9.8 shows the mitochondrially synthesised polypeptide profile in three of the patients in Group II i.e YP, MW, and FB, with PD and EB as controls. An additional patient from this group, MB, is shown in Figure 9.9. From these it can be seen that these profiles are all similar to that of the controls.

![Figure 9.8 Mitochondrial protein synthesis in muscle cell cultures. Details given in text.](image)

The autoradiograph shown in Figure 9.9 also shows the mitochondrially synthesised polypeptide profile in patient JL, who belongs to Group III, together with controls, RC, BD, NT and JB. In contrast to the abnormal polypeptide profile seen in
the in vitro studies his culture synthesised profile is no different to that of the controls.

Figure 9.9 Mitochondrial protein synthesis in muscle cell cultures. Electrophoresis was run using a narrow well comb. Details given in text.
DISCUSSION
CHAPTER 10: DISCUSSION

1. In Vitro Studies; Methodological Considerations

A method for studying in vitro mitochondrial translation was successfully adapted initially using isolated rat muscle mitochondria. The technique has the advantage of requiring less than 200μg of mitochondria per incubation in a total reaction volume of 100μl. This is considerably less than used in previous mammalian mitochondrial studies, clearly an advantage when using scarce patient material. Even with the completing requirements for the isolated mitochondria for polarography, enzyme activity measurements, cytochrome levels, and molecular biological studies it was usually possible to perform several incubations on each patient.

The requirement for freshly isolated mitochondria was highlighted by the decline in the rate of mitochondrial 35S methionine incorporation with time despite storage on ice. This might have caused some variation in the rates of protein synthesis between different samples studied, but the time lapsed between isolation and labelling of the mitochondria was fairly constant. Because cases and controls were studied singly and sometimes at long intervals apart this often meant that direct quantitative comparisons between different patients run on the same gel were not necessarily valid since the activity of the radiolabel used would be different in each case. Particular efforts were made to try and group cases and controls as much as possible.

The incorporation of 35S methionine into mitochondrial protein was consistently
shown to be resistant to cycloheximide but sensitive to chloramphenicol as expected for mitochondrial protein synthesis. The addition of erythromycin did not significantly alter the rate of $^{35}$S methionine incorporation making mycoplasmal incorporation unlikely. Sterile precautions reduced bacterial contamination to a level where it would not contribute to the labelled proteins.

The level of $^{35}$S methionine incorporation achieved with this technique was significantly higher than has been previously reported for mammalian or human mitochondria. The small final reaction volume of incubation meant that a much higher concentration of $^{35}$S methionine label was present, which may have contributed to this higher level of incorporation. The glutamate and atractyloside energy system used here, did seem to be more efficacious than energy systems used by others, such as the succinate or phospho-enol-pyruvate (PEP) based systems assessed here. However the experience of Kroon (1973), Beattie (1979) and Ashwell and Work (1970), suggests that different methods of mitochondrial preparation may show different responses to the various energy systems available. This may reflect the integrity of the respiratory chain and adenyl translocase; those mitochondria retaining an intact outer membrane effectively using self generated ATP while those mitochondria with their outer membrane altered by sonication or digitonin utilising an exogenous ATP supply in preference. Thus it would be necessary to test these responses for mitochondrial isolated in different laboratories.

In past literature on the subject, claims have been made for various exogenous and endogenous energy systems and it has been generally accepted in the past that the
PEP based energy system was an exogenous one, which by definition would not be affected by respiratory chain dysfunction. However, the studies presented here show this is clearly not the case and that this energy system is sensitive to Complex I inhibition by rotenone. On theoretical grounds this might not be too surprising since pyruvate kinase would catalyse the conversion of phosphoenol pyruvate to pyruvate, a Complex I dependant substrate. The succinate based energy system was proportionally less sensitive to Complex I inhibition as one might expect for a Complex II dependant substrate. However the much lower level of radiolabel incorporation achieved with the succinate based system did not recomend its use.

The glutamate based energy system was sensitive to Complex I inhibition by rotenone in a dose dependent manner. The energy requirement for mitochondrial protein synthesis may be considerably less than required for energy export, and so even quite marked defects in respiratory chain function may not cause any fall off in mitochondrial protein synthesis. In support of this was the fact that no significant correlation between the level of mitochondrial protein synthesis and glutamate oxidation rates as measured polaragraphically in either the control or mitochondrial myopathy cases was seen. However, although overall mitochondrial protein synthesis may have been unaffected, in a mixed population of mitochondria with varying respiratory chain function there may have been a greater contribution to the labelled protein pool from more intact mitochondria. This might result in a bias towards the normal translation product profile with under representation of the contribution, or lack of it, from more severely impaired mitochondria.
The level of $^{35}$S methionine incorporation into human skeletal muscle mitochondria was with the exception of one case, less than half and in many cases much less than that seen into rat skeletal muscle mitochondria. Both species and tissue specific variations in mitochondrial protein synthesis are recognised. Some of the factors contributing to such variation are well recognised such as the differing optimal magnesium concentration, but other factors are less well understood. The level of incorporation achieved with rat muscle mitochondria was sufficient to give a good signal to noise ratio in that autoradiographs required only short exposure times resulting in clearly defined mitochondrial protein bands. By contrast the lower rate of incorporation in human muscle mitochondria required longer autoradiography exposure times, and there tended to be more background activity and minor bands present. These minor bands could not be attributed to residual cytoplasmic protein synthesis, since they were completely abolished by the addition of chloramphenicol. Bacterial contamination of the mitochondrial preparation was no more likely in human than in rat mitochondrial preparation and so this too seems unlikely to account for the minor bands.

It was in order to try and improve the quality of the autoradiographs in the human mitochondrial studies that variations to the glutamate based energy system were tried, resulting in significant further increase in the rate of radiolabel incorporation when 1 mM ATP was added.

The assignment of the mitochondrially synthesised polypeptide bands was based on the apparent molecular weights of the subunits which as discussed (Chapter 4 section
can vary considerably with minor differences in electrophoresis conditions making direct comparision between workers difficult. The apparent molecular weights obtained here in fact showed a closer correspondance to the predicted molecular weights than hitherto published. There was clear similarity with the polypeptide profile obtained with the labelled HeLa mitochondria kindly donated by Attardi's laboratory. The faint labelling of the ND5 subunit seen in both rat and human muscle mitochondrial is consistant with the suspected post-transcriptional down regulation of this subunit reported in muscle.

In human mitochondrial disease, mitochondrial DNA abnormalities are usually heteroplasmic. Thus some differences in the mitochondrial translation product profile, are likely to be quantitative rather than qualitative, with reduced synthesis of individual polypeptides relative to others. Visual inspection of the autoradiographs required making allowances for any differences in the overall level of labelling in different cases caused by variations in the rate of incorporation of label and the time lapsed since labelled. Large differences between patients and controls could be detected by this means but more subtle differences may well have been missed. Quantitation of the individual bands was attempted by the use of either laser densitometry of the autoradiographs or in some cases gamma camera scanning of the actual gels. However, in neither case was sufficient resolution obtained that would have allowed consistent results. An alternative method would be to slice each gel lane at intervals down the lane, perform a scintillation count of each slice and integrating the results to produce an activity profile. The latest gamma scanners and associated software probably would be able to give sufficient resolution.
In Vitro Studies In Mitochondrial Diseases

In patients VA and DL, with tRNA\textsuperscript{leu(UUR)} point mutations it was established that both the rate of mitochondrial protein synthesis and the profile of mitochondrial translation products was comparable with that of controls. This would seem to suggest that this point mutation does not in fact alter the ratio between ribosomal and messenger RNA species as has been suggested by Hess et al (1991). A similar conclusion was drawn by King et al (1992) using experiments on Ro\textsuperscript{0} cell lines. Such cell lines showed normal mitochondrial protein synthesis unless the content of mutant mtDNA with the tRNA\textsuperscript{leu(UUR)} mutation exceeded 94\% of total mtDNA.

In cases with mtDNA deletions there was no simple correlation between the percentage mutant mtDNA and the rate of mitochondrial protein synthesis. Several variables may have contributed to this. Cases differed in their respiratory chain function, and their in vitro mitochondrial protein synthesis rates may have been affected by the energy system used. However as stated above there was no correlation between glutamate oxidation and synthesis rates. The mutant to wild type mtDNA proportions were obtained from laser densitometry of the Southern blots performed on the original muscle biopsy rather than on the actual mitochondrial pellets used for in vitro protein synthesis. However, since post mortem studies have shown that the degree of heteroplasmy for mtDNA deletions is the same in different samples from the same tissue, this may not make much difference. In addition, it has been suggested that the deleterious effects of mutant mtDNA may be more related to the absolute amount of wild type mtDNA present, an unknown quantity at present. Cases also differed in the
extent of the deletion present and there is evidence to suggest that more extensive deletions involving a greater number of tRNAs may not be able to complement with wild type mtDNA (Hammons et al 1992). Patient MW with the most extensive deletion involving 8 tRNAs had the highest rate of in vitro mitochondrial protein synthesis, with a normal polypeptide profile and had normal polarography. Perhaps this was the result of increased mitochondrial protein synthesis by mitochondria with wild type mtDNA. However, bearing these factors in mind, lack of simple correlation between percentage mutant mtDNA containing deletion may suggest that complementation is occurring to varying degrees in different cases.

In at least 2 out of the 5 cases with mitochondrial deletions (CS and PS), a reduction in certain mitochondrially encoded subunits was seen which was consistent with a reduction in the number of the reading frames affected by the deletion. This seems likely to represent undersynthesis of the relevant subunits, rather than increased proteolysis, for example, since no increase in low molecular weight proteins was seen. These findings strongly suggest that complementation is occurring in those cases. The absence of such findings in the other 3 cases does not necessarily exclude the possibility of complementation. More detailed quantitation as mentioned above and the influence of the energy system used in vitro may bias the true picture.

In none of the cases with deletion was any novel protein of appropriate molecular weight seen that would have been a candidate for a fusion protein. The common deletion of 4,977bp flanked by a 13bp repeat as seen in patients CS and PS would have produced a fusion protein of about 1.4 kD which would have been difficult
to resolve on the gel system used. The stability of such fusion proteins in vitro is also uncertain. The absence of a fusion protein under these circumstances does not therefore exclude the possibility of complementation.

In 1 of the 5 cases with no known defect of mtDNA (JL), there was a clear undersynthesis of high molecular weight subunits which correlated with the biochemical findings. The mtDNA studies performed, exclude the possibility of a large deletion but a smaller deletion or point mutation remain possible. Such mutation could probably affect either another tRNA gene or perhaps a rRNA gene to account for the picture seen.

3. In Vitro Mitochondrial Protein Synthesis; Future Directions

Although the present micro-technique has made the use of human material easier, the preparation of the mitochondria still required the removal of approximately 6g of muscle under general anaesthetic. This reduces the application of the technique, limits the number that can be studied at one time, and makes control data difficult to obtain. Preliminary studies seem to suggest that a suitable mitochondrial preparation could be acquired using a smaller muscle biopsy sample, such as obtained routinely under local anaesthesia for histological evaluation of muscle disease. This would open the way for more widespread studies in clinically suspected cases of mitochondrial disease which may not have ragged red fibres and do not have any of the recognised mtDNA mutations.
Further adjustments to the energy system may be possible in order to increase still further the level of incorporation and perhaps make it more resistant to respiratory chain dysfunction. However one could argue that such an energy system may obscure the true in vivo lack of energy supply.

Immunoprecipitation studies of the labelled proteins would be desirable in order to more accurately assign the bands to the appropriate protein subunit.

More detailed quantitation by way of gamma scanning would make the technique more sensitive.

A further limitation of the technique is imposed by the necessity to study each patient fresh without being able to label several patients at the same time. It would certainly be worthwhile investigating whether any storage of the mitochondria is possible without loss of synthetic function, perhaps by freezing in liquid nitrogen in the presence of dimethyl sulfoxide (DMSO) to prevent mitochondrial disruption.

4. Cell Culture Studies

In Chapter 5 the importance of characterising cultures to ensure that they expressed the defect of interest was highlighted. In terms of the mitochondrial myopathies there are a variety of ways in which this could be achieved, morphologically, biochemically or genetically.
It appears that the ultra-structural abnormalities of mitochondria, seen in mitochondrial myopathies, while they may be present in culture, are not specific and cannot be regarded as reliable indicators of disease expression. The finding of para-crystalline inclusions may possibly be more specific, but they have not been reported to occur in culture. In any case their relationship to the disease is uncertain. In recent literature there has been a tendency to use cytochrome oxidase negative staining as the morphological marker for the disease. Cytochrome oxidase negative muscle fibres have been shown to correlate with the presence of mutant mtDNA genomes and ragged red fibres using in situ hybridisation studies. The use of other cytochemical stains such as succinate dehydrogenase or the Gomori trichrome stain on cultures has not been reported, despite the latter producing the hitherto morphological hallmark of these diseases, the ragged red fibre.

The possibility of detecting suitably expressed cultures "in vivo", by phase contrast microscopy, would have clear attractions but does not appear tenable.

One of the disadvantages of biochemical characterisation of cultures for this disease is the multiplicity of enzyme assays required to fully appraise the respiratory chain. This in turn requires relatively large amounts of culture material. Since these cultures were not immortalised and had a finite lifespan there was concern about whether sufficient material could be grown for all the studies needed. One solution to this potential problem would be to transform the cultures.

The adaptation of oxygen electrode techniques to cultures in situ as published
by Robinson et al (1986) was assessed for this reason. Unfortunately the method was not transferable from fibroblast cultures to myoblast or myotubule cultures presumably because of different permeability characteristics of the cell membrane. This conclusion was confirmed by others, (Robinson personal communication).

Southern blot analysis of the cultures for deletions of mtDNA was readily performed on small amounts of material. Naturally this was only applicable to those cases known to have an deletion present in their muscle biopsy. Here it was shown that the mutant mtDNA genomes were lost from culture within a short number of cell passages. One explanation for this might be that those myoblasts containing a higher proportion of mutant mtDNA would be selected out of culture by the faster growth rate of those myoblasts whose respiratory chain activity was less impaired. The culture population would thus tend towards cells containing the fewest, possibly no, mutant mtDNA. In vivo this mechanism could not operate in terminally differentiated muscle and neuronal cell lines but could do in continuously dividing haematological cell lines. Indeed this is the proposed mechanism for the retention of mtDNA deletions in the former and their absence in the latter. The shift from the Pearsons syndrome to the Kearnes Sayre phenotype is explicable on this basis (see Chapter 3 section 8a).

If this were the case then preservation of the deletion containing mtDNA would be expected to occur if all cells present in a culture contained the same percentage of mutant mtDNA. In these circumstances there would be no selective advantage operating against any particular cell line. Such a scenario may apply to clonal cell cultures where all the cells originate from a single cell. The results here show that
clonal cell lines do in fact differ in the proportion of mutant mtDNA present, and that some clonal lines retain a significant proportion of mutant mtDNA despite having undergone many more population doublings at harvesting, than the equivalent number of uncloned cultured cells.

That the retention of deletion containing mtDNA in culture is a random process is perhaps illustrated by the fibroblast culture of MW. Fibroblast cultures are not generally thought to express mitochondrial defects in adults. In the paediatric age group fibroblast cultures have represented a useful source material for biochemical study, but these probably result from nuclear genomic defects. One would assume that dividing skin tissue would be unlikely to retain mtDNA with a deletion. Presumably the this particular fibroblast culture derived from a group of cells in which an unusually high number had a significant proportion of mutant mtDNA.

The variable ratio of mutant to wild type mtDNA in the clonal cell lines sheds some light on the possible distribution of the two populations of mtDNA. Hitherto, this distribution has been unknown and in particular the apportionment of genomes within cells, and within mitochondria. The variable degree of heteroplasmy shown in the clonal cultures demonstrates that the mutant mtDNA is distributed amongst different cells of a given tissue, in variable proportion. Both the fibroblast culture, by the nature of the explant technique used to set it up, and the clonal muscle cultures, deriving as they do from a single myoblast would have undergone many more cell doublings prior to harvesting than the non-cloned muscle cell cultures. The retention of deletion containing mtDNA, at a given percentage of the total mtDNA, within such cell lines
might suggest that the percentage of deletion containing mtDNA is constant within all the mitochondria of that cell line. If this were not the case then random assortment of mitochondria within dividing cells would result a mixed heteroplasmic cell population and the selection against the deletion would again occur. However this hypothesis merely begs the question of how replicating mitochondria maintain their constant ratio of the two mtDNA types.

Given the loss of mtDNA with deletion in the uncloned muscle cell cultures it is therefore not surprising that the mitochondrial protein synthesis in these cultures does not differ from that of the control cultures. Under these circumstances no conclusions can be made as to the expression of the mutant mtDNA genomes.

The abnormal profile of mitochondrially synthesised proteins seen in the in vitro studies in patient JL is not reproduced in culture. The genetic basis for the defect of mitochondrial expression in vitro is unknown and it therefore not possible to determine whether it remains present in culture. One might presume it does not.

The results presented here suggest that investigation of the mtDNA deletions in culture will require the establishment of suitable cloned muscle cell lines and that Southern blot analysis of the mtDNA represents the most efficacious way to screen such cell lines. Given the slow growth rate of primary clonal muscle cell cultures, and their finite lifespan, it is tempting to suggest that cell cultures should be transformed with origin deficient SV40 prior to cloning. However such transformed cell lines may not retain full myogenic potential, nor may they be truly immortalised (Hurko personal
The more recently disclosed point mutations of mtDNA have been little studied in culture. However one of the distinguishing features certainly for the MERRF syndrome and the tRNA$^{by}$ point mutation is the evidence for maternal transmission of the defect, in contrast to the situation seen with the deletions of mtDNA. Therefore the mechanisms which mitigate against the transmission of the deletion defect both in vivo and in culture, may not apply to other mtDNA defects.

Detection of point mutations of mtDNA in patients is commonly by PCR techniques. The ability of this technique to visualise the presence of trace amounts of mutant mtDNA is an asset allowing detection in blood samples. However it is more difficult to quantitate the proportion of mutant mtDNA as one would want to do for cell cultures studies.

Abnormalities of expression of the mitochondrial genome which may be secondary to defects of nuclear genomic control may be more likely to persist in culture, and may not require special techniques to preserve them.
APPENDIX 1: MITOCHONDRIAL MYOPATHY PATIENT

DETAILS

VA

She had a two history of progressive limb weakness with muscle twitching and aching. Eighteen months into this symptom she then developed sub acute visual deterioration affecting the left eye more than the right. She had no complaint of diplopia or ptosis.

There was no positive family history.

Visual acuities were 6/12 on the right, 6/36 on the left, with a left central scotoma to a red pin. There was atypical streaky perimacular pigmentary retinopathy with mild bilateral ptosis, severe bilateral ophthalmoplegia, a sensorineural deafness and weakness of sternomastoids and trapezial. There was a proximal myopathy affecting the arms, with proximal wasting, more than the legs. Reflexes were sluggish with an absent left ankle jerk.

The positive finding on initial investigation was of a early sensory peripheral neuropathy on EMG and nerve conduction studies.

Muscle biopsy showed 15 to 20% ragged red fibres with the majority of these being cytochrome oxidase positive, although some additional cytochrome oxidase negative fibres were seen.

FB

She was documented as having retinitis pigmentosa at the age of 16 years and night blindness ensued a year later. Diplopia with ptosis and nasal speech developed at the age of 20 years. Her vision progressively deteriorated to the point where she was registered blind. At the age of 23 years ataxia of gait and limb incoordination became apparent.

There was no family history.

She was of short stature. Visual acuities were reduced to perceiving hand movements only on the right, and perception of light on the left with extensive retinitis pigmentosa with secondary retinal
degeneration. There was an almost complete external ophthalmoplegia. She had bilateral facial weakness, with bilateral deafness and nasal dysarthria. There was mild weakness of neck flexion, in the proximal shoulder muscles and in the legs, with intention tremor of the right arm. Only the right triceps reflex could be obtained, and both plantar responses were flexor.

Nerve conduction studies were normal, ECG showed sinus bradycardia, and EEG showed a slow dominant rhythm. CT scan revealed calcification in both pallidi with some cerebellar and brain stem atrophy. CSF protein had been 0.98g/l when this was measured at the age of 23 years old.

Muscle biopsy revealed ragged red fibres.

SB

Her birth and early milestones were normal but from the age of eight she was noted to be of short stature with normal endocrine investigations. From the age of twelve she has had a progressive ataxia of her arms and of her gait. She continued normal schooling and went on to secretarial college but of late felt that her memory was becoming impaired.

Her parents were first cousins and the related grandmothers were of short stature. Her brother, four years older than her, had a similar progressive neurological illness from infancy with deafness, dementia, pyramidal and cerebellar features.

She was of short stature with normal secondary sexual characteristics. She had bilateral optic disc pallor with normal visual acuities but mildly impaired colour vision. There was a bilateral sensorineural deafness. She had mild proximal weakness of the limbs with generalised ataxia.

Investigations showed normal ECG, generalised cerebral atrophy with calcification of the basal ganglia on CT scan, a slow dominant rhythm on EEG, evidence of a demyelinating polyneuropathy on EMG, marginal delay of visual evoked responses bilaterally, absent brain stem evoked responses and a bilateral high tone cochlear hearing loss. Psychometry confirmed a deterioration in her intellectual function with a verbal IQ of 75 and a performance IQ of 68.

Muscle biopsy: majority of fibres small (30-50um), no RRF or CYTOX negative fibres.
MB

He had visual failure with atypical pigmented retinopathy diagnosed at 15 years old. Bilateral ptosis had been present since childhood. He was never athletic at school and was always poor at sports. In recent years he had developed increasing limb weakness with unsteadiness of gait, a tremor of the hands, slowing and softening of his speech and mild memory impairment.

Two brothers and a two year old daughter were well. A maternal uncle had been given a diagnosis of Lawrence Moon Biedl syndrome. He was confirmed to have typical retinitis pigmentosa with polydactyly but with no evidence of ocular or generalised myopathy.

He appeared slow and vague. He had a bilateral atypical pigmentary retinopathy with optic disc pallor. Vision was reduced to finger counting bilaterally. There was bilateral ptosis with a marked external ophthalomplegia particularly for horizontal gaze. Orbicularis oculi and neck flexion were weak. There was no deafness nor any bulbar signs. He had mild weakness of the arms and proximal weakness of the legs with fatiguability. Both arms and legs were ataxic. Reflexes were symmetrical with bilateral extensor plantars. There were no sensory abnormalities. General physical examination was normal with no cardiac findings.

Investigations showed normal ECG, CSF protein of 0.82g/l, mild cerebral and cerebellar atrophy on CT scan, central delay of brain stem auditory, and somato-sensory, evoked potentials, and generalised slowing on his EEG.

Muscle biopsy: 40% RRF, a few CYTOX negative fibres, a marked type 1 fibre predominance.

MHE

He had delayed motor milestones and from the age six years complained of muscle pains in his legs. From then on he developed progressive fatigue and myalgia.

There was no positive family history.

He had pseudohypertrophy of his muscles which fatigued easily. There was mild fixed weakness of the legs.

CT brain scan was normal. An exercise lactate test showed exaggerated rise in the lactate.

Muscle biopsy showed no clear ragged red fibres but there was peripheral enhanced staining with
Gomori trichrome, succinate dehydrogenase and cytochrome oxidase stains.

**MHO**

Since school days he had generalised muscle weakness and for ten years had had bilateral ptosis together with a complaint of unsteadiness.

He had ophthalmoplegia with bilateral ptosis and generalised limb weakness affecting the arms more than the legs. There were no cerebellar signs.

On muscle biopsy a high proportion, at least 40%, of the total fibres showed a ragged red fibres appearance with markedly enhanced succinate dehydrogenase activity. A small proportion of the fibres were also cytochrome oxidase negative. There were some necrotic fibres with a deficiency of type 2B fibres.

**DL**

This resident of a home for the mentally handicapped had a diagnosis of mitochondrial myopathy made at the age of thirteen at The Hospital For Sick Children, Great Ormond Street. She has a combination of mental retardation, salt and peper type retinopathy, myopathy with proximal arm weakness, a spastic paraparesis with brisk leg reflexes and extensor plantars. She also had insulin dependant diabetes.

There was a strong family history. Her mother died at aged 68 with proximal myopathy, late onset dysarthria and spastic paraparesis but preserved intellect. Electron microscopy of her muscle had shown abnormal mitochondria. A brother died of unknown cause in the neonatal period, and a sister at the age of 7 years with "myocarditis". A surviving sib is known to have a deletion of her muscle mtDNA.

The muscle biopsy showed a mildly myopathic picture with some atrophic fibres, a predominance of type 1 fibres and a deficiency of type 2B fibres. 10 to 15% of the fibres showed a ragged red appearance and there were a few cytochrome oxidase negative fibres.
He first presented at the age of 13 years with generalised seizures occurring while watching television. EEG showed generalised spikes with marked photosensitivity. Treatment with sodium valproate was started with partial response; the generalised seizures reducing to a frequency of once every six months. At aged 14 years old, myoclonic seizures began, but shortly after this he entered a period of remission which was to last two years. Both the generalised and myoclonic seizures then recurred and clonazepam, and subsequently carbamazepine, were added to his anticonvulsant regime. By the age of 24 years intellectual decline, hearing impairment and ataxia were apparent.

He was the only son of non-consanguineous, healthy parents and there was no family history of neurological or metabolic disease.

He had an ataxic gait with Rhomberg testing being positive. He had normal eye movements with no evidence of retinopathy. There was a sensorineural deafness of cochlear origin. He had no muscle weakness or fatigue but did have limb ataxia. Reflexes were intact with flexor plantar responses. There were no sensory findings.

Resting lactate was 1.6 nM/l (normal 1.8 nM/l) and pyruvate was 60 uM/l (normal 45-80 uM/l) but the lactate to pyruvate ratio was raised at 26 (control X). The ECG was normal. EEG showed generalised spike and wave discharges some evoked by photic stimulation. The background activity was mildly abnormal with a dominant rhythm of 6 to 8Hz intermixed with slower components and faster activity particularly in the posterior regions. Myoclonic activity was also seen and confirmed to be cortical in origin with giant cortical sensory evoked potentials and cortical correlate on back averaging. An electroretinogram was normal. Cranial CT scan showed mild cortical atrophy. Psychometric assessment showed a significant decline from his estimated optimal level of functioning with WAIS scores being 82 and 74 for verbal and non-verbal material respectively.

In her mid teens she developed a progressive left ptosis and diplopia. By the age of twenty years she had fatigueable weakness of the legs and this progressed such that two years later her walking tolerance was reduced to half a kilometre and she was having frequent falls.
A paternal cousin was said to have similar symptoms but no further details were available.

She had a salt and pepper pigmentary retinopathy with visual acuities of 6/6 on the right, and 6/12 on the left. There was a fixed left ptosis and a severe external ophthalmoplegia. There was fatigueable weakness of the arms and legs with retained reflexes, and no cerebellar or sensory findings.

ECG and nerve conduction studies were normal while needle EMG showed myopathic changes in right biceps and right vastas medialis.

Muscle biopsy showed 10 to 15% ragged red fibres with a slightly higher proportion of cytochrome oxidase negative fibres. There was a mild deficiency of type 2B fibres and an excess of neutral lipid even in non-ragged red fibres.

**DP**

She was athletic at school but recalled a single episode of collapse following a long distance run. For the last eight years her exercise tolerance had been severely limited by muscle fatigue and pain and in the preceding year she had been having regular episodes of more severe myalgia rendering her bed bound for days at a time. In addition she had had three episodes of reversible encephalopathy of three days or so duration, characterised by pyrexia, coma, meningism, disconjugate gaze, decorticate posturing and spastic tetraparesis with a lactic acidosis and raised CPK.

Her only physical signs were of mild generalised limb fatiguability but with no fixed weakness.

There were mild myopathic changes on EMG. Resting lactate and pyruvate were normal but there was an exaggerated response to sub-anaerobic exercise.

There was no positive family history.

Muscle histology revealed a few fibres with peripheral enhancement of staining for Gomori trichrome and succinate dehydrogenase with some excess of neutral lipid. 90% of the fibres did not stain with cytochrome oxidase.
Her first symptoms were at the age of six years with visual difficulties which have slowly progressed ever since. At eight years old she had bilateral ptosis, unsteadiness with falls, and tremor of the arms. She started having difficulties coping at school, initially because of her physical disabilities but then because of intellectual deterioration so that by the age of eleven she was attending a special school. Her unsteadiness worsened to render her wheelchair-bound from the age of twelve years. There had been two episodes of impaired consciousness accompanied by lactic acidosis. Recently she had been noted to have slurred speech and she had also complained of fluctuating hearing loss.

Her height and weight were below the 3rd centile for her age. She was hirsute and had slow mentation. She had head titubation, a scanning dysarthria, bilateral ptosis, a complete external ophthalmoplegia, bilateral visual acuities of 6/12 with salt and pepper pigmentary retinopathy, and a myopathic facies with mild facial weakness. She had an ataxic, waddling, gait with a proximal myopathy and limb ataxia. Reflexes were absent in the arms, diminished in the legs with equivocal plantar responses.

Routine biochemistry revealed a raised fasting lactate of 3.4mM/l. CT brain scan showed cerebellar atrophy. There was a right axis deviation with a partial right bundle branch block on the ECG. An excess of slow wave activity was apparent on the EEG. Nerve conduction studies showed a peripheral neuropathy, while audiometry confirmed a sensorineural hearing loss.

Muscle biopsy showed type 2 fibre atrophy with randomly scattered ragged red fibres showing enhanced succinate dehydrogenase staining and ocassional cytochrome oxidase negative fibres.

He had a long standing bilateral ptosis, a 20 year history of ophthalmoplegia, a 15 year history of visual failure and a more recent history of hearing impairment.

There were no similarly affected relatives.

Examination confirmed bilateral ptosis, external ophthalmoplegia, pigmentary retinopathy with visual acuities of 6/12 on the right and 6/18 on the left and a sensorineural deafness. There was no muscle weakness apparent but reflexes were diminished inthe arms and absent in the legs.
Myopathic feature were apparent on EMG.

Muscle biopsy showed 5% of the total fibres to have ragged red changes. Many of the ragged red fibres, and some additional fibres were negative for cytochrome oxidase staining.

MW

At the age of 12 he developed complete heart block requiring a pace maker, and it was then his ptosis and ophthalmoplegia were noted. These have both progressed with the subsequent development of muscle wasting and weakness with unsteadiness.

The family had no complaints but the mother was thought to have mild weakness on examination.

He had pigmentary retinopathy with visual acuities of 6/9 on the right and 6/12 on the left, bilateral ptosis, almost complete ophthalmoplegia, wasting and weakness of the facial muscles, sternomastoids and trapezii. There was wasting of the periscapular muscles with winging of the scapulae and generalised arm weakness. Biceps and ankle reflexes were both absent. He had truncal and limb ataxia.

Fasting lactate and pyruvate were raised. CT brain scan revealed cerebral and cerebellar atrophy.

5 to 10% of the muscle fibres showed enhanced succinate dehydrogenase activity with a similar proportion being cytochrome oxidase negative.

PW

At the age of 17 years he required a cardiac pacemaker for episodes of syncope due to complete heart block. Six months later he developed progressive limb tremor and unsteadiness.

He had salt and pepper type retinopathy with gross ophthalmoplegia and bilateral ptosis. There was a cerebellar dysarthria with cerebellar ataxia and dysdiadokinesis.

The otherwise normal muscle biopsy showed a small number of ragged red fibres which were also cytochrome oxidase negative.
## APPENDIX 2: MATERIALS AND SOURCE

### General Chemicals

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Quantity</th>
<th>Supplier</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>1g</td>
<td>Boer. Mann.</td>
<td>236 675</td>
</tr>
<tr>
<td>Amino acids minus methionine</td>
<td>1ml</td>
<td>Amersham</td>
<td>N133</td>
</tr>
<tr>
<td>Aminobenzamidine</td>
<td>5g</td>
<td>Sigma</td>
<td>A 6391</td>
</tr>
<tr>
<td>Aquasol</td>
<td>4l</td>
<td>DuPoint</td>
<td>NEF 934</td>
</tr>
<tr>
<td>ATP</td>
<td>1g</td>
<td>Sigma</td>
<td>A6144</td>
</tr>
<tr>
<td>Atractyloside</td>
<td>50mg</td>
<td>Sigma</td>
<td>A6882</td>
</tr>
<tr>
<td>Bicine</td>
<td>100g</td>
<td>BDH</td>
<td>44109</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>25g</td>
<td>Sigma</td>
<td>C0378</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>5g</td>
<td>Sigma</td>
<td>C6255</td>
</tr>
<tr>
<td>Dimethyl sulfoxide</td>
<td>1l</td>
<td>BDH Scintran</td>
<td>14622</td>
</tr>
<tr>
<td>Diphenyloxazole (PPO)</td>
<td>1kg</td>
<td>BDH Scintran</td>
<td>14615 5W</td>
</tr>
<tr>
<td>EDTA</td>
<td>100g</td>
<td>Sigma</td>
<td>EDS</td>
</tr>
<tr>
<td>Emetine</td>
<td>1g</td>
<td>Sigma</td>
<td>E2375</td>
</tr>
<tr>
<td>Glutamate</td>
<td>100g</td>
<td>Sigma</td>
<td>G1126</td>
</tr>
<tr>
<td>L-35S Methionine</td>
<td>1mCi</td>
<td>Amersham</td>
<td>SJ1515</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>25g</td>
<td>Sigma</td>
<td>M9625</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>500g</td>
<td>Sigma</td>
<td>M7506</td>
</tr>
<tr>
<td>Phosphoenolpyruvate</td>
<td>100mg</td>
<td>Boer.</td>
<td>108278</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>10mg</td>
<td>Boer.</td>
<td>109045</td>
</tr>
<tr>
<td>Succinate</td>
<td>500g</td>
<td>BDH AnalR</td>
<td>10273 4W</td>
</tr>
<tr>
<td>Trichloracetic acid</td>
<td>100g</td>
<td>Sigma</td>
<td>T4885</td>
</tr>
<tr>
<td>Urea</td>
<td>500g</td>
<td>BDH Aristar</td>
<td>45204</td>
</tr>
</tbody>
</table>

### Chemicals For Cultures

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Quantity</th>
<th>Supplier</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine Serum Albumin</td>
<td>1g</td>
<td>Sigma</td>
<td>A6003</td>
</tr>
<tr>
<td>Collagenase Type II</td>
<td>500mg</td>
<td>Sigma</td>
<td>C6885</td>
</tr>
<tr>
<td>Glutamine</td>
<td>100g</td>
<td>Sigma</td>
<td>G3126</td>
</tr>
<tr>
<td>Phosphate Buffered Saline</td>
<td>500ml</td>
<td>Flow</td>
<td>18-604-54</td>
</tr>
<tr>
<td>Trypsin 1:250</td>
<td>100ml</td>
<td>Gibco</td>
<td>043-05090</td>
</tr>
<tr>
<td>Versine 1:5000</td>
<td>100ml</td>
<td>Gibco</td>
<td>043-05040H</td>
</tr>
<tr>
<td>Vitrogen</td>
<td>100ml</td>
<td>Celtrix</td>
<td>0701</td>
</tr>
</tbody>
</table>
Tissue Culture Media

Chick Embryo Extract  20ml Flow  28-501-46
Dulbecco's Modified Eagle's  500ml Gibco  041 01965M
Foetal Calf Serum  100ml Gibco  011 06290H
Horse Serum  100ml Gibco  034 0605H

Chemicals For Electrophoresis

Acrylamide  250g BDH Electran  44299
bis Acrylamide  25g BDH Electran  44300
Ammonium persulphate  100g Sigma  A6761
TEMED  25ml Sigma  T7024

Filters

Acrodisc  Gelman Sci  6144192
Diaflo Ultrafilt. Membr. PM10  Amicon
Nylon Mesh  Kadish & Son
REFERENCES


BATES HM, CRADDOCK VM, SIMPSON MV (1958) The incorporation of valine-1-C14 into cytochrome c by rat liver mitochondria. *Journal American Chemical Society, 80*, 1000.


DOWNER NW, ROBINSON NC, CAPALDI RA (1976) Characterization of a seventh different subunit of beef heart cytochrome c oxidase similarities between the beef heart enzyme and that from other species. *Biochemistry*, 15, 2930-2935.


ENGEL WK, CUNNINGHAM GG (1963) Rapid examination of muscle tissue an improved trichrome staining method for fresh frozen biopsy sections. *Neurology (Minneap)*., 13, 919-923.


HARVEY EB (1946) Structure and development of the c;ear quarter of the arbacia punctulata egg. Experimental Zoology, 102, 253-271.


HOLT IJ, HARDING AE, MORGAN-HUGHES JA (1989b) Deletions of muscle mitochondrial DNA in mitochondrial myopathies: sequence analysis and possible mechanisms. *Nucleic Acids Research*, 17, No.12,


JOHNS DR, CORNBLATH DR (1991) Molecular insight into the asymmetric distribution of pathogenic human mitochondrial DNA deletions. *Biochemical Biophysical Research Communications*, 174, 244-250.


KOBAYASHI T, ASKANAS V, ENGEL WK (1987) Human muscle cultured in monolayer and
cocultured with fetal rat spinal cord: importance of dorsal root ganglia for achieving successful functional
innervation. Neuroscience, 7, 3131-3141.

(1990) A point mutation in the mitochondrial tRNA(Leu)(UUR) gene in MELAS (mitochondrial
myopathy, encephalopathy, lactic acidosis and stroke-like episodes). Biochemical Biophysical Research
Communications, 173, 816-822.


KROON AM (1963) Protein synthesis in heart mitochondria. Biochmica et Biophysica, 72, 391-402.

KRUSE B, NARASIMHAN N, ATTARDI G (1989) Termination of transcription in human mitochondria:
identification and purification of a DNA binding protein factor that promotes termination. Cell, 58,
391-397.

KUHN NENTWIG L, KADENBACH B (1985) Isolation and properties of cytochrome c oxidase from
rat and quantification of immunological differences between from various rat tissues with subunit-specific
antisera. European Journal of Biochemistry, 149, 147-158.


NASS S, NASS MMK (1963) Intramitochondrial fibres with DNA characteristics II. Enzymatic and other hydrolytic treatments. Cell Biology, 19, 613-629.


OLSEN W, ENGEL WK, WALSH FO, ET AL (1972) Oculocraniosomatic neuromuscular disease with ragged red fibres. Archives of Neurology, 26, 193-211.


VASILEVA EA, MINKOV IB, FITIN AF, VINOGRA DOV (1982) Kinetic mechanisms of

VILKKI J, SAVON TAUS ML, NIKOS KELAINEN EK (1989) Genetic heterogeneity in Leber hereditary
optic neuroretinopathy revealed by mitochondrial DNA polymorphism see comments. *American Journal
Human Genetics*, 45, 206-211.

VILKKI JJ, OTT ML, SAVONTAUS PA, NIKOS KELAINEN EK (1991) Optic atrophy in Lebers
hereditary optic neuropathy is probably determined by an X chromosomal gene closely linked to DXS7.

Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. *Science*, 242,
1427-1430.

Familial mitochondrial encephalomyopathy (MERRF): Genetic, Pathophysiological, and Biochemical

mitochondrial DNA. *Cytogenet Cell Genet*, 58, 1103-1123.

in Enzymology*, 10, 245.

WHEELDON LW, LEHNINGER AL (1966) Energy-linked synthesis and decay of membrane proteins


