The University of Manitoba

Genetically Determined Mitochondrial Diseases Studied in Fibroblasts

by

Gisele Scott-Woo

A Thesis

Submitted to the Faculty of Graduate Studies in partial fulfillment of the requirements for the degree of

Master of Science

Department of Biochemistry, Faculty of Medicine

Winnipeg, Manitoba

May, 1984

GENETICALLY DETERMINED MITOCHONDRIAL DISEASES STUDIED IN FIBROBLASTS

by

Gisele Scott-Woo

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

Acknowledgements

I would like to gratefully acknowledge the help and support I have received throughout my graduate training.

I would like to thank Dr. Wrogemann, for accepting me into his lab and providing guidance and support in both my academic training and in my thesis work.

Also, I would like to thank Terri Trottershaw and Barb Nickel, who taught me the rudiments of laboratory research and whose advice and friendship have been invaluable. There are many others who have contributed to my education, notably Dr. Hamerton and the staff of the Department of Genetics, as well as the staff and students of the Department of Biochemistry.

As well, I am grateful for the moral support and encouragement which sustained me throughout my course and thesis work that I received from Glenn, my family and friends.

Finally, I gratefully acknowledge the financial support I have received from the Manitoba Health Research Council and grants-in-aid, from the Medical Research Council of Canada.

Abstract

Genetically Determined Mitochondrial Diseases Studied in Fibroblasts

Mitochondrial diseases are a heterogeneous group of diseases in which the primary biochemical defect resides in the mitochondria causing the mitochondria to be abnormal in either structure or function. Most of the mitochondrial diseases that have been described are myopathies (Luft's Disease (24,36,50), Carnitine Palmitoyltransferase Deficiency (11)) or encephalopathies (Leigh's Disease (15,69,71), Zellweger's Disease (79)), but other manifestations of mitochondrial disturbances, as reported in studies of Cystic Fibrosis (31,32,67,68), are also possible.

Most investigators who study mitochondrial diseases use skeletal muscle tissue obtained post-mortem or from biopsy. This is a very limited tissue source for biochemical studies and use of a renewable tissue source, such as cultured skin fibroblasts would be advantageous. The studies presented here have been restricted to genetically determined mitochondrial diseases that may express the defect in fibroblasts so that this tissue source could be used.

The first step in the study of mitochondrial diseases is to screen many mitochondrial functions simultaneously by following oxidative phosphorylation activity in fibroblast mitochondria. If a defect can be localized to a small

group of putative enzymes, more specific enzyme function tests can be done to identify which enzyme is defective.

A method was developed for isolating fibroblast mitochondria which is superior to currently available methods (43,56). From as few as 12 million cells, mitochondria could be isolated with good respiratory control (RCR, 4.86 $\pm$ 1.1, n = 6, $\bar{x} \pm$ S.D.) and respiration rates (14.1 $\pm$ 6.0 n = 6, μmoles O_2/min/g mitochondrial protein).

This method was then applied to the study of two mitochondrial diseases, Luft's Disease and Carnitine Palmitoyltransferase (CPT) Deficiency. In the study of Luft's Disease, the defect of loosely coupled mitochondria seen in muscle (24,28) was not apparent in fibroblasts. Mitochondria from these fibroblasts were coupled (RCR's of 1.5-3.58) and their respiration could be inhibited by oligomycin, an inhibitor of ADP phosphorylation. CPT deficiency was studied because it is a well characterized mitochondrial disease that is sometimes expressed in fibroblasts (19,21,23,47,62). However, the rate of metabolism of both fatty acid and carbohydrate substrates in mitochondria isolated from fibroblasts from a CPT deficient patient, GM1763, was 70% of the control value. This indicated that this fibroblast strain did not metabolize fatty acids any slower than it could metabolize other substrates and therefore did not express the CPT deficiency.

Finally, the possibility that Cystic Fibrosis (CF) is a mitochondrial disease was explored. Shapiro's group has studied Cystic Fibrosis in fibroblasts and has published many reports (31,32,67,68) that suggest that mitochondria may be the site of a defective protein responsible for Cystic Fibrosis. The most recent investigations (67,68) led to the hypothesis that alterations in NADH dehydrogenase of the electron transport chain may cause increased Ca^{++} sequestration in CF mitochondria and that this Ca^{++} sink is responsible for CF symptoms. CF and control fibroblast pairs were analyzed in a blind study to see if the characteristic Km(NADH) of NADH dehydrogenase, as reported by Shapiro et al (68), could distinguish CF cells from controls. Contrary to the data reported by Shapiro et al (68), it was found that the Km's for this enzyme in Cystic Fibrosis and control cells were not significantly different and therefore it appears that this enzyme is not the defect reponsible for Cystic Fibrosis.

The general approach described here has potential use in the study of mitochondrial diseases. Many more mitochondrial diseases, known or suspected to be expressed in fibroblasts, could be tested by the methods described in this thesis. If expressed in these cultured cells, these diseases could be studied in detail at the molecular level with this renewable tissue source.

List of Figures

List of Tables

Glossary

ADP	adenosine-5'-diphosphate
ATP	adenosine-5'-triphosphate
ADP/O ratio	ratio of molecules of ADP consumed per atom of oxygen utilized
ATPase	an enzyme which hydrolyzes ATP. In mitochondria this enzyme preferentially catalyzes the reverse reaction, synthesizing ATP from ADP and P_i.
BSA	bovine serum albumin
CF	Cystic Fibrosis
CM	cuvette medium
Co A	coenzyme A
Co QH_2	reduced coenzyme Q
CPT	Carnitine Palmitoyltransferase(E.C. 2.3.1.21)
CPT I	located on the outer side of the inner mitochondrial membrane, and catalyzes the conversion of acyl CoA to acylcarnitine.
CPT II	located on the matrix side of the inner mitochondrial membrane, and catalyzes the conversion of acylcarnitine to acyl CoA
DIW	deionized distilled water
DNP	2,4-dinitrophenol, an uncoupler of oxidative phosphorylation
Dounce homogenizer	a hand-held homogenizing glass tube and pestle used to grind fibroblasts
ϵ	epsilon, the molar extinction coefficient
EDTA	ethylenediaminetetra-acetic acid
ETC	electron transport chain
FAD-linked	a substrate which produces $FADH_2$ which can be oxidized by the ETC beginning with Complex II

FMN	flavin mononucleotide
GM	designates those cell strains received from the Genetic Mutant Cell Repository (Camden,NJ) eg. GM1763
G/M	substrates, glutamate (5mM) and malate (1mM) added together
HeLa	human epithelial cell line capable of indefinite replication in tisssue culture and can be grown in suspension.
HSC	Health Sciences Centre (Winnipeg)
Human McCoy's FC10	cell culture medium with 10% fetal calf serum
Km	the Michaelis constant.
MEM FC10	Minimal essential media with 10% fetal calf serum, see Appendix for composition
NAD^+	nicotinamide adenine dinucleotide
NAD^+-linked	a substrate which generates NADH, which can then be oxidized by the ETC beginning at Complex I
Nagarse	a mixture of bacterial proteinases
n	number of experiments
O.D.	optical density
O_2 rate	respiration rate, consumption of oxygen expressed as μmoles O_2/min./g mitochondrial protein
P_i	inorganic phosphate
PC	palmitoylcarnitine
P CoA	palmitoyl Coenzyme A
P/M	substrates, pyruvate (5mM) and malate (1mM) added together
passage	the number times a cell culture has been subcultured

RCR	respiratory control ratio = State 3 rate/State 4 rate
State 3 rate	respiration rate in the presence of ADP
State 4 rate	respiration rate in the absence of ADP
S.D.	standard deviation
TMPD-	N,N,N',N'-tetramethyl-p-phenylene-diamine
Tris-	Tris(hydroxymethyl)aminomethane
$\bar{x}$-	mean

Table of Contents

Genetically Determined Mitochondrial Diseases Studied in Fibroblasts

1. Introduction

1.1 Statement of the Problem

Mitochondrial diseases are a heterogeneous group of diseases consisting primarily of myopathies and encephalopathies. They are defined by Busch et al (57a) as neuromuscular diseases with an increased number of mitochondria having abnormal function or structure. This definition covers most mitochondrial diseases but overlooks those diseases without neuromuscular involvement. For the purpose of this study I have considered any genetically determined disease in which the alterations in DNA (be it nuclear or mitochondrial) are expressed in the mitochondria. While the primary event occurs in DNA, the primary biochemical defect will reside in the mitochondria.

Initially mitochondrial diseases were identified by the appearance of structurally abnormal mitochondria in patients with myopathies (27,50,57a), but now it is apparent that morphological observations alone are not adequate to prove the presence of mitochondrial dysfunction. Ultrastructural changes are also seen in mitochondria from normal (33), myogenic (40,55), or neurogenic (25) tissue, and tissue obtained under experimental conditions (65,83). Therefore, biochemical

studies are also required to prove that mitochondrial dysfunction is part of the pathological process. Biochemical studies may also identify diseases in which structurally normal mitochondria are functionally abnormal and these diseases too, may be classified as mitochondrial diseases.

It has been difficult to establish a relationship between a biochemical defect and the clinical and morphological findings for most of these diseases. Different biochemical deficiencies can produce an identical clinical phenotype and conversely, a given enzyme deficiency may produce different phenotypes (53). Therefore, if the underlying biochemical defect was determined it would lead to a better understanding of the pathological process. Many cases of mitochondrial diseases have been reported, but the specific biochemical defect has been localized in only a few (Menkes, CPT deficiency, see Table 1 for references). One of the aims of this project has been to develop a method by which the biochemical defect of a mitochondrial disease could be located.

Most investigators who study mitochondrial diseases use skeletal muscle tissue obtained post-mortem or from a biopsy. The size of sample is limited and a renewable tissue source for the biochemical study of these diseases would be an advantage. The frequent occurrence of multi-tissue involvement suggests that mitochondria of non-muscle cells may also be affected. Therefore tissues

other than skeletal muscle, such as leukocytes and fibroblasts may also express the defect causing the disease (57a). Fibroblasts are an attractive source for obtaining mitochondria as skin biopsy is a relatively easy procedure, the cells can be grown in large quantities by established tissue culture techniques (2), and cells grown in culture are not subject to the secondary effects of the disease (17). Also, fibroblasts maintain both the chromosomal complement and metabolic uniqueness of their donor including the primary genetic defect (56). The use of such cultures to investigate the molecular basis of inherited diseases is common and has been discussed by Krooth and Sell (44). The studies presented here have been restricted to genetically determined mitochondrial diseases that may express the defect in fibroblasts. If a disease is truly of mitochondrial origin and is expressed in fibroblasts we would be able to search for the biochemical defect using the general methods described herein.

The first step in studying a mitochondrial disease is to screen many mitochondrial functions simultaneously by following oxidative phosphorylation activity. If a defect can be localized to a small group of putative enzymes, more specific enzyme function tests can be done to identify which enzyme is defective.

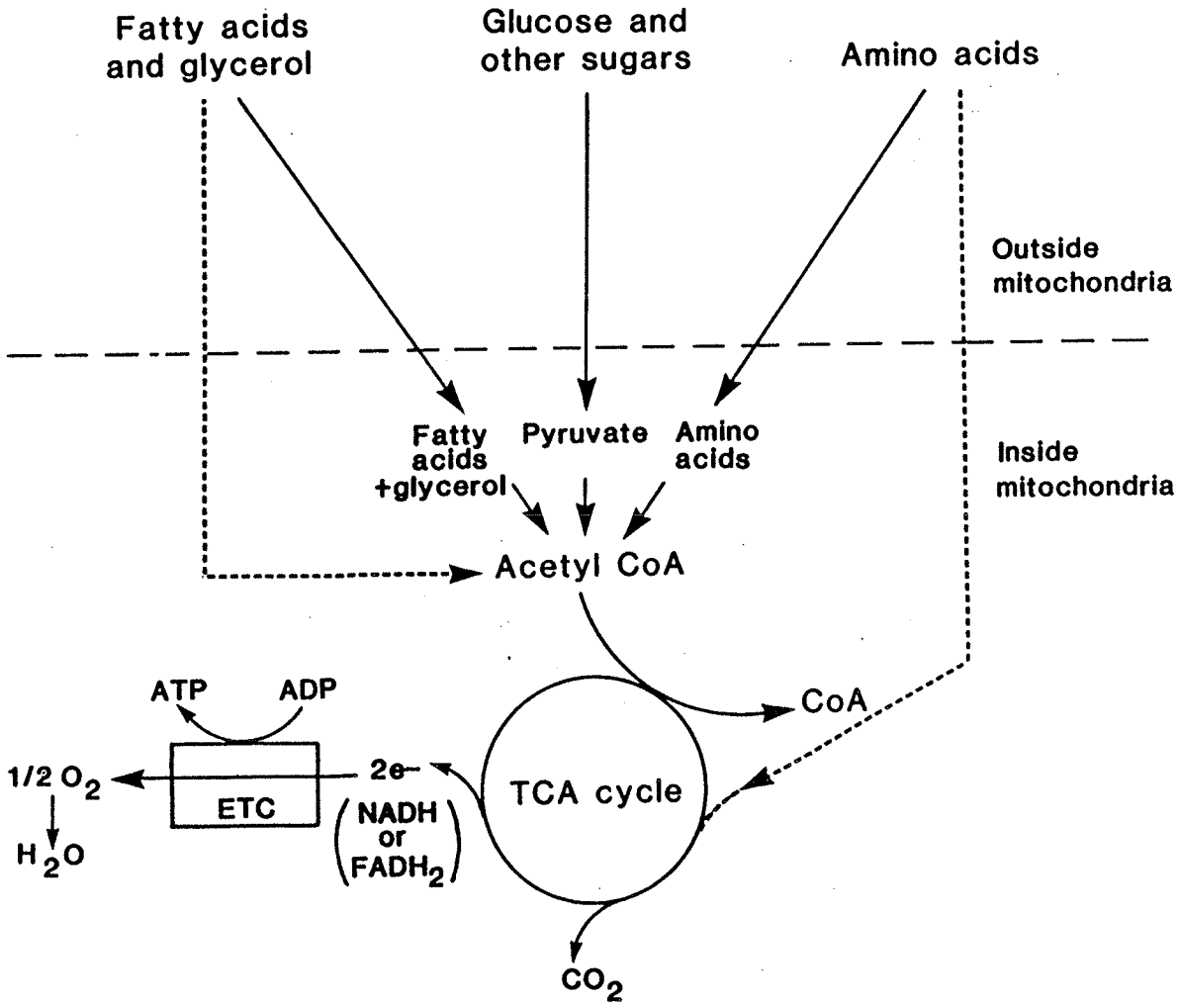
1.2 Literature Review

1.2.1 Studies of Mitochondrial Function

1.2.1.1 Basic Mitochondrial Function

The mitochondrion is a major power source in aerobic cells (74a), whose main function is to produce ATP but will also supply other metabolites and precursors. The major fuels of the body, fats, carbohydrates and proteins are broken down into their constituent molecules outside the mitochondria. These fatty acids, sugars and amino acids are transported into the mitochondria where most are further metabolized through a common intermediate acetyl CoA (81a). As shown in Figure 1, metabolism of acetyl CoA generates electrons in the form of NADH and $FADH_2$ (74a). According to Mitchell's chemiosmotic theory (57), these electrons are transferred between the electron carriers of the electron transport chain (ETC) located in the inner mitochondrial membrane and H^+ ions are released outside the mitochondrial matrix. This process establishes a proton gradient (74a), and is illustrated in Figure 2. Thus the energy from the electrons of NADH has been stored as potential energy in the proton gradient. Respiration will continue if this gradient can be used by some energy-dependent process such as ATP synthesis or ion

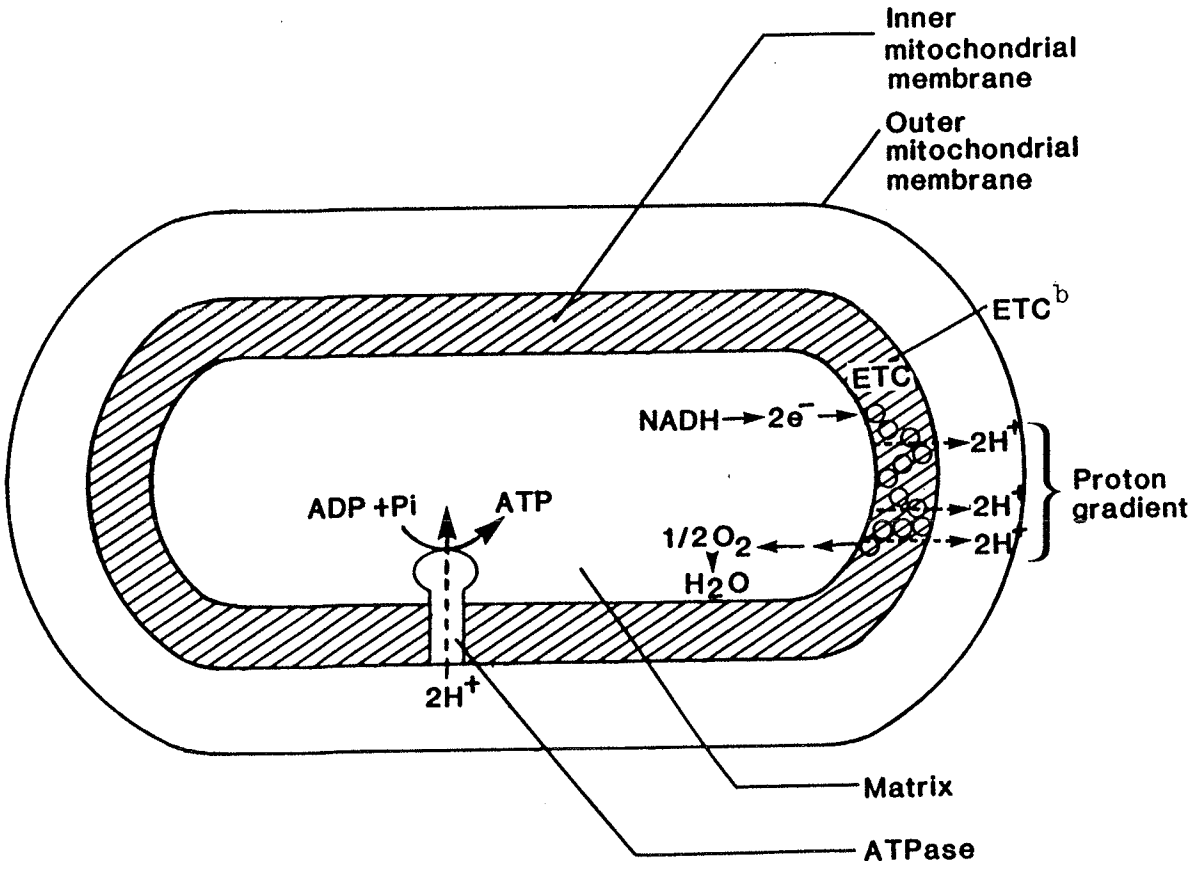
Figure 1
Overview of Mitochondrial Metabolism [a]



a) modified after that of Tzagoloff (81a)

Figure 2

Oxidative Phosphorylation in the Mitochondrion[a]



a) modified after that of Hinkle and McCarty (37)

b) electron transport chain

transport (81b). In oxidative phosphorylation, H^+ ions pass through the ATPase, and tranduce the potential energy of the gradient to form ATP from ADP and P_i (74b). This coupling of respiration to ADP phosphorylation is a regulatory mechanism by which the rate of oxidation of substrates is adjusted to the requirements of energy and is referred to as respiratory control (74c). If the gradient is used exclusively for ATP production the mitochondria are said to be 'tightly coupled' (81b).

Other mechanisms can dissipate the gradient and uncouple oxidation and ADP phosphorylation, thus decreasing the respiratory control. The proton gradient can be used to support ion transport in mitochondria (48) or may be released by uncouplers or ionophores. Uncouplers are usually lipophilic compounds that have the ability to allow respiration to continue in the absence of phosphate acceptor (ADP) (81b), probably by allowing H^+ ions to re-enter the mitochondrial matrix without the use of ATP synthetase (81b). Ionophores promote the transfer of ions such as H^+ ions, through the membrane and so prevent maintenance of the proton gradient (81c).

1.2.1.2 Study of Isolated Mitochondria

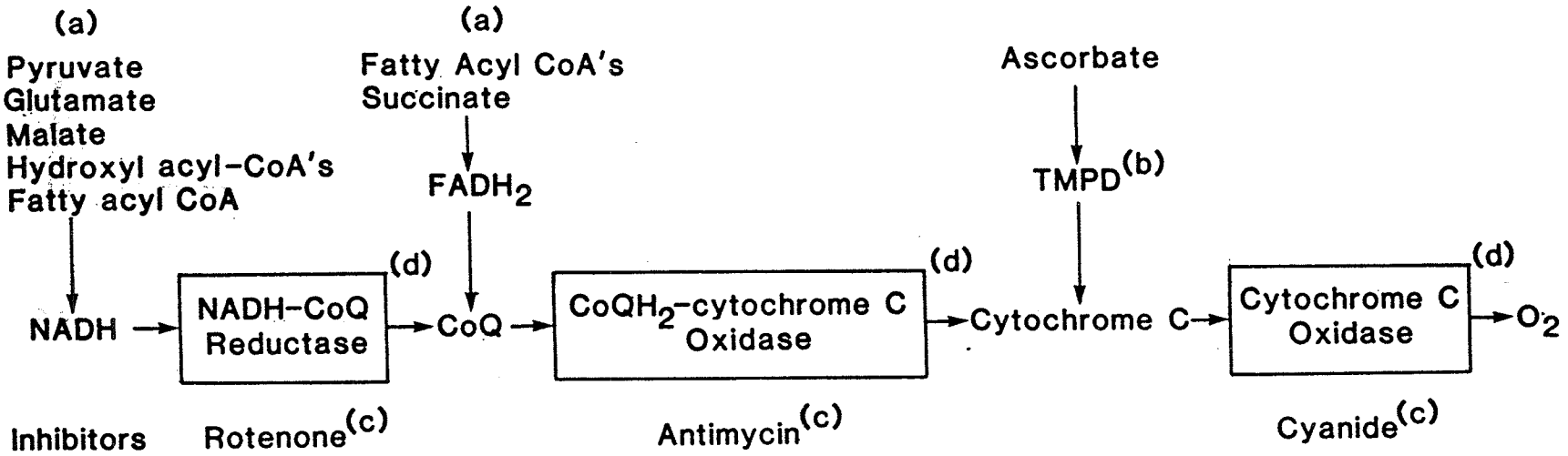
The study of oxidative phosphorylation and respiration in isolated mitochondria using a polarograph and Clark type oxygen electrode was first reported in 1955, by Chance and Williams (13). The apparatus and principles of the

polarograph and oxygen electrode have been reviewed by Estabrook (29) and Lesser and Brierly (48) and are described in the Materials and Methods section. This apparatus measures the concentration of oxygen in a temperature controlled reaction vessel. Mitochondria from a tissue source are incubated in physiological medium in this vessel and are assayed for oxidative phosphorylation activity through the use of various substrates and inhibitors.

Substrates are linked to the ETC either through NADH or $FADH_2$ as illustrated in Figure 3. Glutamate, pyruvate and fatty acids are known as NAD^+-linked substrates because NADH is generated when these substrates are oxidized. However, succinate is linked to the ETC through $FADH_2$. When a substrate is oxidized, the NADH and $FADH_2$ produced are further oxidized by the ETC and, as the final step in the overall reaction, oxygen is reduced. The depletion of oxygen from the medium is monitored by the apparatus and is a reflection of the activity of the enzymes between substrate oxidation and oxygen reduction. Thus the monitoring of oxygen consumption by mitochondria in the presence of a given substrate tests simultaneously the function of many enzymes of a chosen metabolic pathway.

The number of enzymes being monitored at one time can be reduced through the use of inhibitors (74d). Those most commonly used in mitochondrial studies are noted in Figure 3. Rotenone inhibits the first enzyme complex of the ETC,

Figure 3
Substrates and Inhibitors of the Electron Transport Chain[e]



a) substrates that generate reducing equivalents for the ETC

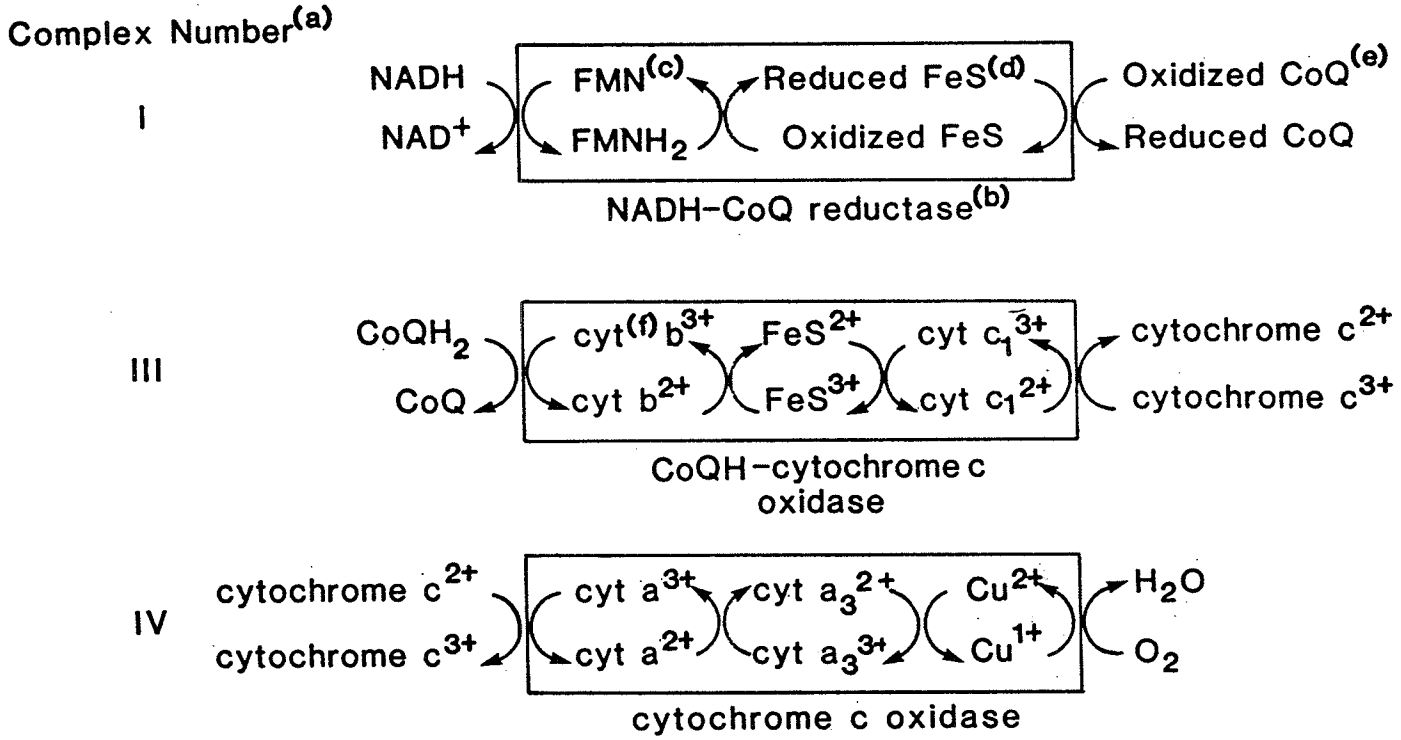
b) TMPD (N,N,N',N',-tetramethyl-p-phenylenediamine) an artificial electron donor

c) inhibitors of the enzyme complexes of the ETC

d) "energy"conserving sites of the ETC (74d)
These are multi-subunit complexes composed of protein and electron carrying prosthetic groups. These complexes are enlarged in Fig 3a

e) this figure is modified from Tzagoloff (81d) and Stryer (74d)

Figure 3a
Electron Transfer in the Major Complexes of the Respiratory Chain[g]



a) Reference (11)
b) included in this complex is NADH dehydrogenase (81i)
c) FMN, Flavin Mononucleotide
d) FeS, iron-sulfur protein
e) CoQ, Coenzyme Q or Ubiquinone
f) cyt, cytochrome (superscript refers to the redox state)
g) based on the reference (74d)

NADH-CoQ reductase. If mitochondria are respiring normally with an NAD^+-linked substrate and rotenone is added, all respiration ceases as NADH can no longer be oxidized (74d). However, the inhibited enzyme can be bypassed by the addition of an FAD-linked substrate like succinate and respiration will continue. Thus inhibitor/substrate combinations can be used to dissect the ETC biochemically. As an example, consider that the ETC is defective only at the NADH-CoQ reductase. The defect would be evident if respiration was altered with an NAD^+-linked substrate and yet normal with succinate as substrate in the presence of rotenone. Antimycin is used similarly to block $CoQH_2$-cytochrome c reductase. This inhibition also can be bypassed, this time through the use of ascorbate which can reduce cytochrome c (74d,81c). TMPD (N,N,N',N'-tetra-methyl-p-phenylenediamine), mediates the reduction (81e,81f) or can be used alone (73). The reaction originates at complex IV (cytochrome c oxidase) and proceeds through to oxygen reduction (81f). Therefore if a sample of mitochondria respires normally with an NAD^+-linked substrate, and with succinate in the presence of rotenone, but the respiration is greatly reduced compared to controls when TMPD is the substrate in the presence of antimycin, then it is possible that cytochrome c oxidase may be defective.

In isolated, tightly coupled, mitochondria, respiration in the presence of excess substrate and P_i is

regulated by the availability of ADP. When ADP is present, the respiration, or O_2 rate, is defined as the State 3 rate (14,81b) and when it has been depleted the O_2 rate slows down to the State 4 rate. Thus the State 4 rate is an indication of how readily the proton gradient can be dissipated without ATP production (81b). In tightly coupled mitochondria, the expected State 4 rate would approach zero (81b). The ratio of the State 3 rate to the State 4 rate is the respiratory control ratio (RCR) (14) and is a measure of the coupled state of the mitochondria which reflects the quality of the mitochondrial preparation. Damaged mitochondria would have a low RCR as a closed compartment is essential (74d) to maintain the proton gradient in oxidative phosphorylation. The preparations may also contain contaminating uncouplers or ionophores, which could increase the State 4 rate by releasing the gradient which stimulates phosphorylation. Thus a large RCR is indicative of intact, uncontaminated mitochondrial preparation.

Oligomycin is an inhibitor of the ATP synthetase and is useful in determining the state of coupling in a mitochondrial preparation (81b). The respiration rate in the presence of oligomycin is similar to the State 4 rate since ADP phosphorylation is blocked. In poorly coupled mitochondria this inhibitor has no effect on slowing the State 3 rate, since respiration is not being regulated by the ability of the mitochondria to phosphorylate ADP. In

coupled mitochondria, inhibited by oligomycin, respiration is restimulated and mimics the State 3 rate by the addition of 2,4-dinitrophenol (DNP) (81b). Thus, the ratio of the O_2 rate in the presence of DNP over the O_2 rate in the presence of oligomycin is similar to the RCR and is another way to determine the degree of coupling in the mitochondrial sample.

Another parameter frequently measured in oxidative phosphorylation assays is the ADP/O ratio. This is an expression of the efficiency of phosphorylation in the mitochondrial sample (81b). Although the molecular details are unknown, many authors agree that the proton gradient generated at each enzyme complex site (I, III, orIV) by the passage of a pair of electrons from NADH is used to synthesize one molecule of ATP (74a,74d,81b). Thus for an NAD^+-linked substrate, three ADP molecules will be phosphorylated per oxygen atom consumed from the medium, and theoretically the ADP/O ratio will approache 3. For an FAD-linked substrate it approaches 2 and if TMPD is used in combination with antimycin, only one molecule of ADP is phosphorylated per oxygen atom (See Figure 3). The stoichiometry of one ADP phosphorylated per site has been challenged by many. Brand (6) suggests that NAD^+-linked substrates yield a mean ADP/O ratio of 2.67, succinate yields a ratio of 2.0 and ascorbate yields a ratio of 1.33. Hinkle (37) finds much lower values for ADP/O ratios of 2.0 for NAD^+-linked substrates, 1.3 for succinate, and 0.7 for

ascorbate. The variability in the ADP/O ratios depends on the method of measurement and so there is some flexibility in the "normal" range for this parameter. For the work presented here, normal values for ADP/O ratios were considered to be between 2.0-3.0 for NAD^+-linked substrates, 1.5-2.0 for succinate and 0.5-1.0 for ascorbate-TMPD.

1.2.1.3 Study of Fibroblast Mitochondria

It is possible to screen many mitochondrial functions simultaneously by following oxidative phosphorylation activity. Mitochondrial diseases could be screened in this manner and if a defect could be localized to a small group of enzymes more specific enzyme function tests could be applied and the defective enzyme identified.

Assays for oxidative phosphorylation activity in muscle mitochondria were developed in the mid-1950's (13,14). The techniques are well established and reliable (60,73,85). However, current methods available for studying oxidative phosphorylation in mitochondria from fibroblasts are not as well developed.

Development of the present methods for isolating mitochondria from cultured cells are based on the method Kobayashi reported in 1966 (43). He found that Nagarse and homogenization treatment of cultured cells (HeLa, amniotic fluid, and conjunctiva cells), released functioning mitochondria which could be enriched by differential

centrifugation. Cells had been grown in large quantities in roller bottles, but only yielded a small amount of mitochondrial protein. The mitochondrial suspension was assayed in a 2 ml reaction vessel and oxidative phosphorylation followed polarographically. Qualitatively, these mitochondria functioned similarly to mitochondria from other tissue sources (muscle, liver), although they respired at a slower rate ($\sim$50 nmoles O/min/mg protein). ADP/O ratios were within the expected range and intactness of the isolated mitochondria was evident by a respiratory control ratio between 1.4-5.4. Also, exogenously added NADH, normally impermeable to intact mitochondria, could not be oxidized by these mitochondria. Inhibitors and uncouplers had normal effects (43).

Millis and Pious reported a similar method for the isolation of mitochondria from fibroblasts (56). Their purpose was to evaluate fibroblasts for potential use in screening for inherited defects in mitochondrial function. They were able to isolate normally functioning mitochondria from cultured skin fibroblasts. As in Kobayashi 's method (43), they started with a large quantity of cells (fibroblasts) grown in roller bottles, and used protease digestion together with homogenization to disrupt the cells and release mitochondria. This was followed by differential centrifugation to obtain mitochondria.

From 10 fibroblast cell lines, they (56) consistently obtained mitochondria with RCR's of 3.4-4.01, ADP/O ratios

within the expected range, and respiration rate of 20-25 μmoles O_2/min/mg protein, similar to those values reported by Kobayashi (43). To date, a superior method for isolating mitochondria from fibroblasts has not been reported.

In our laboratory, Haworth-Hatherell (35) developed a micromethod for isolating mitochondria from fibroblasts in which a mini oxygen electrode and a reaction vessel less than one-tenth the size of that of Millis and Pious (56) was used to assay the mitochondrial preparation. The present work describes improvements to this micromethod yielding tightly coupled intact mitochondria, with expected ADP/O ratios and O_2 rates similar to those previously reported (43,56).

1.2.2 Mitochondrial Diseases

1.2.2.1 General Description of Mitochondrial Diseases

Routine investigation of mitochondrial diseases is difficult, as no single finding clearly defines the disease. Clinically, mitochondrial disease onset can occur from birth to mid-life (4), and follow either a static or progressive course. The degree of impairment is variable, depending on the specific disease, with most patients experiencing some degree of muscle weakness, exercise intolerance, fatigue and sometimes myoglobinuria.

Many investigators have attempted to group and

classify mitochondrial diseases on the basis of clinical, ultrastructural or biochemical findings, alone or in combination (42,46,57a,63). Table 1 following that of Martin (53) shows a provisional classification for mitochondrial diseases on the basis of clinical, histological and biochemical findings.

In this table mitochondrial diseases have been divided into three major classes:

I. CNS disorders in which abnormal mitochondria, present in the CNS, may be involved in the pathogenesis.

II. CNS disorders in which mitochondrial deficiencies, also present in muscle, may be responsible for CNS pathogenesis.

III. Myopathies with abnormal mitochondria.

Table 1 Classification of Mitochondrial Diseases

Class I - CNS disorders in which abnormal mitochondria may be involved in the pathogenesis.

Disease	Description	Ref.	Inheritance	Expressed in fibroblasts
van Bogaert-Bertrand	spongy degeneration of the neuraxis	1	autosomal recessive	?
Reye's syndrome	encephalopathy and fatty degeneration of viscera	18	possibly genetic predisposition	?
Zellweger	hepato-cerebral -renal syndrome	79	autosomal recessive	?
Menkes	kinky hair disease	52	sex linked recessive	no

Class II - CNS disorders in which mitochondrial deficiencies, also present in muscle, may be responsible for CNS pathogenesis.

Cerebral polio-dystrophy	lactic acidemia and mitochondrial myopathy	34,66	?	?
Lactic Acidemia	with mitochondrial myopathies	8,12,51,80	?	?
Leigh's Disease	subacute necrotizing encephalomyelopathy	15,69, 71	autosomal recessive	controversial

Disease	Description	Ref.	Inheritance	Expressed in fibroblasts
Cytochrome disorder of the nervous system and muscle	inherited	72	inherited	?
Familial mitochondrial myopathy with central defect in neural transmission		3	inherited	?
Kearn's-Sayre syndrome	external ophthalmoplegia, heart block, retinus pigmentosa, cerebellar ataxia	45,59	controversial probably autosommal dominant	?
Fatal ataxic encephalopathy with carnitine acyl transferase deficiencies		20	?	?
Friedreich's Disease	ataxia and cardiomyopathy	75,76	autosomal recessive	yes
Class III - Myopathies with abnormal mitochondria				
Luft's Disease	mitochondrial myopathy and hypermetabolism	24,36,50	?	?
Carnitine palmitoyl transferase deficiency	muscle weakness on prolonged exercise and myoglobinuria	11	autosomal recessive	yes
Carnitine deficiency		41	?	?
Fascioscapulohumeral myopathies		40	autosomal dominant	?
ocular myopathies		45	?	?

Disease	Description	Ref.	Inheritance	Expressed in Fibroblasts
Mitochondrial myopathies	morphologically abnormal mitochondria with or without known or suspected biochemical defect	4	?	?
Mitochondrial Diseases	biochemically defective mitochondria without abnormal morphology	4,67,68	?	?

This table was based on the tables by Martin (53). A "?" indicates inheritance or expression in fibroblasts is not known or is controversial.

A biochemical abnormality has been described in some of the diseases listed in Table 1. For example, abnormal pyruvate metabolism has been reported for Leigh's Disease (58,71) while Stumpf et al (75,76) have proposed mitochondrial malic enzyme deficiency to be responsible for Friedreich's Disease. Loose coupling of mitochondria has been suggested to cause hypermetabolism in Luft's Disease (22,24,27,28,36) and Shapiro et al (67,68) suggest that the altered properties of NADH dehydrogenase of the ETC may be the cause of Cystic Fibrosis. However, even for these diseases the relationship between the biochemical abnormality and the primary defect is not certain.

There are very few mitochondrial diseases in which a specific biochemical defect is known to cause the disease. A deficiency in carnitine palmitoyltransferase, an enzyme of the inner mitochondrial membrane, prohibits normal fatty acid metabolism (23,26,39,64), while intestinal malabsorption of copper in patients with Menkes Disease depletes cytochrome c's cofactor and affects the function of the respiratory chain.

More complete biochemical studies are needed to clarify the cause and effects of the biochemical abnormalities that have been associated with certain mitochondrial diseases and whether they could be responsible for the disease. With fibroblasts, a battery of biochemical tests can be used repeatedly on a consistent tissue source to further characterize the abnormalities

found in these diseases. Of those diseases listed in Table 1, only those which are genetically determined and could be expected to be expressed in fibroblasts are included in this study.

1.2.2.2 Diseases of Special Interest

The general method of studying mitochondrial diseases in fibroblasts described here has been applied to Luft's Disease, Carnitine Palmitoyltranferase (CPT) deficiency and Cystic Fibrosis (CF). Following is a brief description of each of these diseases and the rationale behind their inclusion in this study.

A. Luft's Disease

One of the first disorders of mitochondria to be studied in depth was described by Luft and co-workers (27,28,50). The patient suffered from severe hypermetabolism of non-thyroid origin and studies showed that the skeletal muscle mitochondria of the patient differed ultrastructurally from normals. The mitochondria were increased in number, variable in size (up to 5x larger than normal), and contained paracrystalline inclusions. This abnormal mitochondrial morphology initially was the most important diagnostic of mitochondrial diseases. When mitochondria from skin biopsies of the patient were examined by electron microscopy, they appeared normal (50). Biochemical studies on isolated mitochondria from skeletal

muscle showed a lack of respiratory control. It was concluded that the hypermetabolic state of the patient was caused by a defect in the mitochondrial enzyme organization resulting in poor respiratory control (50). The patient's mitochondrial respiration proceeded at a very high rate and energy not coupled to ADP phosphorylation was dissipated as heat.

A second case of Luft's Disease was identified by Haydar et al (36) in 1971, and ultrastructural and biochemical studies later reported by DiMauro et al (22,24). Mitochondria isolated from skeletal muscle from this second patient were examined by electron microscopy and were shown to be increased in number and morphologically abnormal. Biochemically, the mitochondria were loosely coupled, suggesting a defect in respiratory control. DiMauro's group has suggested that in vivo there is a recycling of Ca^{++} between mitochondria and cytosol which results in sustained stimulation of respiration and 'loose' coupling (22). The molecular abnormality responsible for the Ca^{++} recycling in Luft's Disease has not been determined. Also it is not known if expression of the defect in Luft's Disease is restricted to muscle or if all mitochondria and all tissues are affected. Luft's Disease has been included in the present study and if the disease is expressed in fibroblasts, the respiratory control defect should be detected.

B. Carnitine Palmitoyltransferase (CPT) Deficiency

This disease was first described in female twins by Engel et al in 1970 (26). Both women complained of intermittent muscle cramps and myoglobinuria frequently related to exercise, fasting or a high fat diet. Engel et al (26) suggested a defect in utilization of long-chain fatty acids by skeletal muscle but did not investigate the enzymes involved. In 1973, DiMauro et al (23) described a similar patient who had cramps and pigmenturia related to physical exertion. They investigated the enzymes of fatty acid metabolism and found the patient was deficient in carnitine palmitoyltransferase (CPT) activity.

A lack of, or a change in this enzyme affects the patient's ability to oxidize long chain fatty acids by β-oxidation (41). To some extent, compensation is possible but, during prolonged exercise, fasting, or a high fat diet, an energy crisis may develop (41). Depleted of glycogen stores, the patient experiences attacks, consisting of painful muscle stiffness or myoglobinuria, which are probably the result of muscle necrosis (41).

Since this disease was first characterized by DiMauro et al in 1973, (23), many other cases have been reported. It is now clear that CPT deficiency is inherited as an autosomal recessive disease, and is expressed in fibroblasts (19,21,47,62).

The enzyme is located on the inner mitochondrial membrane and is required for the transport of long chain fatty acids into mitochondria for catabolism (41). The enzyme has two functional activities and it has been proposed that these two activities may be the result of the existence of two isoenzymes located at different sites in the mitochondria (39). However, most investigators believe that CPT is actually two different enzymes, CPT I and II (7,23,39,64). CPT I is on the outer face of the inner mitochondrial membrane and catalyzes formation of palmitoylcarnitine so that it can be transported across the inner mitochondrial membrane by acyl carnitine carrier protein (41). On the mitochondrial matrix side, CPT II catalyzes the reverse reaction and regenerates palmitoyl CoA (33) which can be metabolized by β-oxidation. Deficiency of this enzyme prohibits the use of fatty acids as fuel and may precipitate the symptoms previously described.

The disease may actually be a heterogeneous group of diseases (21,41,62), as it has been shown to be caused by deficiencies in CPT I, CPT II, or both. The degree of enzyme deficiency, reflected by the residual CPT activity can vary from 54-100% (41). There is some variability of hepatic involvement in this disease, as well as variability in the relation between clinical manifestations and prolonged exercise (41). It is therefore important that for each patient exhibiting signs of the disease, the exact

nature of the defect be determined.

As it is expressed in fibroblasts and the defect is known to be at CPT I or II, fibroblast mitochondria can be used to test the feasability of the oxidative phosphorylation assay to identify a mitochondrial defect. DiMauro and his group (23) have demonstrated that CPT deficiency in muscle mitochondria, determined by a direct enzymatic method can also be detected by the oxidative phosphorylation assay. Although both assays showed a decrease in CPT activity, the actual amount of CPT activity determined differed. Using the direct enzymatic method the CPT activity of the patient was 20% of controls while with the oxidative phosphorylation assay, the CPT activity was 60% that of controls (23). The oxidative phosphorylation assay has been used in this study on fibroblast mitochondria from a CPT deficient patient.

C. Cystic Fibrosis

Cystic Fibrosis (CF) is an inherited disease of exocrine glands that primarily affects the pancreas, respiratory system and sweat glands (78). The major symptoms of CF, intestinal and pulmonary complications, seem to be caused by viscous secretions found in the organs involved in the pathogenesis. Eventually the patient suffers from pancreatic insufficiency, intestinal obstruction and chronic obstructive lung disease with persistent infection (78). There is no cure for this

disease and currently the only reliable method of diagnosis of CF patients is an elevated Na and Cl level found in sweat (16,77,78). CF is an autosomally recessive inherited disease that primarily affects Caucasian populations (77). It is considered the most common single gene disorder for this population, and for American whites, 1:20 will carry the gene for the disease (77).

CF research has taken many different directions (16) and came to our attention when Shapiro et al (67) suggested on the basis of fibroblast studies, that the primary defect responsible for CF may be found in the mitochondria (67,68). Shapiro's group initially reported increased Ca^{++} in CF homogenates as compared to controls (30). The increased Ca^{++} of CF homogenates could be accounted for by an increased Ca^{++} accumulation by CF mitochondria (31). Release of Ca^{++} by mitochondria gives a rapidly available Ca^{++} source and sequestration by mitochondria provides a major Ca^{++} sink (31). They hypothesize that this increased calcium may affect the composition or viscosity of secretions leading to the clinical signs of the disease (31,32). Calcium uptake by mitochondria is driven by the proton gradient (81b) a fact which led Feigal and Shapiro (31) to speculate that the ETC may be responsible for alteration of Ca^{++} in CF cells. Using an oxygen electrode, they showed that CF whole cells had a greater rate of oxygen uptake than normals, and suggest that the ETC was more active (31), and may have caused increased Ca^{++}

sequestration by these organelles (67). Further studies (67,68) revealed that NADH dehydrogenase (NADH:(acceptor) oxidoreductase, E.C. 1.6.99.3) of the first enzyme complex of the ETC may be affected in CF patients. They showed that this enzyme in CF cells was more sensitive than controls to rotenone inhibition, and that the pH optima for the enzyme differed in CF, heterozygous and control cells (67). Most recently this group has reported distinctive enzyme kinetics for NADH dehydrogenase, where the average Km(NADH) for this enzyme was between 10.9-16.1 μM NADH for CF patients, 20.9-26.3 μM NADH for heterozygous individuals, and 31.8-42.8 μM NADH for controls (68). They suggested this enzyme could be the primary defect responsible for CF (68). One can speculate that an increased affinity for NADH by the ETC enzyme may allow for an increased activity of the respiratory chain. At NADH concentrations below the Km value for controls, CF patients metabolize NADH faster and continue to respire and sequester calcium. Later this Ca^{++} sink can affect secretion and cause CF symptoms.

Although CF's symptomatology is not like that of other mitochondrial diseases, the work of Shapiro et al suggests that the basic defect resides in the mitochondria and therefore CF could be included in these studies of mitochondrial diseases. The initial work of screening for a potential mitochondrial defect had been done by Shapiro et al (67,68) in which they identified NADH dehydrogenase

as the possible primary defect. Their most recent work (68) on NADH dehydrogenase kinetics was followed up on by us and will be presented in the results.

1.3 Aim of the project

Having considered the problems of studying mitochondrial diseases, the general method described here attempts to identify specific biochemical defects systematically by a screening process. The mitochondrion may be suspected to be the site of the defect responsible for a particular disease because of clinical signs, ultrastructural changes or biochemical abnormalities of mitochondrial metabolites. Using the method reported here, the oxidative phosphorylation activity of fibroblast mitochondria from the patient can be screened and if a series of enzymatic steps seems to be defective, each of these enzyme's functions can be tested specifically.

These studies show how this general method has been applied to the study of mitochondrial diseases. Fibroblast mitochondria from both a Luft's Disease and CPT deficient patient were assayed for oxidative phosphorylation activity to determine if the disease was expressed in fibroblasts and could be detected by this method. For both diseases studied no clear mitochondrial abnormality was evident in fibroblasts. Also fibroblasts from CF patients were examined for abnormalities in the mitochondrial enzyme NADH dehydrogenase. Following the protocol of Shapiro *et al*

(68), the CF genotypes could not be distinguished on the basis of NADH dehydrogenase kinetics contrary to Shapiro *et al*'s (68) prediction.

2. Materials and Methods

2.1 Biological Materials

2.1.1 Animals

Golden Syrian hamsters were obtained from the animal care facility of the Faculty of Dentistry, University of Manitoba. Adult animals of both sexes were used.

2.1.2 Fibroblast Cultures

Skin fibroblasts were obtained from the Human Genetic Mutant Cell Repository (Camden, N.J., USA; designated "GM"), Montreal Children's Hospital Repository (Montreal, Canada; designated "MCH"), or from local biopsies (designated "WP"). Cell cultures were grown using standard techniques as described by Adams (2). They were maintained in MEM FC 10 (Minimal Essential Media with Fetal Calf Serum 10%), or Human McCoy's FC 10 cell culture media and when confluent, cells were passaged by trypsinization. The compositions and sources of media and trypsin solutions are given in Appendix I. For all experiments, cultures were grown in tissue culture plates, (60, 100, or 150 mm in diameter, obtained from Lux-Miles labs) and maintained at 37°C., 95%air/5%CO_2, and 90% humidity.

2.2 Chemicals

Adenosine diphosphate (ADP), ethylenediamine

tetraacetic acid, disodium salt (EDTA), L-glutamic acid, mannitol, malic acid, β-NADH (Na and K salts, grade III and IV, respectively), oligomycin, DL-palmitoylcarnitine, palmitoyl CoA (free acid), pyruvic acid, and Trizma base, for making tris buffers, were obtained from Sigma Chemical Company. Copper sulfate, Folin and Ciocalteau phenol reagent, potassium phosphate, sodium tartrate, succinic acid, all of Analar grade , and sucrose of Aristar grade were obtained from BDH. Potassium phosphate and sodium carbonate were obtained from Mallinckdrodt. Sodium chloride and sodium hydroxide were obtained fron Fisher Scientific. Rotenone was obtained from K&K labs, 2,4-dinitrophenol (DNP) from Matheson, L-carnitine from Mann Research labs, bovine serum albumin (BSA) powder (fraction V) from Reheis Chemical Co. (Arizona,USA), "Nagarse" enzymes from the Enzyme Development Corporation (N.Y., USA), trypsin from ICN, and dye reagent concentrate (for protein microassay) from BioRad Laboratories.

The final concentrations of substrate and inhibitors used were as recommended by Stephens and Wrogemann (73), or Pande and Blanchaer (60).

2.3 Polarographic Assay

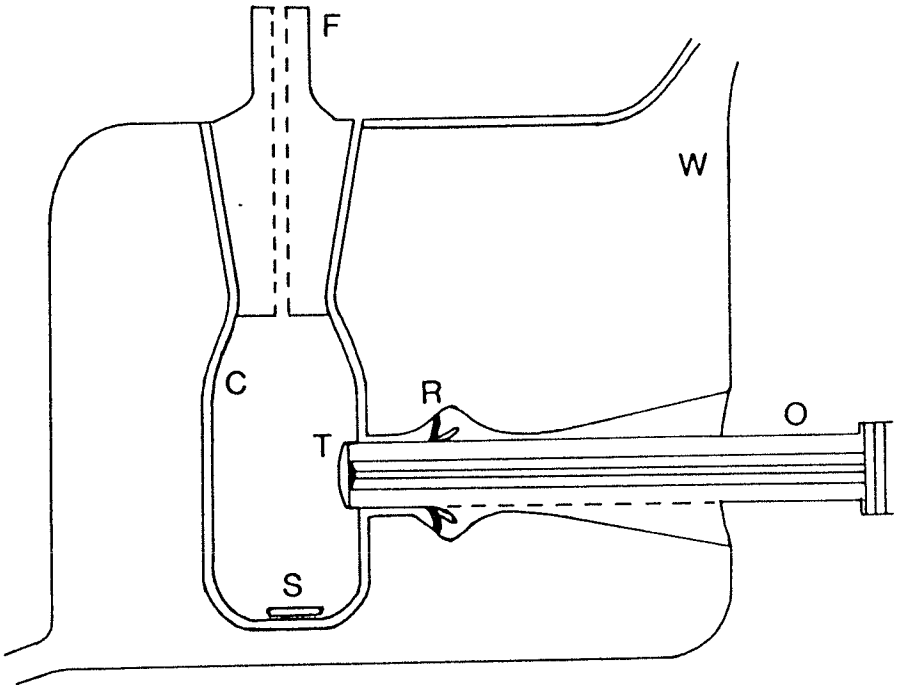
2.3.1 Apparatus

The polarographic determinations were done in reaction vessels of 150, 600, or 1500 µl volume. When reaction

vessels (also called cuvettes) of 600 or 1500 µl volume were used they were fitted with an oxygen electrode (Clark type electrode, Yellow Springs Instrument Co.), and membrane (YSI, Model 5775) and the electrode was connected to an amplifier and recording system. The amplifier was a custom made "Oxygraph" similar to the Gilson Oxygraph, Model KM (Gilson Medical Electronics, Middleton, Wisconsin, USA). For the cuvette of 150 µl volume, a Transidyne mini oxygen electrode (model #730), with membrane, was connected to an amplifier (Transidyne Development Corporation,model #1201,chemical microsensor) from which O_2 concentrations could be monitored. When a polarizing voltage of -0.6 volt is applied to the electrode the current is directly proportional to the oxygen concentration. The current is measured by the "oxygraph" system, amplified and recorded on a strip chart. Isotonic cuvette medium saturated with air, mitochondria and substrates/inhibitors was added to the cuvette. By monitoring oxygen consumption oxidative phosphorylation functions of the mitochondria could be followed. The cuvette, electrode, and stopper form an essentially closed system, except for a small capillary opening through which additions can be made (see Figure 4). During experiments, the cuvette medium would fill the capillary in the stopper by at least one cm. As oxygen diffuses very slowly in stationary fluids (as in the capillary), this forms an essentially closed system.

Figure 4

Schematic Representation of the Oxygraph Cuvette Fitted with an Oxygen Electrode



W) water circulating at 28°C

C) Cuvette or reaction chamber

S) magnetic stirrer

T) Teflon membrane of electrode

F) glass stopper with capillary opening

O) oxygen electrode

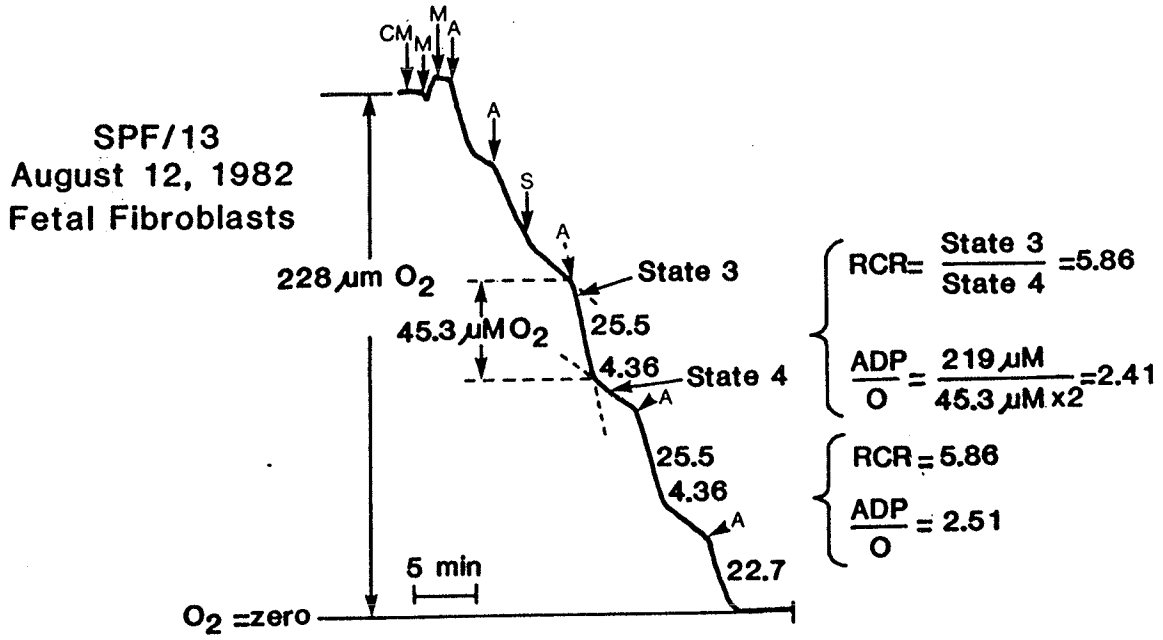
R) o-ring

2.3.2 Oxidative Phosphorylation Assay

The typical polarographic record illustrated in Figure 5 demonstrates the method by which several parameters of oxidative phosphorylation were determined. Mitochondria (M) were added to the cuvette medium (CM, see composition under Solutions for Mitochondrial Isolation and Oxidative Phosphorylation, 2.3.4) saturated with air at 28° C. To avoid respiration due to endogenous substrates ADP (A) was added, respiration allowed to proceed, and oxygen was consumed. When addition of more ADP could no longer stimulate respiration, the endogenous substrates were nearly depleted and the oxygen consumption rate (O_2 rate) slowed down. Then the substrate (S) of choice, eg. pyruvate/malate (5mM/1mM), was added and respiration was stimulated. Respiration in the presence of phosphate acceptor (ADP) is defined as the State 3 rate (14). The rate will decrease when the added ADP has been depleted and this rate is defined as the State 4 rate (14). ADP is added in rate-limiting quantities thus repeating the State 3/State 4 cycle until the oxygen in the cuvette medium is exhausted (O_2 = zero). From such an experiment, the O_2 rates, RCR's, and ADP/O ratios can be calculated. Unless otherwise mentioned the values presented in Results were taken from the second cycle of State 3/State 4 respiration.

Figure 5

Example of a Typical Polarographic Experiment



Additions to cuvette:

CM) cuvette medium
M) mitochondria
A) ADP
S) substrate (5mM pyruvate/1mM malate)

Respiration rates (State 3 and State 4) are expressed in µmoles oxygen per minute per gram of mitochondrial protein

RCR) respiratory control ratio
ADP/O ratio) phosphorylation Efficiency

2.3.3 Calculations

The parameters measured in these oxidative phosphorylation assays were calculated according to Chance and Williams (13,14), and Estabrook (29).

1. RCR = Respiratory Control Ratio

$$= \frac{\text{State 3 respiration rate}}{\text{State 4 respiration rate}}$$

2. O_2 rate (respiration rate) is expressed as

μmoles O_2/min/g mitochondrial protein.

uM concentration of O_2 in CM
= Barometric Pressure (inches) x 7.637

The factor 7.637 was experimentally determined after the method described by Estabrook (29).

3. The ADP/O ratio is an expression of phosphorylation efficiency (81b).

$$= \frac{\text{quantity of ADP added}}{\text{quantity of } O_2 \text{ consumed} \times 2}$$

2.3.4 Solutions for Mitochondrial Isolation and Oxidative Phosphorylation Assay

Stock Solution

0.21 M mannitol
0.07 M sucrose
0.1 mM EDTA

Homogenizing Solution

Stock Solution
0.01 M Tris phosphate, pH 7.6
50.0 mg% Nagarse

Suspending Solution

Stock Solution
1 mM Tris chloride, pH 7.4

Cuvette Medium

0.23 M mannitol
0.07 M sucrose
0.2 M Tris chloride, pH 7.2
0.02 M EDTA
5.0 mM phosphate (from KPO_4 buffer)

2.3.5 Mitochondrial Preparation from Hamster Heart

Mitochondria were isolated from hamster heart according to the methods established by Wrogemann et al (84,85) and modified for heart. The animal was weighed, decapitated, and time of death was recorded. This was considered as Time = 0 for the determination of time required to make the mitochondrial preparation. The following procedures were carried out at 4°C. The heart was removed and rinsed in 50 ml of Stock Solution, four times, trimming off non-muscular material between rinses. The heart muscle was blotted on filter paper and weighed. Using scissors and scalpels, the heart muscle was minced and transfered to a Thomas grinding vessel (size C) and twenty ml of Homogenizing Solution was added. The heart muscle was incubated on ice, and after 5 min into the incubation period, the mixture was homogenized with a loose Teflon pestle for 5-7 passes. This homogenate was allowed to digest a further 5 min and then was diluted with 20 ml of Stock Solution and homogenized again, this time with a tight Teflon pestle for 5-7 passes. The final homogenate was centrifuged at 500g for 5 min at 4°C in a Sorvall

(RC2B,SS-34 rotor) centrifuge. The mitochondria were pelleted from the supernatant by centrifuging at 12,000g for 10 min. The pellet was rinsed with 4 ml Suspending Solution and resuspended with a vortex mixer. This mitochondrial suspension was centrifuged at 8,000g for 5 min to obtain the final mitochondrial pellet. The pellet was suspended in 400 µl Suspending Solution per heart used, with a vortex mixer, and kept on ice until required.

2.3.6 Mitochondrial Preparation from Fibroblasts

The methods for isolating mitochondria from fibroblasts were developed from the method of Haworth-Hatherell (35) which most closely resembles method D described below. Steps were carried out at room temperature except centrifugation which was done at 4°C in a Sorvall centrifuge (RC2B,SS-34 Rotor).

Method A

Fibroblast cultures (from 150 mm plates) were washed twice, with 10 ml Suspending Solution and the cells were scraped off the plate in 2 ml Suspending Solution using a plastic spatula. They were triturated with a Pasteur pipette for 10 passes in order to disperse the cells. The homogenate was centrifuged at 600g for 5 min and the pellet discarded. The supernatant was then centrifuged at 12,000g for 10 min yielding a final mitohondrial pellet. The pellet was dispersed in 50-75 µl Suspending Solution, and

mixed gently with a vortex mixer. An aliquot was removed for protein determinations, and BSA was added to a final concentration of 1%. This suspension was kept on ice until needed.

Method B

Fibroblast cultures were washed twice with 10 ml Suspending Solution, scraped and triturated as in Method A and pooled in a centrifuge tube. Nagarse (0.08 mg%) was added and the suspension was incubated for 7 min. Then the homogenate was diluted to twice the volume with Suspending Solution and centrifuged. The final mitochondrial pellet was suspended as in Method A.

Method C

Fibroblast cultures were washed twice with 10 ml Suspending Solution, scraped and triturated as in Method A, and pooled in a Thomas grinding vessel (size B). The cell suspension was homogenized with a Teflon pestle for 5 passes, then centrifuged and the final mitochondrial pellet was suspended as in Method A.

Method D

Fibroblast cultures were washed twice with 10 ml Suspending Solution, scraped and triturated as in Method A, and pooled in a Thomas grinding vessel (size B). Nagarse (0.08 mg%) was added and the suspension was incubated on

ice for 7 min. The homogenate was diluted to twice the volume with suspending solution and homogenized with a Teflon pestle for 5 passes. The homogenate was centrifuge d and the final mitochondrial pellet was suspended as in Method A.

Method E

Cells from 150 mm plates were harvested with trypsin:EDTA (50mg%:20mg%), 3 ml/plate for a wash, followed by 4 ml for trituration. The cells were pooled into 2 centrifuge tubes containing 10 ml MEM FC-10 culture medium in each, and were pelleted at 500g for 5 min. This cell pellet was washed with 10 ml cold Suspending Solution and pooled into 1 tube. Cells were pelleted again at 500g for 5 min and then were suspended in 10 ml Suspending Solution and triturated with a Pasteur pipette to disperse the cells. Unbroken cells and debris were pelleted at 600g for 5 min and the supernatant saved. The pellet was resuspended in 2 ml Suspending Solution, triturated again and centrifuged at 600g for 5 min. This supernatant was pooled with the first and the mitochondria were pelleted at 12,000g for 10 min. The mitochondrial pellet was resuspended in 100 µl Suspending Solution, an aliquot removed for protein determination, and BSA added to 1% final concentration.

2.4 Enzyme Analysis and Michaelis Constant (Km) Determination

2.4.1 Preparation of Mitochondrial Fraction

A. Solutions

Sucrose A solution

0.25 M sucrose
1.0 mM EDTA
10.0 mM Tris buffer, pH 7.4

Sucrose B solution

0.25 M sucrose
10.0 mM Tris buffer, pH 7.4

NADH dehydrogenase Assay Reaction Mixture

100.0 µM	K_3 Fe(CN)$_6$
0.12 M	Potassium phosphate buffer, pH 7.9
10-100.0 µM	NADH (exact concentration determined spectrophotometrically)
100 µl	sample enzyme (prepared according to method described in section 2.3.1.1)
3.0 ml	Total Volume

B. Procedure

The mitochondrial preparation and NADH dehydrogenase assay was done according to Shapiro et al (68). Confluent fibroblast monolayers were harvested from 8-15, 150 mm tissue culture plates (approximately 1.5 - 2 million

cells/plate) with trypsin:EDTA, and pooled in 2 centrifuge tubes with 10 ml/tube MEM-FC10 cell culture medium. The cells were centrifuged at 500g for 5 min at 4°C. The pellet was washed twice with 10.0 ml NaCl (0.9%), and centrifuged after each wash at 500g for 5 min. In the pilot experiments, an aliquot was taken for cell counting (Haemocytometer), but when the average number of cells/plate was determined, this step was omitted to save time. Cell preparations were kept at 4°C for all subsequent steps until the enzyme assay. The pellet, suspended in 8 ml Sucrose A Solution, was homogenized with 15 -20 strokes in a Dounce homogenizer (Wheaton). A 2 ml rinse with Sucrose A Solution of the homogenizer was added to the homogenate, the total volume measured and a 100 µl aliquot was taken for protein determination. The homogenate was centrifuged at 600g for 10 min and the supernatant containing the mitochondrial fraction was saved. The pellet was washed twice in 2 ml of Sucrose A Solution, each time dispersing the pellet with 10 passes through a Pasteur pipette, and centrifuging at 600g for 10 min. Following each wash, the supernatant was added to the first supernatant containing the mitochondrial fraction. This combined supernatant fraction was centrifuged at 20,000g for 15 min. The pellet was washed once in 5 ml ice-cold Sucrose B Solution and centrifuged again at 20,000g for 15 min. The pellet was suspended in 2 ml of ice-cold 0.03 M potassium phosphate buffer, pH 7.6, and

frozen and thawed 4 times in a dry ice-ethanol bath to disrupt the mitochondria. This solution was then centrifuged at 270g for 5 min. The pellet was discarded and an aliquot of the supernatant was used for protein determination. The remainder was used for mitochondrial NADH dehydrogenase assays. The sample, NADH and phosphate buffer (0.03 M, pH 7.6) were kept on ice, while the ferricyanide and the other phosphate buffer (0.12 M,pH 7.9) were maintained at room temperature (22°C). Enzyme assays were performed on fresh preparations.

2.4.2 NADH Dehydrogenase Assay

The kinetics of the sample enzyme (mitochondrial fraction as prepared by method described in section 2.4.1) were determined by following the oxidation of NADH. The reaction is followed by measuring the decrease in absorbance (Δ O.D.) upon reduction of ferricyanide, an artificial electron acceptor. This was measured at 420 nm in a Beckman DB spectrophotometer for the pilot studies, while a Unicam-SP 1800 spectrophotometer was used for the remainder of the Km determinations. The reaction mixture as given in Section 2.4.1 (A), consisted of potassium ferricyanide (100 μM), potassium phosphate buffer (0.12 M, pH 7.9), and NADH (10-100 μM). The sample enzyme concentrations were adjusted with potassium phosphate buffer(0.03 M,pH 7.6) to yield a 0.10-0.15 ΔO.D./min. The reaction mixture without NADH and sample was pre-incubated

at 30° C for at least 4 min, then NADH was added and the mixture further incubated for 1 min. This was monitored so that no Δ O.D. was observed prior to addition of enzyme preparation (ie. mitochondrial preparation from 2.4.1). The sample was injected into the cuvette without removing the cuvette from the spectrophotometer and mixed by depressing and releasing the pipette push-button several times. The ΔO.D. was recorded during the initial 20-60 s and the reaction rates determined for varying NADH concentrations (10-100 μM). The Michaelis constants (Km) were calculated later using linear regression analysis of Lineweaver-Burk plots (sample shown in Figure 6a). Assays were run in duplicate or triplicate depending on enzyme activity of samples. The Km for each cell line was determined on at least 3 separate experiments, with exceptions as noted in the results.

2.5 Chemical Analysis

2.5.1 Protein Determination

2.5.1.1 Lowry Method

The protein concentrations of mitochondrial suspensions used in polarographic assays were determined by the method of Lowry et al (49). The principle of this assay is that a substance containing two or more peptide bonds will form a purple complex with copper salts in an

alkaline solution. The final color measured at 578 nm in this procedure is the result of that reaction plus the reduction of the Folin reagent by the tyrosine and tryptophan present in the treated protein. The following reagents were used:

A. 1.0 N NaOH

B. 2% Na_2CO_3

C. 1.0 ml 1% $CuSO_4(5H_2O)$, in 1.0 ml 2% NaTartrate. Mix and take 1.0 ml and place in 50 ml 2% $NaCO_3$.

D. Freshly prepared alkaline copper solution: 50 ml B and 1.0 ml C.

E. Dilute Folin reagent, 1:3 of the commercial Folin & Ciocalteau Phenol Reagent.

F. Freshly prepared 0.25% BSA.

Procedure:

Triplicate or quadruplicate samples of each concentration of protein standard (reagent F) were set up in small test tubes and made up to 30 µl final volume with de-ionized distilled water (DIW). To all standards 0.1 ml of NaOH (reagent A) was added and solution was incubated a minimum of 30 min at room temperature. To this, 1.0 ml of reagent D was added and the solution was mixed immediately.

This mixture was allowed to stand for 10 min then 0.1 ml of the dilute Folin reagent (E) was added rapidly with immediate mixing. The standards were left for a minimum of 30 min. then read against a reagent blank at 578 nm using an Eppendorf Photometer (model,100M).

If necessary, mitochondrial suspensions or cell homogenates were diluted with DIW to obtain readings that were within the standard curve. Usually this was a 1:6 or 1:3 dilution from which 10 and 20 µl aliquots were taken for protein determination. All samples were made up to 30 µl starting volume with DIW and then treated exactly as the standards.

2.5.1.2 Protein Microassay (BioRad)

BioRad Laboratories have developed a protein microassay that is based on the protein determination method of Bradford (5). This is a much simpler, faster and more sensitive method than the Lowry assay and is useful for determining protein concentrations less than 1 µg/ul. Therefore, it was used to determine protein concentrations for all NADH dehydrogenase kinetic assays, since the protein concentrations of mitochondrial enzyme samples for these experiments were less than 0.1-0.5 µg/ul. The Bio-Rad protein assay was developed from the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465nm to 595 nm when binding to protein occurs (54,61). Use of this

principle in a protein assay was first demonstrated by Bradford (5). The method was slightly modified from that recommended by the manufacturer so that membrane bound protein could be determined. Mitochondrial and cell homogenate suspensions were incubated a minimum of 1 hour with 1 N NaOH. The final concentration of NaOH after dilution with DIW and reagent is low enough so that it will not interfere with the assay. A comparison of mitochondrial protein determined by both the Lowry method and the Bio-Rad micro assay is included in the results section.

Procedure:

Triplicate samples of each concentration of protein standards were made up to 20 µl final volume with DIW. To each tube, 50 µl 1 N NaOH was added and protein hydrolyzed for 1 hour. To this 730 µl DIW was added, followed by 200 µl of dye reagent concentrate with immediate mixing. The absorbance was read at 578 nm within 15 min after dye reagent had been added.

For most mitochondrial enzyme suspensions, dilution was not necessary. Generally 5 or 10 µl aliquots of sample were required for each assay, and aliquots were made up to 20 µl final volume and treated the same as the standards.

2.5.2 ADP determination

ADP solutions were kept frozen between polarographic

experiments and the concentrations were re-determined monthly. At pH 7 and a wavelength of 259 nm, the molar extinction coefficient (ϵ) was used to calculate the ADP conentration. Under these conditions, $\epsilon = 15.4 \times 10^3\ M^{-1}$ for a 1 cm path (17).

Procedure:

The ADP solution was diluted accurately with 0.02 M phosphate buffer and the absorbance was measured in a Beckman DU spectrophotometer at 259 nm.

Calculation:

$$\frac{\text{Abs. of unknown x dilution factor}}{\text{Molar extinction coefficient } (15.4\ mM^{-1})} = \text{mM ADP}$$

2.5.3 NADH determination

The molar extinction coefficient (ϵ) for NADH at 366nm is $3.3 \times 10^6\ M^{-1}$ for a 1 cm path (70). Therefore the stock NADH solution to be assayed was diluted accurately and the absorbance was measured at 366nm in the Eppendorf Photometer (model,100M).

Calculation:

$$\frac{\text{Abs. of unknown x dilution factor}}{\text{Molar extinction coefficient } (3.3 \times 10^3\ mM^{-1})} = \text{mM NADH}$$

3. Results

3.1 Method Development

3.1.1 Standardization of Technique

The first step in our approach to the study mitochondrial diseases is to isolate mitochondria with good oxidative phosphorylation activity from patient's fibroblasts and then to screen these fibroblast mitochondria for defects. However, the methods available for isolating fibroblast mitochondria needed further development before screening could be done.

I first familiarized myself with the oxidative phosphorylation assay. Mitochondria from hamster hearts were isolated and assayed for oxidative phosphorylation activity. Typically, the state 3 rates were about 100-150 μmoles O_2/min/g, RCR's were around 5-8, and ADP/O ratios were about 2-3 for an NAD^+-linked substrate and 1.5-2 for an FAD-linked substrate. Mitochondria were also isolated from fibroblasts using Haworth-Hatherell's method (35) and O_2 rates of 5-10 μmol O_2/min/g, RCR's of $\sim$ 3.00, and ADP/O ratios of $\sim$ 2-3 were found using a NAD^+-linked substrate. These were somewhat lower than with hamster heart mitochondria and lower than those values reported by Millis and Pious for fibroblast mitochondria (56). Millis and Pious (56) obtained mitochondria with O_2 rates of 13.2-22.4 μmoles O_2/min/g protein, RCR's of 3.4-4.0, and ADP/O ratios

of 2.57 for NAD-linked substrates, 1.73 for FAD-linked substrates.

Three reaction vessels were available for assaying oxidative phosphorylation activity of fibroblast mitochondria. The largest (1500 µl cuvette) is the common size used in oxidative phosphorylation assays (43,56,60,73,84,85). When hamster heart mitochondria were assayed in both the 1500 µl and 600 µl cuvette, results were comparable to each other (see Table 2) and to previously reported results (84,85). However results obtained using the 150 µl cuvette differed from those found using the larger cuvettes (1500, 600 µl). Table 3 shows the differences that were observed when the oxidative phosphorylation parameters for a single mitochondrial preparation were measured in the 150 µl and 600 µl cuvettes. In Table 3, a letter has been assigned to each mitochondrial preparation that was assayed. For a given mitochondrial preparation, the State 3/State 4 cycle was determined 2-4 times per mitochondrial sample and the mean of the parameter values has been reported. It can be seen that regardless of the source of the mitochondria (hamster heart or fibroblast), the State 3 rates and RCR's were lower when measured in the 150 µl cuvette. It can also be seen that for fibroblast mitochondria, the State 4 rate is faster in the smaller cuvette. A comparison of the two cuvettes is shown in Figure 6.

Table 2 Oxidative Phosphorylation Assay of Hamster Heart Mitochondria, Typical Results

Cuvette size	Date of preparation	O_2 rate[a] (State 3)	RCR	ADP/O
1500 µl	21.6.82	149-180	5.72-9.69	2.6 -2.99
600 µl	7.6.82 14.7.82	75-168	5.38-8.85	2.19-2.59
150 µl	7.6.82 14.7.82	38- 86	2.95-5.00	1.82-2.49

a) Respiration rates are expressed as µmoles O_2/min/g. The substrate used was pyruvate/malate (5mM/1mM).

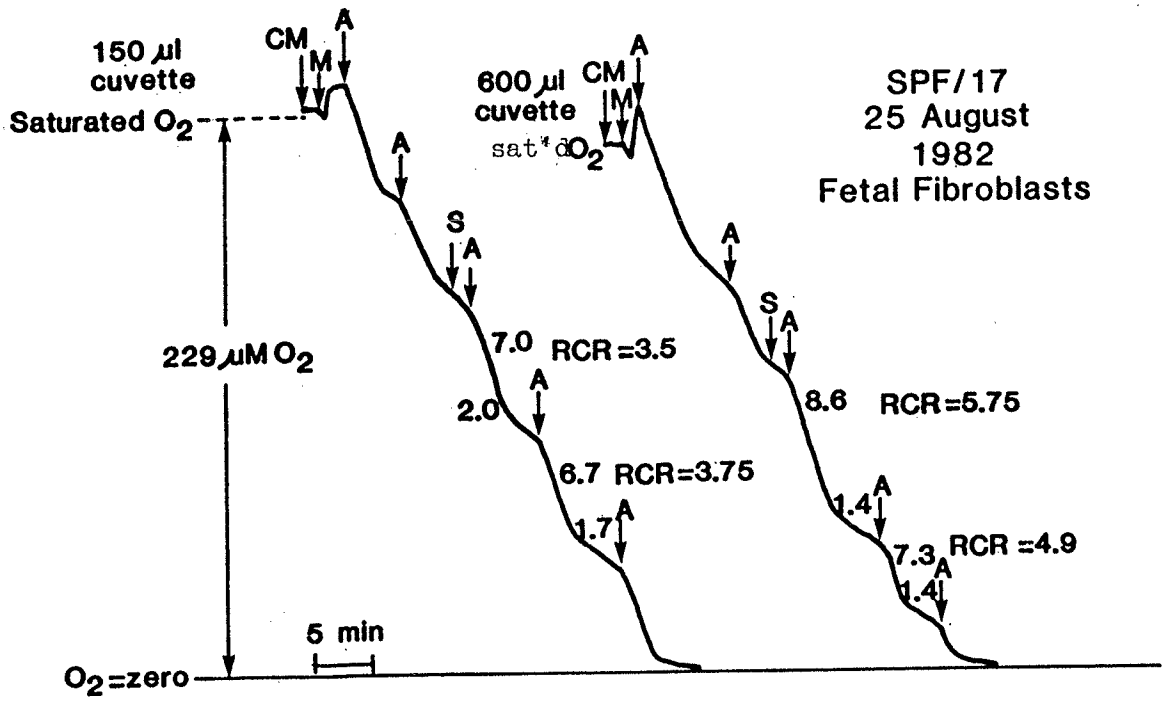
Table 3 Differences in Oxidative Phosphorylation Parameters depending on Cuvette Size

Source of mitochondria	Parameter[a]	Prep.	Cuvette 150 µl	Cuvette 600 µl	% difference[b] +(increase) -(decrease)
Hamster heart	State 3 rate[c]	A	62.1	148	+ 45
		B	36.4	78.3	+ 54
					$\bar{x}$ = + 49
	State 4 rate	A	24.1	25.0	- 4
		B	11.7	10.8	+ 8
					$\bar{x}$ = + 2
	RCR	A	3.4	5.9	+ 42
		B	3.13	7.39	+ 58
					$\bar{x}$ = + 40
Fibroblasts	State 3 rate	C	6.28	6.33	+ 1
		D	6.0	8.0	+ 25
		E	7.1	7.95	+ 11
		F	8.3	16.7	+ 50
					$\bar{x}$ = + 22
	State 4 rate	C	3.1	2.14	- 44
		D	3.6	2.43	- 48
		E	1.85	1.4	- 32
		F	2.23	2.9	+ 33
					$\bar{x}$ = - 25
	RCR	C	2.02	3.24	+ 38
		D	1.08	2.94	+ 43
		E	3.63	5.32	+ 32
		F	4.43	5.8	+ 24
					$\bar{x}$ = + 34

a) The values reported are the mean of the parameter values taken from each State 3/State 4 cycle (usually 2-4 cycles/prep.), for each mitochondrial preparation(A-F).
b) The numerical average ($\bar{x}$) for the % difference between parameter values for each cuvette has been given.
c) State 3 and State 4 rates are given in µmoles/min/g

Figure 6

Comparison of Oxygraph Tracings for Mitochondria Assayed in the 150 µl and 600 µl Cuvette



Additions to cuvette:

CM) cuvette medium

M) mitochodria

A) ADP

S) substrate (5mM pyruvate/1mM malate)

Numbers along the curve are respiration rates and are expressed as:
µmoles oxygen per minute per gram mitochondrial protein

This figure represents the polarographic tracings obtained when a single mitochondrial preparation from fetal fibroblasts was assayed in both the 600 µl cuvette and the 150 µl cuvette.

Since the results obtained in the 150 µl cuvette were lower than expected results, and the 600 µl cuvette required less mitochondrial protein per assay than the 1500 ul cuvette, the 600 µl cuvette was the cuvette used for all polarographic experiments.

3.1.2 Comparison of Methods for Preparation of Fibroblast Mitochondria

Haworth-Hatherell (35) has developed a micro-method for isolating fibroblast mitochondria. By measuring various oxidative phosphorylation parameters, the steps of this method were examined for their effect on the quality of the final mitochondrial suspension. Four methods of preparation, reflecting different combinations of the steps involved in the original method, were studied. The results are summarized in Table 4. It is evident from this table that all of the methods result in mitochondria with comparable State 3 rates. Although there are statistically significant differences between the results obtained by these different methods, the average ADP/O ratio for each method was within the expected range, and the yields were sufficient so that small quantities of cells could be used. However, the RCR's of mitochondria obtained using Method A are significantly higher than those of mitochondria obtained using Method D (that which most closely resembles Haworth-Hatherell's method, (35)). This indicates that Method A yielded better coupled, more intact mitochondria.

Methods A, B and C resulted in comparable mitochondrial preparations but Method A is simpler and faster and therefore the better of these methods for isolating mitochondria from fibroblasts. Later on, Method A was compared with Method E. Mitochondria prepared by Method A had RCR's of 4.5, 6.1, and 7.0 (from individual cycles of a single mitochondrial preparation) while Method E, a time consuming, involved method, did not show improvement over Method A.

Table 4 Comparison of Methods for Isolating Mitochondria from Fibroblasts

Method[a]	RCR[b]	State 3 rate[c] μmoles O_2/min/g	ADP/O[d]	Yield[e] (mg/g)[f]
A	4.86 ± 1.05	14.1 ± 5.99	2.18 ± 0.298	56.6 ± 13.2
B	4.85 ± 1.08	12.2 ± 2.53	2.4 ± 0.276	46.4 ± 8.84
C	4.57 ± 2.47	11.2 ± 4.09	2.06 ± 0.233	63.2 ± 9.71
D	3.35 ± 1.1	12.2 ± 2.1	2.04 ± 0.366	46.5 ± 9.86

The substrate used in all experiments was pyruvate/malate (5mM/1mM). Parameter values are expressed as $\bar{x}$ ± S.D., where the number of experiments (n) = 5

a) details of the different methods are given in the Materials and Methods. They differ in the way in which the fibroblasts are ruptured: Method A involves trituration; Method B involves trituration and protease digestion; Method C involves trituration and homogenization; Method D involves trituration, protease digestion and homogenization.

b) Method D gives a significantly different ($p<0.05$, Student' t test) RCR from Methods A or B

c) all methods not significantly different ($p>0.05$)

d) Method B gives a significantly different ($p<0.05$) ADP/O ratio from Methods C or D

e) Method C gives a significantly different ($p<0.05$) Yield from Methods B or D

f) expressed as mg mitochondrial protein/g starting cellular protein

3.2 Application of the Method

3.2.1 Luft's Disease

Mitochondria isolated from fibroblasts of a patient with Luft's Disease, were examined for coupling of respiration to phosphorylation. It had been shown previously that muscle mitochondria from this same patient are loosely coupled (24). In 4 experiments (A to D), mitochondria from patient fibroblasts were coupled with RCR's between 1.5-3.58 (see Table 5). Also, respiration slowed to approximately the State 4 rate upon addition of oligomycin. As with normally coupled mitochondria (81b), the patient's mitochondria could not respire at the State 3 rate without simultaneously phosphorylating ADP (ie. when oligomycin was present), or releasing the proton gradient by some other mechanism such as ion transport or the addition of the uncoupler, DNP. Therefore mitochondria from fibroblasts from this Luft's Disease patient were coupled and the defect seen in muscle was not expressed in fibroblasts.

Table 5 Oxidative Phosphorylation in Fibroblast Mitochondria from a Luft's Disease Patient[a]

Substrate or Inhibitor[b]	O_2 rate[c] (State3)	RCR	ADP/O
G/M	7.92(A)	1.5 (A)	2.29 (A)
P/M	5.6 (B)	2.11 (B)	-
	10.9 (C)	3.17 (C)	1.73 (C)
	12.5 (D)	3.58 (D)	2.23 (D)
R/S	13.7 (A)	1.73 (A)	1.43(A)
	13.3 (B)	1.9 (B)	
O/DNP	11.1/20.5 (A)		
	7.0/11.2 (B)		
	3.7/ 9.2 (C)		
	2.0/ 9.9 (D)		

Each letter in brackets () denotes individual experiments.
a) Fibroblast strain, GM28, obtained from the Human Genetic Mutant Cell Repository (Camden,NJ,USA)
b) Substrate and inhibitor abbreviations:
G/M,glutamate/malate (5mM/1mM); P/M, pyruvate/malate (5mM/1mM); R/S, succinate(4.5mM) in the presence of rotenone (6.4 μM); O/DNP, oligomycin (80 ug%)/ 2,4 dinitrophenol (50 μM), results given as oligomycin rate/DNP rate.
c) Respiration rates are expressed as μmoles O_2/min/g

3.2.2 Carnitine Palmitoyltransferase Deficiency

The oxidative phosphorylation assay was also used to study fibroblast mitochondria from a CPT deficient patient, to see if a known defect could be identified. Fibroblast mitochondria were isolated by Method A from cell strains derived from both a control (GM495) and CPT deficient (GM1763) individual and oxidative phosphorylation activity was monitored. Table 6 shows the different combinations of substrates that were used and the resulting State 3 rates.

Mitochondria from both strains oxidized pyruvate/malate (P/M), a standard NAD^+-linked substrate, at normal rates, but CPT deficient mitochondria respired at only 70% of the control. As well, GM 1763 fibroblast mitochondria oxidized palmitoylcarnitine (PC) plus malate at 52% the rate control, and palmitoyl CoA at 70 % of control. The reduction to 52% of the control State 3 rate that was seen with PC as a substrate was not that different from the reduced state 3 rates seen with other substrates. Only one State 3 rate determination could be done with the control as the only control available grew very poorly and it was difficult to obtain enough material for an experiment.

These data suggest that the mitochondria from the patient's fibroblasts oxidized substrates at 70% of the control rate and that their ability to use fatty acid substrates was not different from their ability to use carbohydrate substrates (P/M). Therefore, it appeared that both enzymes involved in the transportation of fatty acids into mitochondria, CPT I and II, were functioning in the patient fibroblasts.

Table 6 Oxidative Phosphorylation in Fibroblast Mitochondria from a CPT deficient Patient, Carbohydrate Substrate

Substrate	State 3 Rate[c]		RCR	
	Control (GM495)	Patient (GM1763)	Control (GM495)	Patient (GM1763)
P/M[a]	16.2 (4)[b]	11.2 (10)[b]	3.00(3)	3.08(9)
± S.D.	± 2.42	± 3.5	±0.06	±1.02
Malate	8.92	11.1	1.97(1)	1.86
then Pyruvate	9.77(1)	11.4 (6)		2.95(3)
Pyruvate	9.66	11.9	-	2.18
then Malate	10.2 (1)	11.4 (3)		2.06(3)

The values reported are either from a single experiment or the numerical average, with the number of experiments given in brackets ().
a) P/M, pyruvate (5mM) plus malate (1mM) added together.
b) GM1763 metabolizes P/M at 70% of control(GM495) and is significantly different from control, $p < 0.05$.
c) State 3 ra tes are given in μmoles O_2/min/g

Table 6a Oxidative Phosphorylation in Fibroblast Mitochondria from a CPT deficient Patient, Fatty Acid Substrate

Substrate	State 3 Rate		RCR	
	Control (GM495)	Patient (GM1763)	Control (GM495)	Patient (GM1763)
PC/Malate[a]	-	8.49(1)	-	3.24(1)
Malate	9.24	3.82	-	2.29
then PC[b]	14.5 (1)	7.35(1)	3.24(1)	2.94(1)
PC	-	7.55	-	4.0
then Malate	-	6.97(1)		3.43(1)
P CoA[c]	9.5	8.08	2.33	2.94
then Carnitine	9.78(3)	5.33(1)	2.22(2)	2.21(1)
P CoA	10.4	7.44	-	-
then Carnitine	13.4	8.15	1.88	1.74
then Malate	17.5 (1)	11.9 (2)	4.06(1)	5.4 (1)
Carnitine	-	3.57	-	-
then P CoA		8.3 (1)		2.2 (1)
Malate	-	9.23	-	2.45
then Carnitine		9.73		2.14(1)
then P CoA		8.10(1)		
Malate	-	7.56	-	-
then P CoA		10.4		
then Carnitine		7.66(1)		

The values reported are either from a single experiment or the numerical average, with the number of experiments given in brackets ().
a) PC/Malate, palmitoylcarnitine plus malate, added together
b) PC, palmitoylcarnitine
c) P CoA, palmitoyl Coenzyme A

3.3 Enzymatic Analysis in the Study of a Mitochondrial Disease, Cystic Fibrosis

Other investigators have monitored mitochondrial respiration to try to identify defective enzymatic steps in mitochondria. As discussed in the introduction, Shapiro et al (67,68) have suggested that the defect in NADH dehydrogenase shown in fibroblast mitochondria from CF patients is responsible for Cystic Fibrosis. Their latest findings of distinctive enzyme kinetics of NADH dehydrogenase in CF patients (68), have been followed up here as the first step in studying CF as a mitochondrial disease.

3.3.1 Michaelis Constant (Km) of NADH dehydrogenase in CF patient and Control Fibroblasts

3.3.1.1 Pilot Study

The study began by determining the Km(NADH) of NADH dehydrogenase in mitochondrial preparations from 2 pairs of CF and control fibroblasts that were age and sex matched. The activity of the enzyme preparation decreased about 25% over a period of 3-4 hours, so all enzyme assays were performed within 2-3 hours of obtaining the mitochondrial preparation to minimize loss of enzyme activity and the subsequent effect on the results. Therefore, duplicate assays using 5 substrate concentrations were the maximum

number of assays possible.

The initial results shown in Table 7, seemed to support the findings of Shapiro et al (68) in which the Km for the enzyme in CF patients was less than that in heterozygous individuals which, in turn, was less than that in controls. As shown in Table 7, the Km(NADH) for the enzyme from CF cell strains was lower than that of controls at each passage tested for both CF/control pairs studied. If this trend of lower Km(NADH) for the enzyme was characteristic of CF fibroblasts as Shapiro et al suggested (68) then CF and control fibroblast mitochondria should be distinguishable by this Km value alone. This was examined by determining the Km in four CF/control pairs whose identity had been masked.

The individual Km determinations in the pilot study varied considerably from each other within a cell strain, and from those values reported by Shapiro et al (68). These authors reported that the Km(NADH) for this enzyme in CF patients is 10.9-16.1 μM NADH, in heterozygotes, 20.9-26.3 μM NADH, and in controls, 31.8-42.8 μM NADH (with 3 exceptions in the control group 21.5, 23.7, 22.4 μM NADH). Therefore, before continuing the study, normal values and the effect of passage on Km value were established for two control lines (WP0025S01, WP0027S01) by T. Trottershaw and these are shown in Table 8.

For this work she had used a Unicam spectrophotometer and the reproducibility of the experiments was improved.

It was equipped with several temperature controlled cuvette holders which allowed for triplicate assays using 6 substrate concentrations to be done in about 2.5 hours. Rate determinations were more accurate with this spectrophotometer because the scale could be expanded 5 fold. She found that the mean Km(NADH) values $\pm$ S.D. for the enzyme preparation from two control cell lines were 22.09 $\pm$ 7.3 μM NADH, (n=6) and 20.45 $\pm$ 8.6 μM NADH, (n=6) and these values were close to those reported for heterozygotes by Shapiro *et al* (68).

Table 7 Michaelis Constants for NADH dehydrogenase in CF patients and Controls

Cell Strain[a]	Km(NADH)μM No. of passages 8	9	10	11[b]	$\bar{x}$ ± S.D.(n)[c]
WP0025S01	43.7	104	282	21	112.0 ± 118 (4)
GM4320(CF)	40.3	41.3	42.7	16	35.1 ± 12.8(4)
WP0027S01	82.5	135	65.8	-	94.4 ± 36.1(3)
GM142(CF)	-	73.7	40.9	-	57.3 ± 23.2(2)

a) Fibroblast strains WP0025S01 and WP0027S01, were obtained locally by biopsy of normal individuals, while fibroblast strains GM4320 and GM142, were obtained through the Human Genetic Mutant Cell Repository (Camden, NJ, USA) and are from CF patients.
b) Enzyme assays of these 2 cell strains at passage 11 were done on a Unicam spectrophotometer. All others were done on a Beckman DB spectrophotometer.
c) The mean Km values in each CF/control pair are not significantly different ($p>0.05$).

Table 8 Michaelis Constants for NADH dehydrogenase in Fibroblasts from Controls

Cell Strain (a)	Passage	Starting material #cells x10^6	Yield (b)	protein conc.[c] of mitochondrial sample	Km(NADH) µM NADH	Vmax (c)
WP0025S01	9	38.0	73.0	25.0	10.7	2.31
	10	22.9	81.8	36.0	19.9	2.59
	11	18.4	88.6	35.0	24.5	2.83
	12	12.8	116.7	18.0	35.4	4.93
	13	13.4	145.4	44.0	22.0	2.11
	14[e]	y)16.0	72.1	6.1	20.7	6.46
		z)14.1	135.5	8.8	21.5	7.77
$\bar{x}$		19.4	101.9	24.0	22.1	4.10
S.D.		8.92	30.4	10.0	7.3	2.3
WP0027S01	8	19.0	122.3	47.0	34.6	2.99
	9	32.6	104.0	23.0	17.9	2.70
	10	49.4	91.7	41.0	16.2	2.16
	11	39.2	86.8	29.0	15.7	2.75
	12	27.0	58.5	20.0	31.4	3.83
	13[d]	y)20.7	101.7	26.0	14.1	2.11
		z)20.7	75.0	36.0	13.2	1.23
$\bar{x}$		29.8	91.4	31.0	20.4	2.54
S.D.		11.3	20.8	9.0	8.6	0.82

Summary

Cell Line	Km Range(uM NADH)	No. of exp.	Km (NADH) $\bar{x} \pm$ S.D.
WP0025S01	10.69-35.39	6	22.1 ± 7.3
WP0027S01	14.11-34.63	6	20.4 ± 8.6

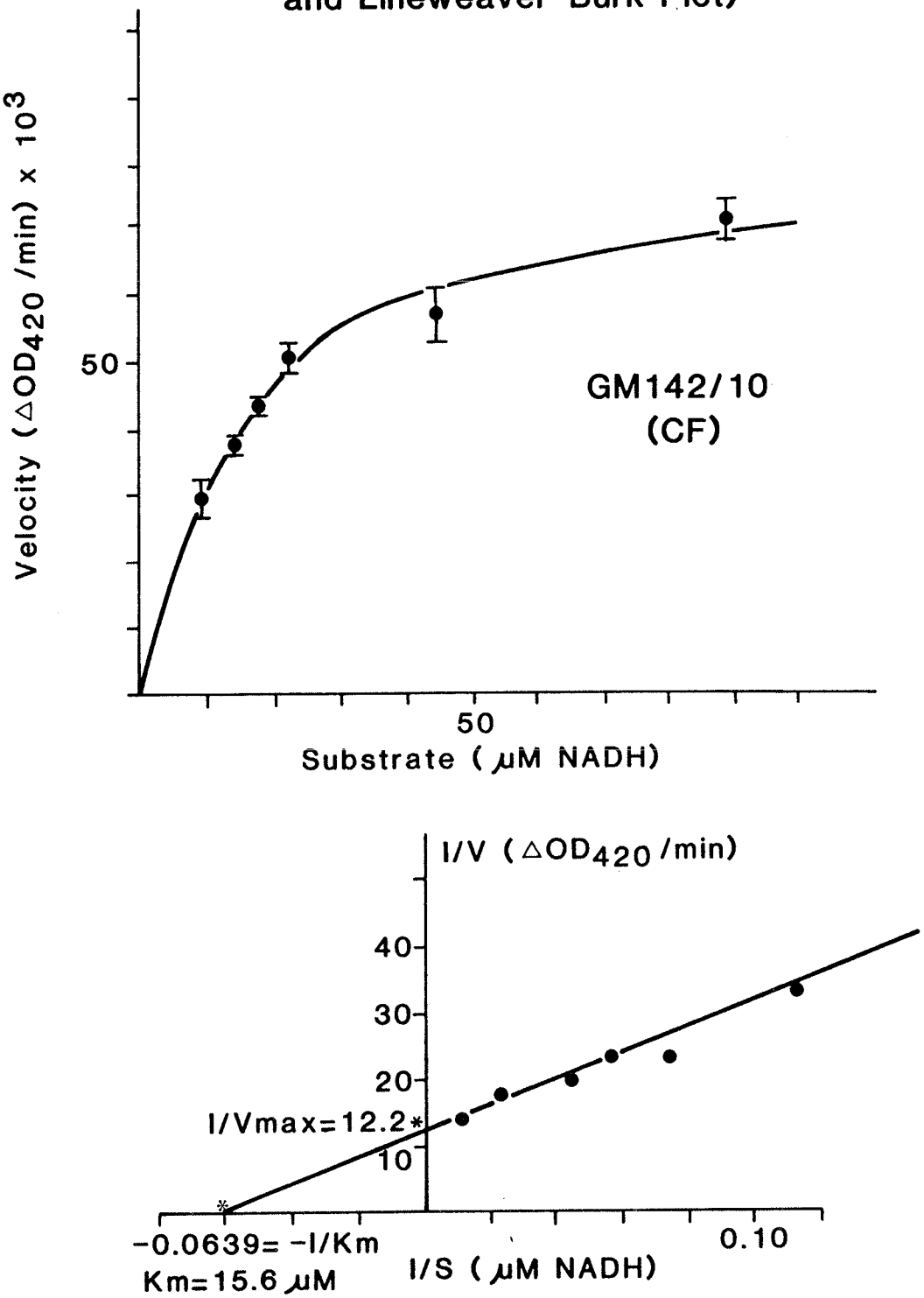
a) Cell strains were obtained from local biopsies.
b) Expressed as µg mitochondrial protein/mg starting cellular protein.
c) Expressed as µg/ 100 ul.
d) Expressed as Δ O.D./min/mg mitochondrial protein.
e) Cell suspension was divided after harvesting and 2 separate preparations (y&z) were made and analyzed.

3.3.1.2 Blind Study

This work was followed by a study in which 4 more CF/control fibroblast pairs were analyzed for differences in Km(NADH) for NADH dehydrogenase in a blind study. As predicted by Shapiro et al (68), the Km should be characteristic of CF and should distinguish CF mitochondria from controls. In this study the Km was determined at three successive passages for each cell strain. The strains were analyzed in CF/control pairs that had been coded to mask their identity. Assays were done in triplicate for 6 substrate concentrations (see Figure 6a) and the results are shown in Table 9. This study indicates that CF and control fibroblasts could not be distinguished on the basis of the Km(NADH) for NADH dehydrogenase, shown in Table 10, as the mean Km for the enzyme of CF cell strains was not statistically different from controls ($p > 0.05$, Student's t test).

Figure 6a

Determination of Km (NADH) for NADH dehydrogenase
(Velocity vs substrate concentration
and Lineweaver-Burk Plot)



Legend next page

Figure 6a Determination of Km(NADH) for NADH dehydrogenase
(Velocity vs. Substrate concentration
and Lineweaver-Burk Plot)

The Km(NADH) for NADH dehydrogenase was determined in mitochondria from the fibroblast strain GM142/10. These fibroblasts were derived from a 14 year old male CF patient.

I Velocity vs. Substrate concentration

The reaction rate (ΔO.D.$_{420}$/min) was determined at least 3 times for each substrate concentration. The mean of these reaction rates has been plotted and the S.E. illustrated by the bars (I) shown.

The units for the velocity of the reaction have been amplified 1,000 fold for this graph.

II Lineweaver-Burk Plot

A double reciprocal plot of the data from I(above) is shown here and the Km(NADH) for NADH dehydrogenase has been determined. The best fitting line was determined by least-squares analysis and the intercepts, 1/Vmax and -1/Km, are marked (*).

Table 9 Properties of NADH dehydrogenase in CF and Control Fibroblast Mitochondria

Parameter	Pair No.[a]	Fibroblast Strain CF	Control
Km $\bar{x} \pm$ S.D.(n)	1	13.2 ± 3.85(3)	17.2 ± 0.07(3)
	2	17.5 ± 4.29(3)	10.8 ± 5.2 (2)
	3	19.2 ± 8.91(3)	16.9 ± 9.67(3)
	4	13.3 ± 0.07(2)	13.2 ± 7.14(2)
Overall Mean ± S.D.(n)		15.8 ± 3.02(4)	14.52 ± 3.07(4)
Apparent Vmax[b] $\bar{x} \pm$ S.D.(n)	1	4.86 ± 2.58(3)	18.3 ±15.1 (3)
	2	6.74 ± 2.32(3)	9.41 ± 8.32(2)
	3	6.12 ± 1.71(3)	7.27 ± 3.68(3)
	4	4.84 ± 0.84(2)	6.30 ± 0.84(2)
Overall Mean ± S.D.(n)		5.64 ± 0.094(4)	10.3 ± 5.47(4)
Yield[c] $\bar{x} \pm$ S.D.(n)	1	51.1 ± 24.2 (3)	37.4 ± 50.2(2)
	2	111.3 ± 7.09(3)	53.7 ± 51.7(3)
	3	98.8 ± 25.7(3)	61.0 ± 29.3(3)
	4	154.5 ± 57.3(2)	61.3 ± 9.7(2)
Overall Mean ± S.D.(n)		103.9 ± 42.5(4)	53.4 ± 11.2(4)

a) Pair No. refers to the CF/control pair studied, ie.pair 1 is A_1/B_1. The strain numbers are given in Table 10.
b) Expressed as Δ OD/min./mg mitochondrial protein.
c) Expressed as mg mitochondrial protein/g starting cellular protein.

Table 10 Michaelis Constants for NADH dehydrogenase in CF and Control Fibroblasts, Blind Study

Cell Strain	Km(NADH), μM NADH[a]				
	Week 1	Week 2	Week 3	Mean (S.D.,n)[d]	Mean (S.D.)[b] (weighted)
A_1(GM142)	12.9	9.5	17.2	13.2 (3.9, 3)[c]	11.8 (2.6)
B_1(WP0027)	17.9	17.1	16.5	17.2 (0.7, 3)	14.2 (1.8)
A_2(GM1864)	14.5	-	7.14	10.8 (5.2, 2)	9.7 (3.8)
B_2(GM768)	15.4	22.4	14.6	17.5 (4.3, 3)[c]	14.7 (2.7)
A_3(GM2987)	12.1	10.5	28.0	16.9 (9.7, 3)	14.1 (3.1)
B_3(GM4320)	10.6	18.5	28.4	19.2 (8.9, 3)[c]	16.5 (3.7)
A_4(GM4339)	-	13.8	12.8	13.3 (0.7, 2)[c]	10.5 (1.1)
B_4(GM3652)	18.2	-	8.1	13.2 (7.1, 2)	13.6 (6.9)

a) Km's were determined using a Lineweaver-Burk plot (see Figure 6a). Triplicate assays for each of 6 concentrations were performed to determine each Km shown. The best fitting line was determined by least squares analysis for these Km determinations, except where noted (b).
b) The best fitting line was also determined by a modified least squares analysis (2a) for the same data. This analysis statistically weights the data points so that those points determined at the lowest substrate consentrations (where experimental error is greatest) are given less weight. Only the mean Km(NADH) for each cell strain is shown.
c) Denotes CF cell line
d) Mean Km for this enzyme in each of the pair of CF/control strains are not significantly different, $p>0.05$, Student's t test.

The Km's were not given at some passages since those fibroblast cultures were contaminated with fungus.

3.3.2 Substrate Accessibility Study

Reasons for discrepancies between Shapiro's results and those shown here will be discussed. One of these reasons, substrate accessibilty, was explored in more detail and the results are given here.

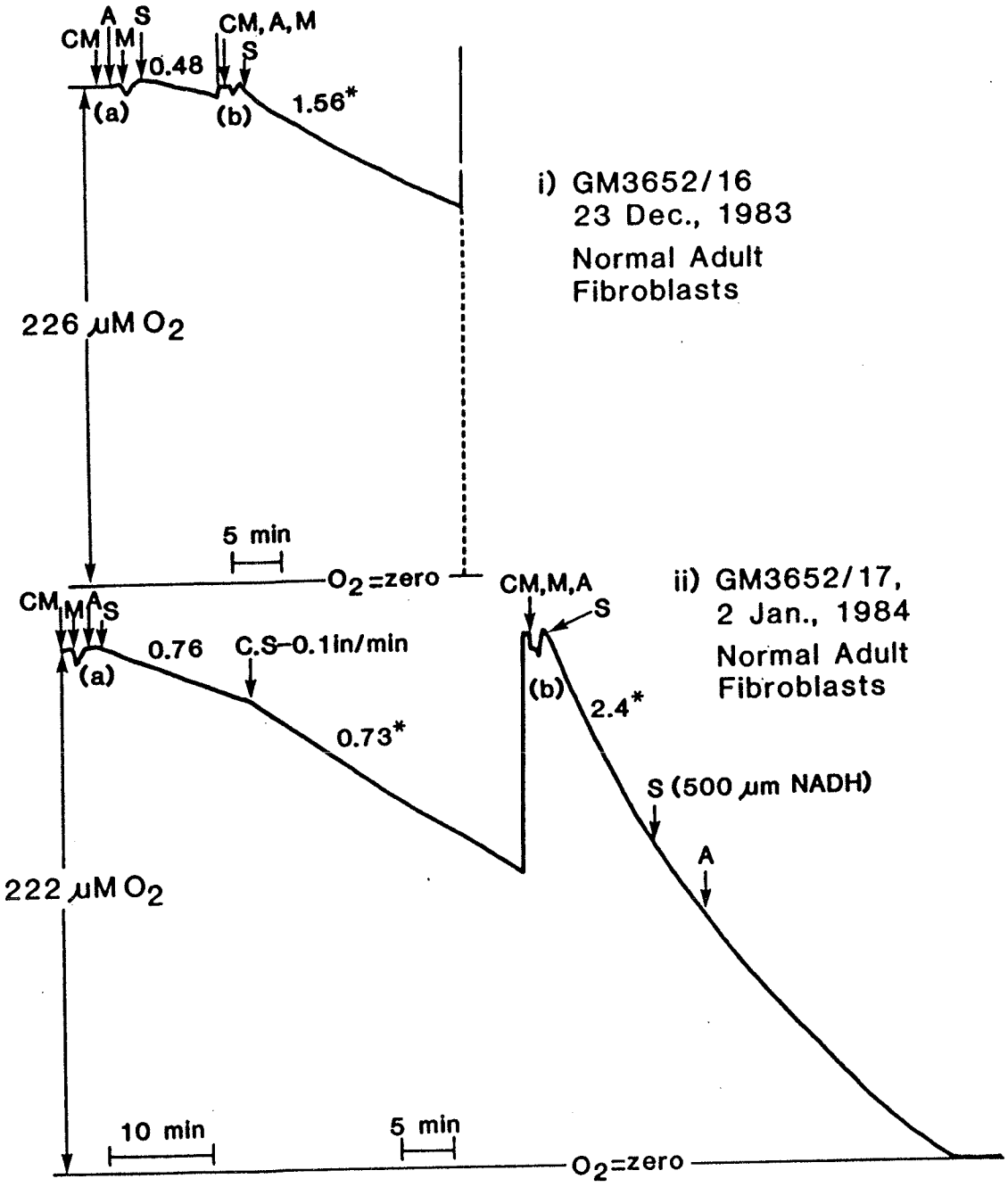
Respiration is started at the inner face of the mitochondrial membrane (38), by the transfer of electrons from NADH to FMN (flavin mononucleotide, a part of NADH dehydrogenase,(81g)). Since the inner mitochondrial membrane is impermeable to NADH (81h), variability in mitochondrial disruption could lead to a difference in NADH availability to the enzyme. Subtle changes in the membranes of CF mitochondria might result in a difference in the mitochondrial membrane disruption leading to a reduced substrate availability which may be misconstrued as reduced enzyme affinity for the substrate (ie. higher Km).

Table 11 NADH Accessibility to NADH dehydrogenase of Isolated Fibroblast Mitochondria

Method of Isolating Mitochondria	Method of Disruption to allow NADH access	Km (NADH) μM NADH A[a]	Km (NADH) μM NADH B	O_2 rate div/min. A	O_2 rate div/min. B
Shapiro (68) et al	Freeze-thawing(FT)	24.2	21.6	-	-
Shapiro (68)	Hypotonic lysis	14.4	17.0	-	-
Method A	Sonication	-	-	0.48	1.56
Method A	Sonication	-	-	0.73	2.4
Method A	Sonication	29.4	20.1	-	-
Shapiro (68)	Sonication	35.1	21.6	-	-

a) "A" refers to the half of the mitochondrial preparation that was not treated to disrupt the mitochondria while "B" refers to the half that was treated.

Figure 7



Legend next page

Figure 7 NADH Oxidation in Non-sonicated and Sonicated Mitochondria

Two preparations were made:
i) from GM3652 fibroblasts, passage 16

ii) from GM3652 fibroblasts, passage 17

* Numbers along the curve are respiration rates and are expressed in divisions per minute as concentration of mitochondrial protein could not be determined in these experiments.

C.S. chart speed

Additions to the cuvette:

CM) cuvette medium

M) mitochondria
a) Non-sonicated
b) Sonicated

A) ADP

S) substrate (250 μM NADH, except where noted)

Three methods of disruption, freeze-thawing, osmotic lysis and sonication, were examined (Table 11). When mitochondria were isolated according to the method of Shapiro (68), half of the mitochondria were frozen and thawed while the remainder were kept in 0.03 M phosphate buffer on ice until assayed. Freeze-thawing (FT) did not have any effect on Km since Km(NADH) with FT was 21.55 μM NADH, and Km(NADH) with no FT was 24.2 μM NADH.

It was possible that the hypotonic suspending solution (0.03 M phosphate buffer), might disrupt the CF and control mitochondria differentially and therefore affect the Km. This was examined by putting half the isolated mitochondria in 0.03 M phosphate buffer (hypotonic) and half in 0.12 M phosphate buffer (iso/hypertonic). The Km did not seem to be affected by storing the mitochondria in hypotonic buffers as the Km(NADH) was 17.0 μM in hypotonic medium and 14.4 μM NADH in iso/hypertonic medium.

Both freeze-thawing and hypotonic lysis did not affect the Km, suggesting that NADH may be fully acccessible to the mitochondria. However, if they were not fully accessible, sonication should lower the Km. After sonication, more NADH would be available to the enzyme when added to the reaction mixture at a given concentration. Also, respiration would be stimulated following sonication since more complete disruption of the mitochondria would allow the substrate access to the enzyme. This hypothesis was tested by following the effect of sonication on both Km

and oxidation of NADH as measured polarographically. A Bronwill sonicator was used at 60 cycles for three 10 s bursts with 10 s cooling on ice in between. Sonication increased the oxidation rate of NADH three fold in mitochondria isolated from fibroblasts by Method A in two separate preparations (see Figure 7 and Table 11). The small amount of mitochondria available from this method of preparation prohibited a Km assay and oxygraph analysis to be done on the same preparation. Subsequent Km determinations were done on mitochondria isolated by Method A and Shapiro's method (Table 11), and in both cases, the sonicated mitochondria had a lower Km value. The Km(NADH) for mitochondria isolated by Method A was 29.4 μM NADH (unsonicated-US) and 20.1 μM NADH (sonicated-S) and for mitochondria isolated by Shapiro's method the Km's were 35.1 μM NADH (US), and 21.6 μM NADH (S). This dependence of Km on membrane disruption may explain the discrepancies between the results of Shapiro (55) and our own findings of Km's for the CF genotypes and will be discussed.

3.4 Comparison of Lowry and BioRad Protein Assays

Most investigators determine mitochondrial protein using the Lowry Assay (49). For those mitochondrial preparations used in NADH dehydrogenase Km determinations, the protein concentration was so low that too large a sample would be required for a Lowry protein assay. This was not practical and therefore the microassay developed by

BioRad Laboratories, which is 10 times more sensitive, was used. This is possible since the mitochondrial protein can be solubilized from membranes by NaOH hydrolysis. The two assays were compared for the determination of mitochondrial protein and the results are given in Table 12.

Table 12 Comparison of Protein Assays[a]

Sample	Lowry(ug/ul)	BioRad(ug/ul)	Lowry/BioRad
1	7.76	5.96	1.30
2	9.23	6.37	1.45
3	10.80	7.27	1.49
4	9.79	7.34	1.33
5	9.71	6.82	1.42
		$\bar{x}$ =	1.40

a) Five mitochondrial samples were assayed by both the Lowry and BioRad Assays as described in Materials and Methods. The ratio of the value for protein concentration obtained with the Lowry over those values from the BioRad is given in the final column and represents a correction factor.

Although the Lowry (49) and BioRad (section 2.4.1.2) assays give different results, they can be related by using a correction factor of 1.4. As shown in Table 12 the value for the protein concentration determined by the Lowry assay was 1.4 times that obtained by the BioRad assay of the same mitochondrial sample. The Lowry assay was used to determine protein for oxidative phosphorylation assays included in these results, since most published data (19,20,59,60,67) on oxidative phosphorylation in mitochondria use the Lowry method for protein determination which is then calculated into the O_2 rates reported (μmoles O_2/min/g mitochondrial protein). However, the Lowry assay was not practical for determining protein in the mitochondrial samples used in NADH dehydrogenase assays and the BioRad assay was used to determine protein for this group of experiments. The correction factor was not applied to the results of the NADH dehydrogenase studies because it did not affect the overall conclusions. For example, the primary result, Km(NADH), did not contain a protein concentration value in its calculation. Also, the apparent Vmax values reported in Table 9 could be compared between CF and control without using a protein assay correction factor, provided the same protein assay (BioRad or Lowry) was used throughout the experiments.

4. Discussion

4.1 Oxidative Phosphorylation Assay of Fibroblast Mitochondria

4.1.1 Method Development

Once the system for measuring oxidative phosphorylation of fibroblast mitochondria was established, work began on developing an improved method for the isolation of mitochondria. The method described by Millis and Pious (56) gave good results but required a lot of starting material (40 mg cellular protein). Haworth-Hatherell (35), developed a micro-method in our laboratory which required twenty fold less starting material than the method of Millis and Pious (56). However the results were unsatisfactory as RCR's and O_2 rates were low. Method A, described here, is a compromise between the two. From approximately one-half the starting material that Millis and Pious used (56), mitochondria that were intact and well coupled could be isolated in high yield.

There are a number of alterations to the original micro-method which improved the preparation. The first was to use a larger cuvette and electrode. The size of the capillary opening (10-15 µl) is 10% of the total reaction volume in a 150 µl cuvette versus 2% and less significant in the 600 µl cuvette. Back diffusion of oxygen through this port may occur and slow down the State 3 rate

resulting in a decrease in the RCR, as shown in Table 3. The electrode used in the 150 µl cuvette has a smaller tip at which the O_2 concentration is measured. This means that it samples a smaller amount of cuvette medium in order to make its O_2 determination and therefore may be less sensitive than the larger Clarke type electrode that is used with the larger cuvettes. Thus by using a larger cuvette (600µl) and electrode the parameters measured showed significant improvement and were more comparable to those obtained by established methods (85).

The mitochondrial isolation procedure was also changed to obtain a better preparation. The starting material was increased from 6 million to 15-20 million cells (approximately 20 mg protein), and some of the steps of the protocol were deleted. The original micro-method involved scraping the cells from 150 mm tissue culture plates in Suspending Solution dispersing the cells with a Pasteur pipette and then breaking the cells open by digesting with Nagarse protease and followed by homogenization. At this step BSA was added to help prevent mitochondrial damage and DNAase to decrease the viscosity of the final mitochondrial suspension. Both the BSA and the DNAase additions were deleted from the protocol in this study with no apparent effect. Following cell breakage, unbroken cells or debris were removed by centrifugation. The mitochondria in the supernatant were pelleted, resuspended and BSA added as a protectant in the final step.

To examine the effect of homogenization and protease digestion, four methods, A to D, were studied. It appears from the data in Table 4 that mitochondria were released from cells by simple trituration with a Pasteur pipette. As well as being partially ruptured when scraped off the plate, forceful expulsion of the cell suspension against the plate surface can open the cells. Harms et al (33a) have reported that fibroblasts can be lysed by a very similar procedure in which a cell supension in isotonic sucrose buffer is drawn into and then expelled from a 10 ml glass pipette. Further homogenization and protease digestion did not improve the yield or the quality of the mitochondria. In fact, the combination of homogenization and protease digestion was detrimental to the quality of the preparation perhaps because of damage of the mitochondria by these "cell disruption" procedures.

Method A was the simplest and fastest of these methods of preparation. It yielded intact and coupled mitochondria (RCR's, 4.86 $\pm$ 1.05, n=5, $\bar{x} \pm$ S.D.) with normal oxidative phosphorylation activity (14.1 $\pm$ 5.99 μmoles O_2/min/mg). All methods gave sufficient yield that 2 runs could be done on a single mitochondrial preparation starting with 12 million cells or 20 mg cellular protein.

Method A has been used a number of times to isolate mitochondria for oxidative phosphorylation assays. During the analysis of a CPT deficient patient's fibroblast mitochondria the RCR's of 2.5-3.0 were lower than previous

results (Table 4), but since that time, mitochondria have been isolated successfully with RCR's as high as 7.0 (data not shown).

A micromethod is important when fibroblasts are used as the tissue source because culturing is costly. Furthermore, culturing in plates yields less material than roller bottles but it is simpler and takes less space, and some fibroblast lines do not grow well and cannot be cultured in large quantities. This method is an improvement over that reported by Millis and Pious (56) who started with twice the amount of cellular material and obtained RCR's of 3.4-4.0 and O_2 rates of 13.2-22.4 μmoles O_2/min/g). For the study of mitochondrial diseases, this was the method I used to isolate mitochondria.

4.1.2 Use of the Oxidative Phosphorylation Assay

4.1.2.1 Luft's Disease

It has been shown that skeletal muscle mitochondria from a patient with Luft's Disease are loosely coupled (24) but it was not known if this defect was also expressed in fibroblasts. If it was, further biochemical studies could be done using this renewable tissue source to study the exact mechanism responsible for the disease.

Studies were carried out on fibroblasts from the patient described by Haydar et al (36). It has been shown (Table 5) that unlike mitochondria obtained from the

patient's skeletal muscle, mitochondria isolated from these fibroblasts were coupled. These mitochondria exhibited respiratory control (RCR's, 1.5 to 3.58) and respiration was decreased in the presence of oligomycin, an inhibitor of the mitochondrial ATPase. Consequently, H^+ cannot use this inhibited ATPase as a re-entry route into the mitochondria. Once the proton gradient has been established, respiration ceases since the gradient cannot be dissipated. This phenomenon is known as respiratory control. This is evidence that Luft's Disease is not expressed in fibroblast mitochondria.

4.1.2.2 CPT Deficiency

Studies were carried out on fibroblasts from a CPT deficient patient. The patient had recurrent myoglobinuria and CPT deficiency. However, it was not clear whether the CPT deficiency was expressed in the fibroblasts from the patient as the cell repository catalogue did not give any published references. However if this deficiency was present it should be detectable by following respiration with fatty acid substrates. Both the patient (GM1763) and an age and sex matched control (GM495) cell strain were assayed for oxidative phosphorylation activity and the results are given in Table 6. As a control, P/M was a substrate for mitochondria from both strains. They respired with State 3 rates of 16.2 $\pm$ 2.42 μmoles O_2/min/g (4) ($\bar{x} \pm$ S.D.) in the control, and 11.2 $\pm$ 3.5 μmoles

O_2/min/g (10) in the CPT deficient patient. If the patient was deficient in CPT activity, it was predicted that either palmitoylcarnitine, palmitoyl CoA plus carnitine or both would be metabolized more slowly by the patient cell strain than by the control. Malate was a co-substrate so that TCA cycle intermediates were not limiting. When palmitoylcarnitine was the substrate, the State 3 rates of the patient were 52% those of the control cell strains and were not appreciably different from the 70% control rate seen with P/M or palmitoyl CoA plus carnitine as substrates. This would indicate that this cell strain does not express a CPT deficiency.

There are a number of reasons why CPT deficiency was not detected in this cell strain. The only available age and sex matched control strain grew poorly and very few experiments could be done to determine the normal oxidation rate for these substrates. The mitochondrial preparation used in these experiments was within the acceptable range (ie. RCR> 3.0 for NAD^+-linked substrate), but less than optimum (RCR> 5.0). The poorer quality of the preparation may have affected the sensitivity of the assay. In addition, malate acts as a substrate itself and when added in combination with a fatty acid substrate, it may mask small changes in fatty acid oxidation due to CPT deficiency. Finally, CPT deficiency may be a heterogeneous disease (41) as there is some variabilty in the clinical manifestations of the disease. Although the patient has

the enzyme deficiency, expression of this defect in fibroblasts is not guaranteed and may explain why the defect was not detected in this cell strain (GM1763).

Some experiments could be done to resolve this ambiguity. The first is to use a fibroblast cell strain in which the CPT deficiency has been determined by specific enzyme analysis, and then to polarographically screen the fibroblast mitochondria from the same cell strain for CPT deficiency. Should the polarographic screen confirm the enzyme analysis, this CPT deficient cell strain (GM1763) could then be retested with an increased number of experiments on both control and patient lines. Statistical analysis of these results may be able to identify a defect in CPT I or II. However, GM 1763 was the only commercially available cell strain listed as having CPT deficiency and we did not have access to any other CPT deficient strains.

4.2 Enzymatic Analysis in the Study of a Mitochondrial Disease, Cystic Fibrosis

4.2.1 Michaelis Constant (Km) of NADH dehydrogenase in CF patients and Control Fibroblasts

4.2.1.1 Pilot Study

Respiration and enzyme analysis of CF fibroblasts by Shapiro *et al* (67,68), revealed that mitochondria may be the site of the expressed primary defect in CF. Their

hypothesis was that a change in the Km for NADH in NADH dehydrogenase was responsible for an increased calcium uptake which they had seen in mitochondria from CF patients (67,68). They explained that it was this increased cellular calcium that could affect both the viscosity of secretions and the secretion process in CF patients (67). These viscous secretions can be found in the affected organs of CF patients (78) and probably contribute to the cardinal signs of the disease, meconium ileus (viscous obstruction of the bowels at birth), pulmonary complications and pancreatic insufficiency (78). The work of Shapiro and Feigal (30,31,67,68) on fibroblast mitochondria was relevant to these studies because of the possible involvement of mitochondria in causing CF.

Specific enzymatic analysis was applied to the study of this mitochondrial disease because the potential defect had been identified. The latest results of Shapiro et al (68) were examined before further characterization of this "mutant" protein could be carried out.

A pilot study was set up and the initial results were encouraging. For two CF/control pairs, the Km(NADH) for NADH dehydrogenase was always lower in mitochondria from CF fibroblasts. Variation in Km values was reduced when we gained expertise in doing the assay and we changed to equipment that allowed for more repetitions of rate determinations. The mean Km(NADH) $\pm$ S.E. for enzyme preparations from two control cell lines were 22.09 $\pm$ 2.76

μM NADH (n=6) and 20.45 ± 3.31 μM NADH (n=6). This was within the range of values for heterozygous individuals reported by Shapiro et al (68).

4.2.1.2 Blind Study

The preparation of the mitochondrial enzyme suspension may be subject to bias as many steps in the protcol involved hand manipulation of the cell suspension, for example Dounce homogenization or dispersion of cells with a Pasteur pipette. More vigourous treatment of one cell suspension over another may damage the mitochondria and affect the availability of the substrate, NADH. Therefore, in a blind study in which 4 pairs of CF/control cell strains had been coded, the Km(NADH) for NADH dehydrogenase was determined. The mean Km was determined for each cell strain and was compared to its counterpart. Data for pair 4 (A_4,B_4) were included in Table 10 but direct comparison of the Km's for same day preparations was not possible on 2 of 3 occasions. In one week, the A_4 fibroblast culture had been lost to fungal contamination while in the second week, B_4 was contaminated.

The Km's for these 6 different cell strains (pairs 1 to 3) do not differ significantly and in only one pair, the CF cell strain had a lower mean Km than the control line. Data were difficult to obtain for the blind study, as experiments and analysis were laborious. Perhaps subtle differences in Km's would be detectable if more CF/control

pairs were analyzed. From this study it was concluded that the CF and control lines could not be distinguished on the basis of Km(NADH) for NADH dehydrogenase and that this enzyme is not the site of the primary defect.

4.2.3 Reasons for the Discrepancies

There are some probable reasons for discrepancies between the data obtained from the blind study and those reported by Shapiro et al (68). The Km(NADH) for the enzyme is an apparent Km since the enzyme has not been purified and Km determinations are subject to a variable environment. This is especially true for the Km(NADH) of NADH dehydrogenase since this enzyme is on the matrix side of the inner mitochondrial membrane (38, 81g), and is inaccessible to its substrate, NADH (81h). Thus, if the membranes are intact, the Km will be dependent on the availabilty of NADH. It follows that differential rupture and resealing of mitochondrial membranes, might account for different Km's between CF and control samples. Membrane rupture and resealing is difficult to control and therefore it may not be possible to reproduce the findings of Shapiro et al (68).

To examine this possibility, the effect of mitochondrial disruption on Km was determined. Neither freeze-thawing nor osmotic lysis affected the Km (Table 11), a fact which suggests that the substrate was fully accessible to the enzyme. It was shown polarographically

that NADH was impermeable to the fully intact, coupled mitochondria isolated by Method A. These mitochondria respired very slowly in the presence of this substrate, and upon sonication respiration increased 3 fold (Table 11, Figure 7). This indicated that NADH was now accessible to the first enzyme of the respiratory chain. Increased substrate accessibility also decreased the Km by one-third whether the mitochondria were isolated by Method A or by the method of Shapiro et al (68); this supports the hypothesis that this Km is dependent on NADH accessibility. Therefore, if the enzyme environment generated in Shapiro's laboratory could not be reproduced by us, substrate accessiblity and therefore Km determinations would not be the same in both laboratories.

There are other reasons which may also explain these discrepancies. It has been proposed (70a) that CF may be a heterogeneous disease in which additional genes may modify the expression of the CF gene. This would help to explain the different manifestations of the disease (onset, severity)(70a). Therefore if there is more than one gene that can affect the CF phenotype, the discrepancies in the results could be understood. The cell strains used in the experiments of Shapiro et al (68), were derived from a select group of patients who attend a clinic in Minneapolis (68) and may have a common CF characteristic. The cell strains used in these studies were obtained from a commercial cell repository and could not be selected based

on clinical signs. Therefore, the CF genotype with altered enzyme kinetics seen in studies reported by Shapiro's group (30,31,67,68) may be a sub-population of CF patients.

Finally, it might be that there is no difference the in NADH dehydrogenase of CF patients and controls. One group(82) has published an abstract confirming the results of Shapiro et al (68), while another group (63a) has failed to confirm the pH profile of NADH dehydrogenase reported by Shapiro et al (67). The evidence presented here indicates that this enzyme is not the primary defect in Cystic Fibrosis.

4.3 Conclusions

The focus of this project has been the study of mitochondrial diseases that may be expressed in fibroblasts. Potential defects were screened by an oxidative phosphorylation assay. This was followed by specific enzyme analysis to determine the nature of the suspected defect.

This approach has been applied to the study of three mitochondrial diseases. Luft's Disease, the prototype mitochondrial disease, did not express its well known defect of uncoupled mitochondria in fibroblasts. Therefore, further studies of this disease in fibroblasts were unwarranted. The oxidative phosphorylation assay was also applied to the study of a CPT deficient fibroblast

strain. The work presented here indicates that there was no CPT deficiency in this cell strain. However, the possibility remains that this assay is not sensitive enough to detect a small CPT deficiency without statistical analysis of a large amount of data. Finally, specific enzyme analysis was applied to the study of Cystic Fibrosis, possibly a "new" mitochondrial disease. Shapiro, Feigal and co-workers (30,31,67,68) have reported that a defect in the NADH dehydrogenase of the electron transport chain may be responsible for CF. This study was expanded in our laboratory by attempting to verify their latest findings of distinctive enzyme kinetics for CF fibroblasts (68). In a blind study, NADH dehydrogenase of CF and control fibroblast mitochondria could not be distinguished by Michaelis constants as predicted by Shapiro et al (68). Therefore, this enzyme is probably not the site of the primary defect in CF.

The general method described here has potential use in the study of mitochondrial diseases. Others, such as Stumpf et al (75,76) have demonstrated its effectiveness in locating a potential defect in Friedreich's Disease and Shapiro et al have studied cellular respiration, polarographically (67) in CF fibroblast mitochondria. Since we now have a good micro-method for the isolation of fibroblast mitochondria, any mitochondrial disease that may be expressed incultured fibroblasts could be screened using the oxidative phosphorylation assay. Diseases so

identified could then be studied in more detail at the molecular level with this renewable tissue source.

BIBLIOGRAPHY

1. Adachi, M., L. Schneck, J. Cara, B.W. Volk. Spongy degeneration of the central nervous system (Van Bogaert and Bertrand type: Canavan's Disease): A review. Human Pathol. 4: 331-347 (1973).

2. Adams, R.L.P.. Cell culture for biochemists. In Laboratory techniques in biochemistry and molecular biology. Edited by T.S. Work, R.H. Burdon. Elsevier/North Holland Biomedical Press, New York (1980).

2a. Barns, J.E., A.J. Waring. Pocket programmable calculators in Biochemistry. John Wiley and Sons. New York (1980). p.204-206

3. Barron, S.A., R.R. Heffner, R. Zwirecki. A familial mitochondrial myopathy with central defect in neural transmission. Arch. Neurol. 36:553-556 (1979).

4. Bethlem, J.. The classification of myopathies with abnormal mitochondria. In Mitochondria and musclar diseases. Edited by H.F.M. Busch, F.G.I. Jennekens, H.R. Scholte. Mefar b.v., Beetsterzwaag, The Netherlands, p. 147-149 (1981).

5. Bradford, M.M.. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. Anal. Biochem. 72:248-254 (1976).

6. Brand, M.D. Stoichiometry of charge and proton translocation in mitochondria: Steady-state measurement of charge/O and P/O ratios. Biochem Soc. Trans. 583rd meeting, 7:874-880 (1979).

7. Bressler, R. Carnitine and the twins. New Eng. J. Med. 282:745-746 (1970).

8. Britton, D.E., J.M. Pellock, R.M. Eiben. Acute hemiplegia of childhood, lactate-pyruvate acidemia, and mitochondrial disorder. Ann. Neurol. 2:265 (1977).

9. Bottacchi, E., S. Di Donato. Skeletal muscle $NAD^+(P)$ and $NADP^+$-dependent malic enzyme in Friedreich's ataxia. Neurology 33:712-716 (1983).

10. Brucher, J.M., S. Tassin, G.F. Walter, H.R.Scholte, T. de Barsy. Myopathic carnitine deficiency. Clinical, morphological and biochemical findings in three cases. In Mitochondria and musclar diseases. Edited by H.F.M. Busch, F.G.I. Jennekens, H.R. Scholte. Mefar b.v., Beetsterzwaag, The Netherlands, p.199-205 (1981).

11. Capaldi, R.A. Arrangement of proteins in the mitochondrial inner membrane. Biochem. Biophys. Acta 694:291-306 (1982).

12. Challa, V.R., W.R. Markesbery, R.J. Baumann, J.A. Noonan. Lactic acidosis associated with cerebellar vermal atrophy and cardiomyopathy. Neuropaditrie 9:277-284 (1978).

13. Chance, B., G.R. Williams. A simple and rapid assay of acid phosphorylation. Nature 175:1120-1121 (1955).

14. Chance, B., G.R. Williams. The respiratory chain and oxidative phosphorylation. Adv. Enzymol. 17:65-134 (1956).

15. Crosby, T.W., S.M. Chou. "Ragged-red" fibers in Leigh's disease. Neurology 24:49-54 (1974).

16. Data for Biochemical Research. Second edition. Edited by R.M.C. Dawson, D.C. Elliot, W.H. Elliot, K.M. James. Oxford University Press, Oxford, G.B.,1969, p.170.

17. Davis P.B., P.A. Di Sant'Agnese. A review. Cystic fibrosis at forty - Quo Vadis? Pediatr. Res. 14:83-97 (1980).

18. De Vivo, D.C.. Reye syndrome: A metabolic response to an acute mitochondrial insult? Neurology 28:105-108 (1978).

19. Di Donato, S., F. Cornelio, L. Pacini, D. Pelluchetti, M. Rimoldi, S. Sprecifico. Muscle carnitine palmityltransferase deficiency: A case with enzyme deficiency in cultured fibroblasts. Ann. Neurol. 4:465-467 (1978).

20. Di Donato, S., M. Rimoldi, A. Moise, B. Bertagnoglio, G. Uziel. Fatal ataxic encephalopathy and carnitine acetyltransferase deficiency: A functional defect in pyruvate oxidation? Neurology 29:1578-1583 (1979).

21. Di Donato, S., A. Castiglione, M. Rimoldi, F. Cornelio, F. Vendemia, G. Cardace, B. Bertagnolio. Heterogeneity of carnitine-palmitoyltransferase deficiency. J. Neurol. Sci. 50:207-215 (1981).

22. Di Mauro, S., D.L. Schotland, C.P. Lee, E. Bonilla, H. Conn, Jr.. Biochemical and ultrastructural studies of mitochondria in Luft's Disease: Implications for "mitochondrial myopathies". Trans. Am. Neuro. Ass. 97:265-267 (1972).

23. Di Mauro, S., P.M. Di Mauro. Muscle carnitine palmityltransferase deficiency and myoglobinuria. Science 182:929-931 (1973).

24. Di Mauro, S., E. Bonilla, C.P. Lee, D.L. Schotland, A. Scarpa, H. Conn, Jr., B. Chance. Luft's Disease: Further biochemical and ultrastructural studies of skeletal muscle in the second case. J. Neurol. Sci. 27:217-232 (1976).

25. Dobkin, B.H., M.A. Verity. Familial progressive bulbar and spinal muscular atrophy. Neurology 26:754-763 (1976).

26. Engel, W.K., N.A. Vick, C.J. Glueck, R.I. Levy. A skeletal muscle disorder associated with intermittent symptoms and a possible defect of lipid metabolism. N.E.J.M. 282:697-704 (1970).

27. Ernster, L., D. Ikkos, R. Luft. Enzymatic activities of human skeletal muscle mitochondria: A tool in clinical metabolic research. Nature 184:1851-1854 (1959).

28. Ernster, L., R. Luft. Further studies on a population of human skeletal muscle mitochondria lacking resiratory control. Exp. Cell Res. 32:26-35 (1963).

29. Estabrook, R.W.. Mitochondrial respiratory control and the polarographic measurement of ADP:O ratios. In Methods in enzymology. Edited by R.W. Estabrook, M.E. Pullman. Vol. 10, Academic Press, New York, USA, p.41-47 (1967).

30. Feigal, R.J., B.L. Shapiro. Altered intracellular calcium in fibroblasts from patients with Cystic Fibrosis and heterozygotes. Pediat. Res. 13:764-768 (1979).

31. Feigal, R.J., B.L. Shapiro. Mitochondrial calcium uptake and oxygen consumption in Cystic Fibrosis. Nature 278:276-277 (1979).

32. Forstner, J.F., G.G. Forstner. Effects of calcium on intestinal mucin: Implications for Cystic Fibrosis. Pediat. Res. 10:609-613 (1976).

32a. Freund, J.E.. Modern Elementary Statistics. 6th Edition.
Prentice-Hall Inc., Englewood, N.J., USA, 1984.

33. Hammersen, F., A. Gidlof, J. Larsson, D.H. Lewis. The occurrence of paracrystalline mitochondrial inclusions in normal human skeletal muscle.
Acta Neuropathol. 49:35-41 (1980).

33a. Harms, E., H. Kern, J.A. Schneider. Human lysosomes can be purified from diploid skin fibroblasts by free-flow electrophoresis.
Proc. Natl. Acad. Sci. 77:6139-6143 (1980).

34. Hart, Z.H., C.H. Chang, E.V.D. Perrin, J.S. Neerunjun, R. Ayyar. Familial poliodystrophy, mitochondrial myopathy, and lactate acidemia.
Arch. Neurol. 34:180-185 (1977).

35. Haworth-Hatherell, E. -personal communication, 1982.

36. Haydar, N.A., H.L. Conn, A. Affi, N. Wakid, S. Ballas, K. Fawaz. Severe hypermetabolism with primary abnormality of skeletal muscle mitochondria.
Ann. Intern. Med. 74:548-558 (1971).

37. Hinkle, P.C., R.E. McCarty. How cells make ATP.
Sci. Amer. 238(3):104-123 (1978).

38. Hinkle, P.C.. The maximum P/O ratio of mitochondrial oxidative phosphorylation is 2.
Fed. Proc. 38:776 (1979).

39. Hoppel, C.. Carnitine and carnitine palmitoyltransferase in fatty acid oxidation and ketosis.
Fed. Proc. 41(12):2853-2857 (1982).

40. Hudgson, P., W.G. Bradley, M. Jenkison. Familial "mitochondrial" myopathy. A myopathy associated with disordered oxidative metabolism in muscle fibers. Part 1. Clinical, electrophysiological and pathological findings.
J. Neurol. Sci. 16:343-370 (1972).

41. Jennekens, F.G.I., H.R. Scholte, J.T. Stinis, I.E.M. Luyt-Houwen. Carnitine palmitoyltransferase deficiency: Variations in clinical expression, differences between CPT I and II and mode of inheritance.
In Mitochondria and musclar diseases. Edited by H.F.M. Busch, F.G.I. Jennekens, H.R. Scholte.
Mefar b.v., Beetsterzwaag, The Netherlands, p. 213-218 (1981).

42. Kamieniecka, Z., H. Schmalbruch. Neuromuscular disorders with abnormal muscle mitochondria.
Int. Rev. Cytol. 65:321-357 (1980).

43. Kobayashi, S., B. Hagihara, M. Masuzumi, K. Okunuki. Preparation and properties of mitochondria from mammalian cells cultured in vitro.
Biochim. Biophys. Acta 113:421-437 (1966).

44. Krooth, R.S., E.K. Sell. The action of mendelian genes in human diploid cell strains.
J. Cell Physiol. 76:311-330 (1970).

45. Laak, H.J.T., H.H.J. Jaspar, A.M. Stadhouders, L.A.K. Bastiaensen, E.M.G. Joosten. Chronic progressive opthalmoplegia (CPEO) and mitochondrial myopathy.
In Mitochondria and musclar diseases. Edited by H.F.M. Busch, F.G.I. Jennekens, H.R. Scholte.
Mefar b.v., Beetsterzwaag, The Netherlands, p. 187-186 (1981).

46. Land, J.M., J.B. Clark. Mitochondrial myopathies.
Biochem. Soc. Trans. 7:231-245 (1979).

47. Layzer, R.B., R.J. Havel, M.B. McIlroy. Partial deficiency of carnitine palmityltransferase: Physiologic and biochemical consequences.
Neurology 30:627-633 (1980).

48. Lessler, M.A., G.P. Brierly. Oxygen electrode measurements in biochemical analysis.
Methods of Biochem. analysis 17:1-29 (1969).

49. Lowry, O.H., N.J. Rosenbrough, A.L. Farr, R.J. Randall. Protein measurement with the Folin phenol reagent.
J. Biol. Chem. 193:265-275 (1951).

50. Luft, R., D. Ikkos, G. Palmieri, L. Ernster, B. Afzelius. A case of severe hypermetabolism of nonthyroid origin with a defect in the maintenance of mitochondrial respiratory control: A correlated clinical, biochemical, and morphological study. J. Clin. Invest. 41:1776-1804 (1962).

51. Markesbery, W.R.. Lactic acidemia, mitochondrial myopathy, and basal ganglia calcification. Neurology 29:1057-1061 (1979).

52. Martin, J.J., J. Flament-Durand, J.P. Farriaux, N.Buyssens, P. Ketelbant-Balasse, C. Jansen. Menkes kinky hair disease. A report on its pathology. Acta Neuropath. 42:25-32 (1978).

53. Martin, J.J.. Generalized mitochondrial disturbances and myopathies: Concluding remarks. In Mitochondria and musclar diseases. Edited by H.F.M. Busch, F.G.I. Jennekens, H.R. Scholte. Mefar b.v., Beetsterzwaag, The Netherlands, p. 219-223 (1981).

54. McIntosh, J.C.. Application of a dye-binding method to the determination of protein in urine and cerebrospinal fluid. Clin. Chem. 23:1939-1940 (1977).

55. McLeod, J.G., W.D.C. Baker, C.D. Shorey, C.B. Kerr. Mitochondrial myopathy with multisystem abnormalities and normal ocular movements. J. Neurol. Sci. 24:39-52 (1975).

56. Millis, A.J.T., D.A. Pious. Oxidative phosphorylation in mitochondria isolated from human fibroblasts. Biochim. Biophys. Acta 292:73-77 (1973).

57. Mitchell, P.. Coupling of phosphorylation to electron and hydrogen transfer by a chemiosmotic type of mechanism. Nature 191:144-148 (1961).

57a. Mitochondria and musclar diseases. Edited by H.F.M. Busch, F.G.I. Jennekens, H.R. Scholte. Mefar b.v., Beetsterzwaag, The Netherlands, (1981).

58. Montpetit, V.J.A., F. Andermann, S. Carpenter, J.S. Fawcett, D. Zborowska-Sluis, H.R. Giberson. Subacute necrotizing encephalomyelopathy. A review and a study of two families. Brain 94:1-30 (1971).

59. Palmucci, L., A. Berttolotto, D. Cavicchioli, G. Monga, D. Schiffer. Sporadic oculopharyngeal myopathy with abnormal mitochondria.
Acta Neurol. Belg. 78:373-382 (1978).

60. Pande, S.V., M.C. Blanchaer. Reversible inhibition of mitochondrial adenosine diphosphate phosphorylation by long chain acyl coenzyme A esters.
J. Biol. Chem. 246:402-411 (1971).

61. Pierce, J., C.H. Suelter. An evaluation of the Coomassie Brilliant Blue G-250 dye binding method for quantitative protein determination.
Anal. Biochem. 81:478-480 (1977).

62. Pula, T.P., S.R. Max, H.R. Zielke, M. Chacon, P. Baab, M. Gumbinas, W.D. Reed. Selective carnitine palmitoyltransferase deficiency in fibroblasts from a patient with muscle CPT deficiency.
Ann. Neurol. 10:196-198 (1981).

63. Samaha, F.J.. Mitochondrial myopathies: Emergence of new clinical entities.
In Mol. Asp. Med. 2:325-335 (1979).

63a. Sanguinetti-Briceno, N.R., D.J.H. Brock. NADH dehydrogenase in Cystic Fibrosis.
Clin. Genet. 22:308-311 (1982).

64. Scholte, H.R., F.G.I. Jennekens, J.J.B.J. Bouvy. Carnitine palmitoyltransferase II deficiency with normal carnitne palmitoyltransferase I in skeletal muscle and leucocytes.
J.Neurol. Sci. 40:39-51 (1979).

65. Shah, A.J., V. Sahgal, G. Muschler, V. Subramani, H. Singh. Morphogenesis of the mitochondrial alterations in muscle diseases.
J. Neurol. Sci. 55:25-37 (1982).

66. Shapira, Y., S.D. Cedrbaum, P.A. Cancilla, D. Nielsen, B.M. Lippe. Familial poliodystrophy mitochondrial myopathy and lactate acidemia.
Neurology 25:614-621 (1975).

67. Shapiro, B.L., R.J. Feigal, L.F.H. Lam. Mitochondrial NADH dehydrogenase in Cystis Fibrosis.
Proc. Natl. Acad. Sci., USA 76:2979-2983 (1979).

68. Shapiro, B.L., L.F.H.Lam, R.J. Feigal. Mitochondrial NADH dehydrogenase in Cystic Fibrosis: Enzyme kinetics in cultured fibroblasts.
Am. J. Hu. Genet. 34:846-852 (1982).

69. Sheu, K.F.R., C.W.C. Hu, M.F. Utter. Pyruvate dehydrogenase complex activity in normal and deficient fibroblasts.
J. Clin. Invest. 67:1463-1471 (1981).

70. Siegal, J.M., G.A. Montgomery, R.M. Bock. Ultraviolet absorbtion spectra of DPN and analogs of DPN.
Arch. Biochem. Biophys. 82:288-299 (1959).

70a. Sing, C.F., D.R. Risser, W.F. Howatt, R.P. Erikson. Phenotypic heterogeneity in Cystic Fibrosis.
Am. J. Med. Genet. 13:179-195 (1982).

71. Sorbi, S., J.P. Blass. Abnormal activation of pyruvate dehydrogenase in Leigh disease fibroblasts.
Neurol 32:555-558 (1982).

72. Spiro, A.J., C.L. Moore, J.W. Prineas, P.M. Strasberg, I. Rapin. A cytochrome-related inherited disorder of the nervous system and muscle.
Arch. Neurol. 23:103-112 (1970).

73. Stephens, N.L., K. Wrogemann. Oxidative phosphorylation in smooth muscle.
Am. J. Physiol. 219:1796-1801 (1970).

74. Stryer, L.. Biochemistry.2nd Edition.
W.H. Freeman and Company, San Fransico. (1981).

74a. ibid. p.307

74b. ibid. p.321

74c. ibid. p.325

74d. ibid. p.311-318

75. Stumpf, D.A., J.K. Parks, L.A. Eguren, R. Haas. Friedreich's Ataxia III. Mitochondrial malic enzyme deficiency.
Neurology 32:221-227 (1982).

76. Stumpf, D.A., J.K. Parks, W.D. Parker. Friedreich's disease IV: Reduced mitochondrial malic enzyme activity in heterozygotes.
Neurology 33:780-783 (1983).

77. The Merck Manual of Diagnosis and Therapy. 13th Edition. Editors R. Berkow, J.H. Talbott.
Merck, Sharp, and Dohme Reasearch Laboratories, Rahway, NJ,USA (1978).

78. Talamo, R.C., B.J. Rosenstein, R.W. Berninger. Cystic Fibrosis.
In The metabolic basis of inherited disease. Fifth edition. Edited by J.B. Stanbury, J.B. Wyngaarden, D.S. Fredrickson, J.L. Goldstein, M.S. Brown.
McGraw-Hill Book Company, New York, USA, p.1889-1917 (1983).

79. Trijbels, J.M.F., L.A.H. Monnens, J.A.J.M. Bakkeren, J.L. Willems, R.C.A. Sengers. Mitochondrial abnormalities in the cerebro-hepato-renal syndrome of Zellweger.
In Mitochondria and muscular diseases. Edited by H.F.M. Busch, F.G.I. Jennekens, H.R.Scholte.
Mefar b.v., Beetsterzwaag, The Netherlands, p. 187-190 (1981).

80. Tsairis, P., K.W. Engel, P.Kark. Familial myoclonic epilepsy syndrome associated with skeletal muscle mitochondrial abnormalities.
Neurology 23:408 (1973).

81. Tzagoloff, A.. Mitochondria.
Plenum Press, New York (1982).

81a. ibid. p.40

81b. ibid. p.131-135

81c. ibid. p.328

81d. ibid. p.107

81e. ibid. p.112

81f. ibid. p.137

81g. ibid. p.63

81h. ibid. p.219

81i. ibid. p.99

82. von Reuker, A., R. Bauer, Y.S. Shin, W. Enders, R. Bertele, K. Harms. Cystic Fibrosis - A mitochondrial defect? Abstr.
In 18th Workshop for Pediatric Research.
Eur. J. Pediatr. 138:94 (1982).

83. Walter, G.F., J.M. Brucher, S. Tassin, J. Bergmaans. Experimental changes in muscle mitochondria induced by electric stimulation and inhibition of energy metabolism.
In Mitochondria and muscular diseases. Edited by H.F.M. Busch, F.G.I. Jennekens, H.R.Scholte. Mefar b.v., Beetsterzwaag, The Netherlands, p. 107-111 (1981).

84. Wrogemann, K., M.C. Blanchaer. Oxidative Phosphorylation by muscle mitochondria of dystrophic mice.
Can. J. Biochem. 45:323-329 (1968).

85. Wrogemann, K., M.C. Blanchaer. Respiration and oxidative phosphorylation by muscle and heart mitochondria of hamsters with hereditary myocardiopathy and polymyopathy.
Can. J. Biochem. 46:323-329 (1968).

Appendix 1 Composition of Media and Trypsin Solutions

Media	Composition	Source	Quantity required for 10 l
Human McCoy's FC-10	Human McCoy's 5a Medium -HSC-modified -without $NaHCO_3$ -Formula#78-5265	Gibco	1 package
	FBS-Fetal Bovine Serum	Gibco, Flow, or M.A.Bio-products	1 l.
	$NaHCO_3$	Fisher	22 g.
	100x Antibiotics-	see below	100 ml.
MEM FC-10 package	MEM-Eagle's modified -with Earle's salts -with glutamine -without $NaHCO_3$	Flow labs	1
	FBS	Gibco, Flow or M.A.Bio-products	1 l.
	$NaHCO_3$	Fisher	22 g.
	100x Antibiotics	see below	100 ml.
100x Anti-	Streptomycin	Allen &	10 g.
biotics	Sulphate	Hanbury's	per 1.
	Penicillin G	Glaxo	$10x10^6$
I.U./l.			
			per 5 l
0.05%Trypsin	Trypsin	ICN	2.5 g.
	Dextrose	Fisher	5.0g.
	$NaHCO_3$	Fisher	0.65g.
	100x Antibiotics	as before	50ml.
	Saline D concentrate	see below	250 ml.
			per 1.
Saline D concentrate	NaCl	Fisher	160.0 g
	KCl	Fisher	8.0 g.
	$Na_2HPO_4.7H_2O$	Fisher	0.9g.
	KH_2PO_4	Analar-BDH	0.6g.
	Phenol Red	stock	20 ml.