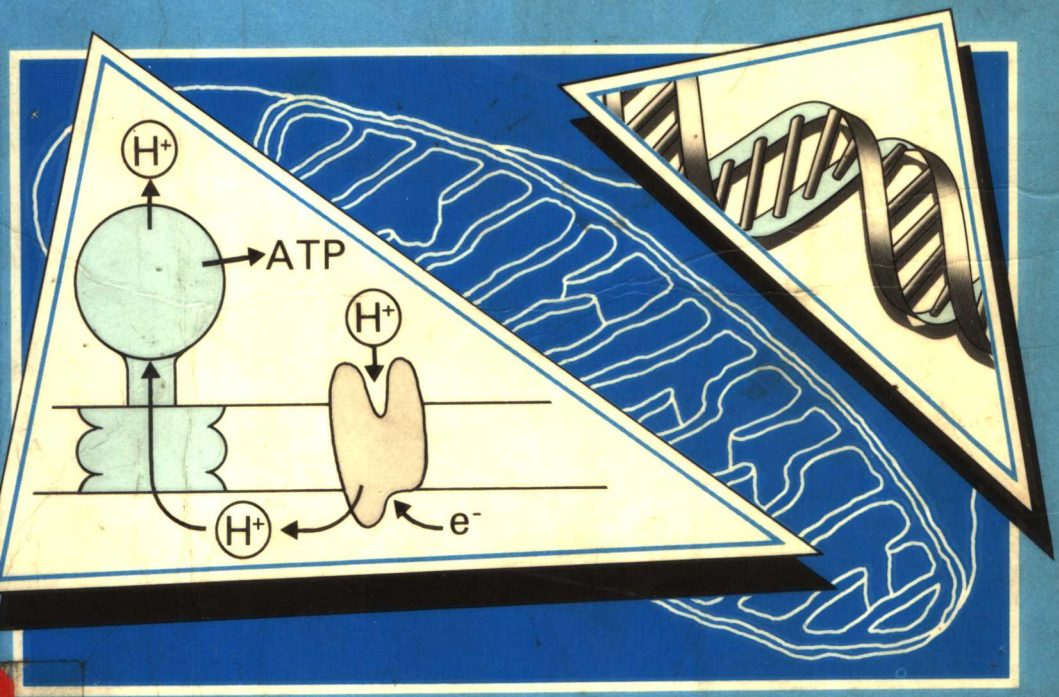
Mitochondria

a practical approach

Edited by
V M Darley-Usmar, D Rickwood & M T Wilson



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Preface

Studies of the mitochondrion have always been at the heart of biochemistry and its associated areas of research. This is hardly surprising considering the functions that this organelle performs, functions which are central to the life of most eukaryotic cells. Although we have learned a great deal about mitochondria over the last decades, the field of mitochondrial research remains a fertile one and there is no doubt there is still much to learn using the new techniques that are now available.

This book is intended to help all those people who wish to do experiments using either mitochondria or systems derived from mitochondria. Such research workers may be relative novices to the field, postgraduates or scientists coming into the area of mitochondrial investigations from other disciplines. They may, on the other hand, be researchers ('mitochondriacs' even) who are widely experienced in some aspects of mitochondrial biochemistry but who now wish to broaden the scope of their research interests. Indeed, such shifts of emphasis are quite likely to occur in mitochondrial research as there has grown up, over the years, a dichotomy in the approach to working on this most important of organelles. On the one hand there have been intensive studies of the components of the major metabolic pathways associated with the mitochondrion and on the other a great deal of work has been carried out on the biogenesis of mitochondria in terms of the molecular biology and origin of the various components. The aim of this book is to present in a single volume the most important techniques in both these areas of mitochondrial research.

In the course of compiling these methods some techniques have inevitably been related to mitochondria from a particular species or cell type. However, this should not pose a serious problem in view of the similarity of mitochondrial functions in a wide range of organisms. What we have tried to do is to give a representative method for all the important investigative procedures.

Unfortunately, constraints of space have made it necessary for us to leave out some important topics. Probably the foremost of these is a full discussion of the lipid composition of mitochondria. This we have, with regret, relegated to Appendix II.

We thank the contributors to this volume who have not only written in a way we hope and believe is clear and informative, but who have also made our job as editors so much easier than it could otherwise have been.

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Abbreviations

ARS	autonomous replication sequence
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	bovine serum albumin
COV	cytochrome oxidase vesicles
DAPI	4,6-diamidine-2-phenylindole hydrochloride
DBM-paper	diazobenzyloxymethyl paper
DEPC	diethylpyrocarbonate
DMO	5,5′-dimethyloxazolidine-2,4-dione
DTT	dithiothreitol
EGTA	ethyleneglycobis(β-aminoethyl)ether tetraacetic acid
ETC	1-ethyl-3(-3-[^{14}C]trimethylaminopropyl) carbodiimide
FAME	fatty acid methyl ester
FITC	fluorescein isothiocyanate
HEDTA	*N*-hydroxyethyl ethylenediamine triacetic acid
HRP	horseradish peroxidase
MM	minimal medium
MNNG	*N*′-methyl-*N*′-nitro-*N*′-nitrosoguanidine
Mops	3-(*N*-morpholino) propanesulphonic acid
mtDNA	mitochondrial DNA
mtRNA	mitochondrial RNA
NBT	nitroblue tetrazolium
NM	nutrient medium
NTA	nitrilotriacetate
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCA	perchloric acid
PEG	polyethylene glycol
PVC	polyvinyl chloride
PVP	polyvinyl pyrrolidone
RC	respiratory competent
RCR	respiratory control ratio
RD	respiratory deficient
SDS	sodium dodecylsulphate
SMP	sub-mitochondrial particle
SSC	standard saline citrate
TAME	*N*α-*p*′-tofyl-L-arginine methyl ester
TPMP	triphenylmethylphosphonium
TPP	tetraphenylphosphonium
TMPD	tetramethyl-*p*-phenylenediamine dihydrochloride
Xgal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside

Contents

CHAPTER 1

Isolation and characteristics of intact mitochondria

D.RICKWOOD, M.T.WILSON and V.M.DARLEY-USMAR

1. INTRODUCTION

Eukaryotic cells are extremely diverse, covering the plant and animal kingdoms as well as the fungi. Whether the eukaryotes are single-celled or complex multi-cellular organisms it is remarkable that the structure and functions of mitochondria are so similar. There are four major structural regions, these are the outer and inner membranes and the two spatial regions delineated by these membranes, namely the intermembrane space and the matrix. These structural regions are broadly associated with specific and different functions. *Table 1* lists the major metabolic functions of the mitochondrion, their associated macromolecules and intra-mitochondrial location. In this book we have concentrated on the practical aspects of those functions shown in *Table 1* which form major areas of current research interest. For this reason some topics have not been covered here; for example rather little is known of outer membrane structure and function. In other cases, for example the proteases and proteins which guide the import and processing of those mitochondrial proteins coded for and synthesized in the cytosol, their presence has been implied but, except in a few cases (e.g. ref. 1), they have not been isolated. For a more detailed theoretical discussion of mitochondrial function the reader is referred to a text that reviews many aspects of mitochondria (2).

Differences between mitochondria from different species and between different tissues in the same organism reflect, in the most part, the relative importance of the different metabolic pathways contained within the mitochondrion to a given cell or organism. Morphologically and genetically all mitochondria appear to be strikingly similar. This fact has allowed researchers to generalize both methods of preparation and analysis for mitochondria from a wide variety of sources. Deeper analysis invariably reveals important and intriguing differences between mitochondria from different sources, some of which are of considerable significance to the metabolism of the organism. A case in point is the way in which mitochondria from brown adipose tissue have been modified specifically to generate heat (3).

In order to assess any of the properties of mitochondria it is important to be able to purify them free of contaminants that may interfere with the activity of interest. However, the 'purity' of mitochondria depends on the area of interest and this also determines the method of assay used to determine the purity. For example, if one is interested in electron transport processes then usually one is not interested in whether or not the mitochondria are contaminated with nuclear DNA, while such contamina-

Table 1. Compartments of the mitochondrion and their associated metabolic functions.

Outer membrane	
Function	*Associated enzyme(s)*
Oxidation of neuroactive aromatic amines	Monoamine oxidase (4)
Cardiolipin biosynthesis	e.g. glycerol phosphate acyl transferase (5)
Transport of nuclear coded and cytoplasmically synthesized proteins	Not yet identified (6)
Electron transfer	NADH cytochrome *c* reductase (7) (rotenone-insensitive). The function of this protein is not yet fully defined.
Intermembrane space	
Function	*Associated enzymes*
Maintenance of adenine nucleotide balance	Adenylate kinase Nucleoside diphosphokinase Nucleoside monophosphokinase (8)
Electron transfer from complex III to complex IV of the respiratory chain	Cytochrome *c* (see Chapter 5)
Processing of proteins imported from cytoplasm	Not yet isolated (see Chapter 9)
Inner membrane	
Function	*Associated macromolecules*
Oxidative phosphorylation	The phospholipid bilayer which is essential to maintain the proton gradient.
	Four electron transfer complexes, three of which couple electron transfer to formation of a proton gradient, and a proton-driven ATP synthetase (see Chapters 4,5).
Transport of pyridine nucleotides	ADP/ATP translocase (see Chapter 3)
Ca^{2+} ion transport	e.g. Ca^{2+} ATPase (see Chapter 3)
Transport of metabolites	Pyruvate carrier, $H_2PO_4^-/OH^-$ antiport, dicarboxylate carriers, citrate/malate antiport, carnitine shuttle
Matrix	
Function	*Associated macromolecules*
Oxidation of pyruvate to acetyl CoA	The pyruvate dehydrogenase complex (9)
Oxidation of ketone bodies	e.g. 3-ketoacid CoA transferase (10)
Oxidation of amino acids	e.g. glutaminase, glutamate dehydrogenase, aspartate aminotransferase, α-ketoglutarate transaminases (11)
Part of the urea cycle	Carbamylphosphate synthetase, ornithine transcarbamylase (12)
Oxidation of fatty acids to acetyl CoA	Fatty acyl-CoA dehydrogenase, enoyl hydratase, β-hydroxyacyl-CoA dehydrogenase, β-ketoacyl-CoA thiolase (13)
Protection against oxidative stress	Superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase (14)
Processing of proteins imported from the cytoplasm	Proteases for specific signal peptides of imported proteins (see Chapter 9)
Inheritance of genes coding for mitochondrial RNA and some proteins	Mitochondrial DNA, DNA polymerase and primase (see Chapters 7 and 8)
Synthesis of 13 membrane components of the proteins of oxidative phosphorylation	Ribosomes and apparatus for transcription and translation (see Chapters 8 and 9)

Table 2. Purification of mitochondria by isopycnic sucrose gradients.

1.	Prepare continuous sucrose gradients from 1–2 M sucrose containing 1 mM EDTA, 0.1% BSA and 10 mM Tris-HCl, pH 7.5. Prepare gradients either by using a simple gradient maker or, more easily, by allowing four solutions of 1.0, 1.3, 1.6 and 2.0 M sucrose buffered and containing EDTA and BSA to diffuse overnight. Cool the gradients to 5°C before use.
2.	Gently but thoroughly resuspend the pellet of crude mitochondria in 0.8 M sucrose buffered and containing EDTA and BSA; it is often convenient to use a loose-fitting Potter homogenizer for this step.
3.	Centrifuge the gradients for 2 h at 80 000 *g* at 5°C. The intact mitochondria form a brown band at about 1.19 g/ml. On occasions brown bands denser and lighter than the intact mitochondria are also found; these represent damaged mitochondria.
4.	It is possible to unload the whole gradient into fractions but usually one removes the band of intact mitochondria using a Pasteur pipette.
5.	Dilute the gradient solution containing the mitochondria by the addition of 2 vols of 1 mM EDTA, 10 mM Tris-HCl, pH 7.4 and pellet the pure mitochondria by centrifugation at 20 000 *g* for 10 min at 5°C.

tion could be prejudicial for studies of the transcription of mitochondrial DNA.

This chapter describes in detail the isolation of mitochondria from different types of cells and the methods that can be used to assess their integrity and purity.

2. ISOLATION OF MITOCHONDRIA

2.1. General principles

The experimental approach for isolating mitochondria is basically the same irrespective of the source of tissue; plant or animal. The first steps involve rupture of the cell membrane while maintaining the structural integrity of the mitochondria. After breaking open the cells, differential centrifugation is used sometimes together with isopycnic density gradients, to separate the mitochondria from other organelles and cell debris. However, the yield of mitochondria and the ease with which pure preparations may be obtained depends very much on the type of tissue and, of course, the amount of tissue available. The exact details of the method chosen will depend not only on the tissue source but also on the type of experiment for which the mitochondria are to be isolated. For example, in some instances, such as the isolation of electron transport complexes, mitochondrial integrity may be sacrificed to obtain high yield. In this chapter we describe experimental protocols for preparing mitochondria from a wide range of organisms; in addition, in the subsequent chapters, the methods most suitable for a specific application are also described.

2.1.1 *Purification of mitochondria by isopycnic centrifugation*

After lysis of the cells, low-speed centrifugation is used to remove unlysed cells, nuclei and large membrane fragments prior to pelleting the mitochondria by centrifugation at 10 000 *g* for 10 min. The 'mitochondrial' pellet in fact contains a wide range of components including lysosomes, peroxisomes and membrane fragments. Although repeated washing of this crude pellet can be used as a method of purifying mitochondria, it is not very efficient either in terms of the final purity or yield. Usually the preferred method of purifying the mitochondria is to use isopycnic centrifugation. Usually one uses a 1–2 M sucrose gradient centrifuged at 80 000 *g* for 2 h at 5°C (*Table 2*). In some instances it may be advantageous to use one of the newer types of gradient