ABSTRACT

Optimal conditions for amino acid incorporation into protein by isolated plant mitochondria were established. This amino acid incorporation was completely dependent upon the intactness of the mitochondrion and coupled oxidative phosphorylation. It also displayed characteristic sensitivity towards inhibitors of respiration and organellar protein synthesis.

Analysis of the labelled products of mitochondrial protein synthesis by one dimensional sodium-lauryl sulphate polyacrylamide tube gel electrophoresis revealed eight distinct polypeptides. However, with the improved resolution obtained by slab gel electrophoresis and subsequent autoradiography, it was possible to discern at least 17 radioactively labelled polypeptides, ranging in molecular weight from 10,500 to 57,000 daltons.

The polypeptides synthesised by mitochondria isolated from artichoke tuber were shown to be very similar to those synthesised by the organelle in vivo.

Polypeptides synthesised by mitochondria isolated from mung bean hypocotyl and artichoke tuber were compared and again found to be similar. Subsequent fractionation of the mitochondria showed that the labelled polypeptides were associated with the mitochondrial membrane system.

An investigation of the solubility in organic solvents of the polypeptides suggested a method of purifying one of the low molecular weight mitochondrially synthesised polypeptides.

The effect of anaerobiosis on mitochondrial protein synthesis in vitro was examined, but no conclusive results were obtained.

A preliminary investigation of the ability of isolated mitochondria to incorporate radioactive uridine into RNA suggested that this system might be of use in the direct characterisation of the products of mitochondrial transcription.
PLANT MITOCHONDRIAL PROTEIN SYNTHESIS

by

PETER A. K. POPE

Thesis presented for the Degree of Doctor of Philosophy
of the University of Edinburgh in the Faculty of Science.

August, 1976
I declare that this thesis has been composed entirely by myself and the experiments and results described herein are a product of my own work.

Peter A. K. Pope.
ABSTRACT

Optimal conditions of amino acid incorporation into protein by isolated plant mitochondria were established. This amino acid incorporation was completely dependent upon the intactness of the mitochondrion and coupled oxidative phosphorylation. It also displayed characteristic sensitivity towards inhibitors of respiration and organellar protein synthesis.

Analysis of the labelled products of mitochondrial protein synthesis by one dimensional sodium lauryl sulphate polyacrylamide tube gel electrophoresis revealed eight distinct polypeptides. However, with the improved resolution obtained by slab gel electrophoresis and subsequent autoradiography, it was possible to discern at least seventeen radioactively labelled polypeptides, ranging in molecular weight from 10,500 to 57,000 daltons.

The polypeptides synthesised by mitochondria isolated from artichoke tuber were shown to be very similar to those synthesised by the organelle in vivo.

Polypeptides synthesised by mitochondria isolated from mung bean hypocotyl and artichoke tuber were compared and again found to be similar. Subsequent fractionation of the mitochondria showed that the labelled polypeptides were associated with the mitochondrial membrane system.

An investigation of the solubility in organic solvents of the polypeptides suggested a method of purifying one of the low molecular weight mitochondrially synthesised polypeptides.

The effect of anaerobiosis on mitochondrial protein synthesis in vitro was examined, but no conclusive results were obtained.

A preliminary investigation of the ability of isolated mitochondria to incorporate radioactive uridine into RNA suggested that this system might be of use in the direct characterisation of the products of mitochondrial transcription.
ACKNOWLEDGMENTS

I thank my supervisor, Dr C. J. Leaver, for his help and advice during the course of this study, and his constructive criticism of my draft thesis.

I would also like to thank the academic staff, technicians and research students of the Department of Botany for useful help and discussion. I am also grateful to Derek Denham for taking the electron micrographs of mitochondria, and Bill Foster for processing the photographs.

My thanks also to Mr T. McGowran for organising the printing of this thesis and to Mr G. Lloyd for setting the print.

Lastly, I am indebted to my wife, Carol, for constant encouragement and great patience.

This research was financed by a post-graduate studentship from the S.R.C.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>adenosine disphosphate.</td>
</tr>
<tr>
<td>ATPase</td>
<td>adenosine triphosphatase.</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CAP</td>
<td>chloramphenicol.</td>
</tr>
<tr>
<td>Cl</td>
<td>Curie (3.7 x 10^10 disintegrations per second).</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute.</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetra-acetic acid.</td>
</tr>
<tr>
<td>E265</td>
<td>extinction at 265nm.</td>
</tr>
<tr>
<td>mA</td>
<td>milliamps.</td>
</tr>
<tr>
<td>mol. wt.</td>
<td>molecular weight.</td>
</tr>
<tr>
<td>mtDNA</td>
<td>mitochondrial DNA.</td>
</tr>
<tr>
<td>mt-rRNA</td>
<td>mitochondrial ribosomal RNA.</td>
</tr>
<tr>
<td>RNAase</td>
<td>ribonuclease.</td>
</tr>
<tr>
<td>SLS</td>
<td>sodium lauryl sulphate.</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid.</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N,N'-tetramethylenediamine.</td>
</tr>
<tr>
<td>Tris</td>
<td>2-amino-2-hydroxymethylpropane-1:3 diol.</td>
</tr>
<tr>
<td>Triton-X-100</td>
<td>octylphenoxypolyethoxy ethanol.</td>
</tr>
</tbody>
</table>
## CONTENTS

### CHAPTER 1: INTRODUCTION

A. INTRODUCTION ........................................ 1

B. THE STRUCTURE AND FUNCTION OF THE MITOCHONDRION ..........1

C. MITOCHONDRIAL DNA ..................................3

   1. Physical and chemical properties of mitochondrial DNA ..........3
   2. Function of mitochondrial DNA ................................4
   3. Mitochondrial DNA replication and transcription .................5

D. MITOCHONDRIAL RIBOSOMES AND RIBOSOMAL RNA ...............5

E. MITOCHONDRIAL PROTEIN SYNTHESIS ........................7

F. CONTROL OF MITOCHONDRIAL DEVELOPMENT ...................10

### CHAPTER 2: MATERIALS AND METHODS

A. MATERIALS ........................................12

   1. Plant material ......................................12
   2. Chemicals and radioisotopes ..............................12
   3. Enzymes and substrates .................................13

B. METHODS ..........................................13

   1. Growth of plant material ................................13
   2. Preparation of plant tissue for mitochondrial isolation .................13
   3. Ageing of the artichoke tissue .............................14
   4. Isolation of mitochondria ................................14
   5. Incorporation of radioactive amino acids by isolated mitochondria ........15
      i. Assay of amino acid incorporation ..............................15
      ii. Analysis of the labelled products of protein synthesis ..............16
      iii. Fractionation of proteins by polyacrylamide gel electrophoresis ...16
      iv. Radioactive analysis of the gels ................................18
   6. Measurement of oxygen uptake ................................19
   7. Protein estimation ......................................19
   8. Estimation of bacterial and fungal contamination of the mitochondrial preparation 19
   9. Analysis of mitochondrial nucleic acids .......................19
      i. Extraction of nucleic acids ..................................19
      ii. Fractionation of RNA by polyacrylamide gel electrophoresis .......20
iii. Buoyant-density analysis of mitochondrial DNA ................................ 21
10. Electron microscopic analysis of mitochondrial preparations .......... 21

CHAPTER 3: PRELIMINARY CHARACTERISATION OF THE ISOLATION MITOCHONDRIA

A. INTRODUCTION ..................................... 23
B. PURITY OF STANDARD PREPARATIONS .......................... 23
  1. Electron microscopy .................................. 23
  2. Buoyant density centrifugation of DNA ...................... 24
  3. Estimation of mitochondrial ribosomal RNA during purification of mitochondria . 24
     i. Mitochondria purified by existing techniques ............... 24
     ii. Mitochondria purified by modified techniques ............. 25
C. BACTERIAL CONTAMINATION .................................. 26
D. PHYSIOLOGICAL INTEGRITY OF MITOCHONDRIAL PREPARATIONS ...... 26
E. THE QUALITY OF THE MITOCHONDRIAL PREPARATIONS .............. 27

CHAPTER 4: CHARACTERISATION OF AMINO ACID INCORPORATION INTO PROTEIN BY ISOLATED MITOCHONDRIA

A. INTRODUCTION ..................................... 28
B. CHARACTERISATION OF THE CONDITIONS OF INCORPORATION OF RADIOACTIVE AMINO ACIDS ..................................... 28
C. THE EFFECT OF TEMPERATURE ON AMINO ACID INCORPORATION BY ISOLATED MITOCHONDRIA ..................................... 30
D. THE EFFECT OF DIFFERENT AMINO ACIDS ON THE FINAL SPECIFIC ACTIVITY OF THE MITOCHONDRIAL PROTEIN .............. 30
E. THE EFFECT OF ADDITION OF A NON-RADIOACTIVE AMINO ACID MIXTURE ON AMINO ACID INCORPORATION BY ISOLATED MITOCHONDRIA ..................................... 30
F. COMPARISON OF THE ABILITY OF MITOCHONDRIA ISOLATED FROM DIFFERENT TISSUES TO INCORPORATE RADIOACTIVE AMINO ACIDS INTO PROTEIN ..................................... 31
G. DISCUSSION ........................................ 32

CHAPTER 5: THE EFFECTS OF INHIBITORS ON AMINO ACID INCORPORATION BY ISOLATED MITOCHONDRIA

A. INTRODUCTION ..................................... 33
CHAPTER 6: CHARACTERISATION OF THE PRODUCTS OF MITOCHONDRIAL PROTEIN SYNTHESIS

A. INTRODUCTION ........................................36

B. ESTIMATION OF THE NUMBER OF POLYPEPTIDES SYNTHESISED BY ISOLATED ARTICHOKE TUBER MITOCHONDRIA ........................................36

C. MOLECULAR WEIGHTS OF THE POLYPEPTIDES SYNTHESISED BY ISOLATED ARTICHOKE MITOCHONDRIA ........................................36

D. INVESTIGATION OF A "TAILING" PHENOMENON ....................37

E. THE EFFECT OF PROTEASE ON THE PRODUCTS OF PROTEIN SYNTHESIS BY MITOCHONDRIA ISOLATED FROM ARTICHOKE TUBER .....................38

F. COMPARISON OF THE PRODUCTS OF PROTEIN SYNTHESIS BY MITOCHONDRIA ISOLATED FROM DIFFERENT TISSUES ........................38

1. A comparison of the products of protein synthesis by mitochondria isolated from mung bean and artichoke tuber ........................................38

2. Comparison of the products of protein synthesis by mitochondria isolated from fresh and aged artichoke tuber .................................39

G. THE IN VIVO PRODUCTS OF MITOCHONDRIAL PROTEIN SYNTHESIS ........39

H. LOCATION OF PROTEINS SYNTHESISED BY MITOCHONDRIA ISOLATED FROM MUNG BEAN ................................................41

I. EXTRACTION WITH ORGANIC SOLVENTS OF RADIOACTIVE PROTEIN FROM MITOCHONDRIA ........................................42
J. THE EFFECT OF TREATING THE LABELLED MITOCHONDRIAL POLYPEPTIDE PRODUCTS WITH SODIUM HYDROXIDE .................................. 43
K. EFFECT OF ANAEROBISIS ON PROTEIN SYNTHESIS BY ISOLATED MITOCHONDRIA .............................................................. 43
L. THE EFFECT OF DIFFERENT INCUBATION MEDIA ON THE PRODUCTS OF ISOLATED MITOCHONDRIAL PROTEIN SYNTHESIS ................ 45
M. DISCUSSION ............................................................................. 46

CHAPTER 7: RNA SYNTHESIS BY ISOLATED MITOCHONDRIA
A. INTRODUCTION ........................................................................ 47
B. CHARACTERISTICS OF MITOCHONDRIAL RNA SYNTHESIS ........ 47
C. THE PRODUCTS OF RNA SYNTHESIS ........................................ 48

CHAPTER 8: DISCUSSION .............................................................. 49

REFERENCES ............................................................................ 59
A. INTRODUCTION

Mitochondria are the sites of cellular respiration in eucaryotic cells and were first described by Altman in 1890. Although visible under the light microscope, the advent of electron microscopy allowed a more detailed description of the internal structure (Palade, 1953; Sjöstrand, 1953).

The development of cell fractionation techniques by differential centrifugation enabled the mitochondria to be studied in isolation. The association of the mitochondrion with catabolic biochemical reactions and with oxidative phosphorylation confirmed the earlier suggestions of its involvement in energy production (Hackett, 1955) and earned it the title of “powerhouse of the cell”. In the 1950’s there were several reports suggesting mitochondria could synthesise proteins (McLean, 1958). The significance of these observations was not appreciated until the 1960’s after it was shown that the organelle contained DNA (Nass and Nass, 1963). This led to the concept of the mitochondrion as a semi-autonomous organelle, not only having a separate genetic system to that of the nucleus, but also the ability to express it independently of cytoplasmic protein synthesis. Over the last 10 years, the degree of autonomy of the organelle has been assessed. It has emerged that the great majority of the mitochondrial proteins are coded by nuclear genes, synthesised on cytoplasmic ribosomes and subsequently integrated into the mitochondrion. Mitochondria are thus similar to chloroplasts in that they contain the macro-molecular components for autonomy but are not autonomous in any meaningful sense (Ellis, 1975).

B. THE STRUCTURE AND FUNCTION OF THE MITOCHONDRION

A recent book on this subject (Munn, 1974) emphasises the diversity of forms adopted by the organelle. However, the basic structure of the mitochondrion of both plants and animals is a continuous, smooth outer membrane, within which is an inner membrane, the latter enclosing an inner space or matrix. The surface area of the inner membrane is increased by invaginations, which in animals and some plants appear plate-like and are referred to as cristae. In some higher plants they appear finger-like and are therefore called microvilli (Opik, 1968).

In electron micrographs, the mitochondria often have a circular or rod-like outline but published electron micrographs show them in almost every conceivable shape; circular (Fuller, 1966), cup-shaped, dumbell (Kessel, 1966) and branched (Munn, 1974). Generally, they are considered to be 0.5-1.0 μm in diameter, and from 1-6 μm long. The size of mitochondria and the number per cell varies according to the tissue under examination, but anything from one (Fuller, 1966) to over a thousand...
have been reported. Also, the number of mitochondria per cell increases as the cell grows, so the number of mitochondria per unit volume does not vary greatly. For example, in maize root cap the initial cells have around 50 mitochondria per cell, whereas the mature cells have about 175 (Clowes and Juniper, 1964). Both the number of mitochondria per cell, and the number of cristae per mitochondrion are considered approximate indicators of that part of the energy requirements of the cell met by aerobic respiration.

In negatively stained preparations of mitochondria, the cristal membranes are characterised by regular arrays of stalked particles on the side facing the matrix (Fernández-Morán, 1963). The particles have a roughly spherical head 7-11nm in diameter subtended by a stalk 4-6nm long.

The light microscope enables one to appreciate the dynamic aspect of mitochondrial structure. Their shape appears to be much more labile than electron micrographs suggest, and the light microscope shows them undergoing movement independent of that caused by cytoplasmic streaming (Honda et al, 1966).

Cytoplasmic streaming in large plant cells distributes mitochondria randomly throughout the cell and prevents any specific spacial relationship forming between the mitochondria and any other cell component. However, in animal cells and a few plant cells, regional specialisation is found, mitochondria being distributed according to localised energy requirements. For example the close association seen between mitochondria and the contractile filaments of muscle cells. Mitochondria are sometimes seen associated with chloroplasts, although the functional significance of this is unclear.

The structure of the isolated organelle as revealed by the electron microscope, differs from its in vivo counterpart in that it is almost invariably spherical. The intracristal space appears to be swollen, the cristae apparently having fused into a net-like arrangement in the interior of the organelle (Baker et al, 1968). The degree of swelling appears to be determined in part by the osmolarity of the suspending medium (Laties and Treffry, 1969).

As previously indicated, the mitochondrion is the centre of cellular energy metabolism, the enzymes of the Kreb's cycle, fatty acid oxidation, the cytochrome electron transport chain, and the system of oxidative phosphorylation are all associated with the organelle. There is compartmentation of these activities within the mitochondrion. The enzymes of fatty acid oxidation and the Kreb's cycle are found in the soluble matrix. The electron transport chain, including succinic dehydrogenase are located in the inner membrane. An NADH dehydrogenase is found on the outer membrane of plant cells (Douce et al, 1973). Most of the other dehydrogenases, e.g. malate and glutamate dehydrogenase are thought to be in the matrix. The stalked particles attached to the inner membrane have been shown to have ATPase activity, which is probably active in ATP synthesis under normal conditions. Not only do the inner and outer membranes display different enzymatic properties, they
also behave very differently osmotically. Only the inner membrane is a permeability barrier to small ions and solutes such as sucrose or mannitol, and can expand and contract in response to changes in concentration of these substances. The outer membrane is permeable to these solutes, while the inner membrane is the site of a variety of specific solute transport systems, for example active calcium uptake, organic acids, amino acids, phosphate and an ADP/ATP exchange.

The majority of evidence points to mitochondria increasing in number by division of pre-existing mitochondria. Division and fusion of mitochondria have been observed in living cells during the pleomorphic shape changes of the organelle. Many electron micrographs contain constricted mitochondrial profiles suggestive of fission or budding of small mitochondria from larger ones (Kessel, 1966). Biochemical evidence for division of mitochondria comes from radioactive tracer experiments. Luck (1963) labelled the mitochondria of a choline-requiring Neurospora mutant with radioactive choline, and during subsequent growth in unlabelled choline found the label distributed over all the mitochondria with a continuous decrease of label per mitochondrion. This result is compatible only with formation of mitochondria by division of pre-existing ones.

The following review of mitochondrial DNA (mtDNA), ribosomes, and protein synthesis, describes results obtained mainly from fungal and animal studies. Reports concerning plant mitochondria are few and have been largely of a confirmatory nature. Part of the reason for this imbalance of information is the greater technical problem posed when fractionating plant cells, i.e. the presence of a tough cell wall and a large vacuole. In addition, plants do not lend themselves to genetic manipulation in the same way that fungi do. However, it is advisable to be cautious in the extrapolation of results obtained with mitochondria from different organisms.

C. MITOCHONDRIAL DNA

1. PHYSICAL AND CHEMICAL PROPERTIES OF MITOCHONDRIAL DNA

The first convincing demonstration of the presence of DNA in mitochondria was by Nass and Nass (1963). Previous claims for its presence had been attributed to nuclear contamination (Stahl et al., 1963). However, this criticism could not be levelled at Nass and Nass who saw fibres in electron micrographs of mitochondria of duck embryo in vivo. The behaviour of these fibres paralleled that of known preparations of DNA under various fixative and staining conditions and in addition the fibres were shown to be sensitive to DNAase. Since this date, DNA has been found in mitochondria from a variety of organisms.

The base composition and therefore the buoyant density of mtDNA is frequently different to that of homologous nuclear DNA (Borst and Kroon, 1969). In a range of higher plants
the density is remarkably constant at around \(1.706-1.707 \text{ g/cm}^3\) (Suyama and Bonner, 1966; Wells and Ingle, 1970; Leaver and Harmey, 1972), in contrast to the nuclear DNA’s which vary in density between 1.691 and 1.702 g/cm\(^3\).

Animal mtDNA is circular, these circles having a contour length of about 5\(\mu\)m. The basic contour length of mtDNA from different animals is found to vary within narrow limits, from 4.5-5.9\(\mu\)m (Borst, 1972). Some of the circles contain superhelical turns, this being a reflection of the circles being closed. Those not containing such “super-twists” are thought to be “nicked”. A small percentage of the circles are larger and appear to be multiples, or oligomers of the basic 5\(\mu\)m circle. Also some of the circles are occasionally seen to be interlocked, as in the links of a chain. Although there were several reports of linear mtDNA in fungi (Schafer et al, 1971) and plants (Wolstenholme and Gross, 1968), later reports using more delicate techniques have shown they also contain circular mtDNA. The contour length of these DNA’s appears to be substantially greater than the 5\(\mu\)m reported for animals and in Pisum sativum, Kolodner and Tewari (1972) found circular mtDNA with a contour length of 30\(\mu\)m. In yeasts a circular mtDNA with a contour length of 25\(\mu\)m has been found (Hollenberg et al, 1970). Table 1.1 shows some characteristics of mtDNA from a range of organisms.

Quantitative renaturation studies of denatured DNA are thought to be indicative of the complexity of DNA. Mitochondrial DNA is found to renature relatively rapidly, suggesting the complexity to be low (Borst, 1972). The 2 to 10 circles of DNA found within one mitochondrion and the mtDNA’s found in different mitochondria of the same organism are considered to be very similar, if not identical. This limits the information content of the animal mtDNA to 5\(\mu\)m of DNA, which is equivalent to around 15,000 base pairs. Further limitations on the coding potential of mtDNA have been suggested by Bultman and Laird (1973). They found a stepwise melting profile of mtDNA isolated from Drosophila melanogaster. The relatively low temperature of the melt suggested a region rich in adenine and thymine (AT), considered to be spacer region, and only the remainder of the DNA (perhaps 70%) coding for meaningful products. Similarly, Bernardi and Drunel (1974) found in yeast mitochondria substantial regions of the mtDNA rich in AT and considered only 50% of the mtDNA to be informational.

2. FUNCTION OF MITOCHONDRIAL DNA

In an attempt to explain cytoplasmic inheritance in micro-organisms, Ephrussi (1953) suggested that the mitochondrion had a genetic function. Certain respiratory mutants and later mutants of mitochondrial protein synthesis, were shown (Linnane et al, 1968) to be inherited in a manner which could not be explained by classical nuclear inheritance. The finding of DNA in the mitochondrion
<table>
<thead>
<tr>
<th>Source of mitochondria</th>
<th>Conformation</th>
<th>Size (μm)</th>
<th>Genetic complexity based on quantitative renaturation experiments</th>
<th>Buoyant density in CsCl (g/cm²)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANIMAL TISSUES</td>
<td>Circular</td>
<td>4.6-5.9</td>
<td>1.0 x 10⁷</td>
<td>1.686-1.711 1.694-1.707</td>
<td>Borst and Flavell (1972)</td>
</tr>
<tr>
<td>PROTOZOA</td>
<td>Linear</td>
<td>15</td>
<td>3.0-4.0 x 10⁷</td>
<td>1.684 1.688</td>
<td>Suyama and Miura (1968) Flavell and Jones (1970)</td>
</tr>
<tr>
<td>FUNGI</td>
<td>Linear</td>
<td>26</td>
<td>6.6 x 10⁷</td>
<td>1.701 1.712</td>
<td>Luck and Reich (1964) Agsterribbe et al (1972)</td>
</tr>
<tr>
<td></td>
<td>Linear</td>
<td>19</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Circular</td>
<td>30</td>
<td>7.4 x 10⁷</td>
<td>1.706 1.698</td>
<td>Kolodner and Tewari (1972)</td>
</tr>
<tr>
<td>HIGHER PLANTS</td>
<td>Linear</td>
<td>19.5</td>
<td>1.0 x 10⁷</td>
<td>1.707 1.693</td>
<td>Wolstenholme and Gross (1968) Suyama and Bonner (1966) Wells and Birnstiel (1969)</td>
</tr>
<tr>
<td>Phaseolus vulgaris</td>
<td>Linear</td>
<td>-</td>
<td>1.4 x 10⁶</td>
<td>1.706 1.706</td>
<td>-</td>
</tr>
<tr>
<td>Lactuca sp.</td>
<td>Linear</td>
<td>25</td>
<td>5 x 10⁷</td>
<td>1.706 1.698</td>
<td>-</td>
</tr>
<tr>
<td>Pisum sativum</td>
<td>Circular</td>
<td>30</td>
<td>7.4 x 10⁷</td>
<td>1.706 1.698</td>
<td>-</td>
</tr>
</tbody>
</table>
suggested a vehicle for this cytoplasmic inheritance. The coding capacity of mtDNA is fairly limited. In animals it could code for about 30 proteins which represents only a small fraction of the total number of different proteins found within the organelle, and suggests that the remainder are coded for by nuclear genes. In fungi and plants, the mtDNA could potentially code for between 150 and 180 proteins. However, as already noted, there are indications that not all the plant or fungal mtDNA is informational so this potential coding figure is reduced considerably.

In *Helia*, *Xenopus* and *Tetrahymena* cells, it has been shown by hybridisation studies that mt-rRNA's are transcribed from mtDNA (Aloni and Attardi, 1971; Dawid, 1970; and Suyama, 1967). Some mitochondrial tRNA's were shown to hybridise with mtDNA in *Helia* (Aloni and Attardi, 1971). These results constitute the most direct evidence of mtDNA function. Although several proteins have been shown to be synthesised on the mitochondrial ribosomes (mt-ribosomes), no proof of where they are coded has been reported. It is possible that they could be coded by nuclear genes and then the mRNA imported into mitochondria and translated by mitochondrial ribosomes.

3. MITOCHONDRIAL DNA REPLICATION AND TRANSCRIPTION

Isolated mitochondria will incorporate radioactively labelled deoxyribonucleotide triphosphates into a product which has been identified as mtDNA (Wintersberger, 1966). A specific mitochondrial DNA polymerase has been purified and partially characterised in yeast (Iwashima and Rabinowitz, 1969; Wintersberger and Wintersberger, 1970), and in rat liver (Meyer and Simpson, 1968).

The synthesis of mtRNA by isolated mitochondria has been reported by several workers (see Borst, 1972), although the product has been poorly characterised. Kuriyama and Luck (1973) demonstrated the synthesis of a high molecular weight rRNA which is subsequently processed into the two stable mt-rRNA's in mitochondria of *Neurospora*. Using the same organism, Klintzel and Schafer (1971) have purified a mitochondrial RNA polymerase with a strong preference for *Neurospora* mtDNA as template.

D. MITOCHONDRIAL RIBOSOMES AND RIBOSOMAL RNA

The cytoplasm of plants and animals contain ribosomes which sediment at approximately 80S and contain two high molecular weight ribosomal RNA's (25-28S, 1.3-1.75 x 10^6 mol. wt. and 18S, 0.7 x 10^6). The ribosomes of blue green algae, bacteria and chloroplasts on the other hand sediment at approximately 70S and contain 23S (1.1 x 10^6) and 16S (0.56 x 10^6) rRNA's.

It has become apparent that mt-ribosomes cannot be characterised as being either of the 70S or the 80S type; their sedimentation coefficients range from 55-60S in animal mitochondria, and from 70-80S in fungi and higher plants.
Table 1.2 gives the sedimentation characteristics of mt-ribosomes and their component rRNA's from a variety of organisms. It is apparent that the progressive decrease in size of the mtDNA from higher plants to animals is reflected in the size of the ribosomes which in animals are the smallest ever reported.

**TABLE 1.2**

Sedimentation characteristics (S values) of mitochondrial ribosomes and their RNA components compared with *Escherichia coli*

<table>
<thead>
<tr>
<th>Organism</th>
<th>Mitochondrial ribosomes</th>
<th>Mitochondrial RNA's</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ANIMALS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Xenopus laevis</em></td>
<td>60</td>
<td>21/13</td>
<td>Dawid (1970)</td>
</tr>
<tr>
<td><em>HeLa cells</em></td>
<td>60</td>
<td>16/12</td>
<td>Attardi et al (1970)</td>
</tr>
<tr>
<td><em>Rat (liver)</em></td>
<td>55</td>
<td>16/13</td>
<td>O'Brien and Kalf (1967)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ashwell and Work (1970)</td>
</tr>
<tr>
<td><strong>FUNGI</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Neurospora crassa</em></td>
<td>73</td>
<td>23/16</td>
<td>Kuntzel and Noll (1967)</td>
</tr>
<tr>
<td><em>Aspergillus nidulans</em></td>
<td>67</td>
<td>23.5/15.5</td>
<td>Edelman et al (1970)</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>74+1</td>
<td>21-22/14-15</td>
<td>Stegeman et al (1970)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Grivell et al (1971)</td>
</tr>
<tr>
<td><strong>PROTOZOA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Tetrahymena</em></td>
<td>&quot;80&quot;</td>
<td>21/14</td>
<td>Chi and Suyama (1970)</td>
</tr>
<tr>
<td><em>Euglena gracilis</em></td>
<td>&quot;71&quot;</td>
<td>21/16</td>
<td>Avadhani and Buetow (1972)</td>
</tr>
<tr>
<td><strong>HIGHER PLANTS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Brassica rapa L.</em></td>
<td>&quot;78&quot;</td>
<td>24/18.5</td>
<td>Leaver and Harmey (1972)</td>
</tr>
<tr>
<td><em>Zea mays L.</em></td>
<td>&quot;78&quot;</td>
<td>24-25/18-19</td>
<td>Pring (1973)</td>
</tr>
</tbody>
</table>

A number of unusual physical properties of the mt-rRNA's have in the past led to difficulties in characterising the precise size of these molecules. The low percentage guanine plus cytosine content (from 26% in yeasts to 44% in some animals) of mt-rRNA contrasts with most bacterial and cytoplasmic rRNA, and may account for their secondary structure being very sensitive to ionic strength and temperature (Groot et al, 1970). The molecular weights of the mt-rRNA's of animals have been calculated to be $0.3-0.36$ and $0.35-0.56 \times 10^6$ daltons. This contrasts with the substantially higher
values reported for fungi, 0.63-0.72 and 1.23-1.28 x 10^6 daltons, or plants 0.69-0.78 and 1.12-1.18 x 10^6 daltons (Leaver and Harmey, 1972; Pring, 1974).

The SS rRNA (ca 40,000 molecular weight) normally associated with the large sub-unit of ribosomes from bacteria, chloroplasts and the cytoplasm of eucaryotic cells has only been reported in plant mt-ribosomes (Leaver, 1975). Apparently it is absent in Neurospora (Lizardi and Luck, 1971), yeast and animal mitochondria (Borst and Grivell, 1971).

Several studies have shown the presence of tRNA’s or 4S RNA (molecular weight of ca 25,000) in animal, plant and fungal mitochondria, which are distinct from those of the cytoplasmic tRNA. Like the rRNA’s these molecules hybridise with mt-DNA of the same cell (Borst, 1972). It is of considerable interest that a methionyl-tRNA which can be formylated by a transformylase has been extracted from animal and plant mitochondria (Guillemaut et al, 1973). This suggests that like bacteria and chloroplasts, the initiation of protein synthesis in mitochondria requires a formylated methionyl-tRNA. This contrasts with the process in the cytoplasm of eucaryotic cells where a nonformylated tRNA is required.

E. MITOCHONDRIAL PROTEIN SYNTHESIS

The ability of mitochondria to synthesise protein has been the cause of much debate. Initially regarded with scepticism, it has now attained wide acceptance, and an appreciation of its importance in mitochondrial biogenesis is beginning to emerge (Schatz and Mason, 1974).

There are four major aspects of mitochondrial protein synthesis which should be examined. Firstly, there is the mechanism of protein synthesis. Secondly, there are the products of mitochondrial protein synthesis. Thirdly, there is the control of this protein synthesis, and lastly, there is the integration of proteins synthesised by both the mitochondria and cytoplasm during the biogenesis of the organelle.

The mechanism of mitochondrial protein synthesis is basically similar to that of the cytoplasm of eucaryotic cells, and to that of procaryotes. The mitochondrial system resembles the latter in several respects. For example in its sensitivity to certain inhibitors of protein synthesis, its use of formylated methionyl-tRNA for the initiation of protein synthesis and in its interchangeability of protein factors required for protein synthesis (Boulter et al, 1972).

Both in vivo and in vitro studies have contributed to our understanding of mitochondrial protein synthesis. In vitro studies first suggested the existence of mitochondrial protein synthesis (MacLean, 1958) and showed the organelle’s protein synthesis, in contrast to that of cytoplasm, was inhibited by chloramphenicol, and was insensitive to cycloheximide and RNAase (Mager, 1960).
However, the incorporation of radioactive amino acids by isolated mitochondria was generally low, and responded unpredictably to slight changes in the incubation medium (Schatz and Mason, 1974). Examination of the products of protein synthesis by the isolated organelle did not, until recently, produce very satisfactory results. (For example, contrast Poyton and Groot, 1975, with Coote and Work, 1971, or Ledermann and Attardi, 1973.)

The in vivo experiments have frequently exploited the differences in the sensitivities of the cytoplasmic and mitochondrial protein synthetic machinery to various inhibitors. This has allowed either system to be inhibited while the other continues to synthesise protein. The inclusion of radioactive amino acid in the incubation with the inhibitor increases the sensitivity of this method. The advantage of this technique over that of the in vitro system is that the mitochondrion has not suffered the inevitable damage that occurs during isolation, and the mitochondrion is in an environment which is not as drastically different from the normal situation. However, there are disadvantages. The inhibitor must be 100% effective. Mitochondrial protein synthesis constitutes a small proportion of the total cellular protein synthesis. Therefore even a low residual cytoplasmic incorporation would complicate the results. The inhibitor must be specific for protein synthesis and not have any side effects, for example on respiration (Wilson and Moore, 1973; MacDonald and Ellis, 1969).

The use of the in vivo technique has proved the most productive and it has been shown that some of the sub-units of 3 inner mitochondrial membrane proteins are synthesised on mt-ribosomes. Rubin and Tzagoloff (1973) and Mason and Schatz (1973) showed in yeast the synthesis of 3 of the 7 subunits of cytochrome oxidase was insensitive to cycloheximide, but sensitive to chloramphenicol. They concluded that 3 subunits are therefore synthesised on mt-ribosomes. Sebald et al (1973) obtained similar results with Neurospora. Using a similar technique, Tzagoloff and Meagher (1972) demonstrated in yeast that 4 of the 10 subunits of the oligomycin-sensitive ATPase are synthesised on mt-ribosomes. Lastly, Weiss (1972) showed that one of the two subunits of cytochrome-b was synthesised by the mitochondria of Neurospora.

Selective inhibition of mitochondrial or cytoplasmic protein synthesis in vivo has also yielded information regarding the synthesis of mitochondrial proteins by the cytoplasmic protein synthetic machinery. Their synthesis is inhibited by cycloheximide, or anisomycin but not by chloramphenicol or erythromycin. Proteins falling into this category include most if not all of the mitochondrial ribosomal proteins (Schmitt, 1972; Lizardi and Luck, 1972) a mt-DNA polymerase (Ch'ih and Kalf, 1969), four subunits of cytochrome oxidase (Mason and Schatz, 1973) and proteins of the mitochondrial outer membrane (Schatz and Mason, 1974).

In vitro studies of the products of mitochondrial protein synthesis have been disappointing.
Initially they suggested the mitochondrion was responsible for the synthesis of "structural" protein of the inner mitochondrial membrane (Beattie et al., 1967). This structural protein was considered to be a fairly homogeneous, insoluble protein lacking in any enzymatic function. However, when improved techniques were used to characterise this protein, it was found to consist of many different components, some of which were denatured enzymes (Schatz and Saltzgaber, 1969). Poyton and Groot (1975) have shown that isolated mitochondria can synthesise recognisable products. Their results suggest the isolated organelle from yeast can synthesise the 3 large subunits of cytochrome oxidase. This agrees well with the results discussed above.

While identification of mitochondrially synthesised proteins was advancing, it was emerging from studies of cytoplasmically inherited petite mutants of yeast, that the majority of mitochondrial proteins were coded for and synthesised by, the nucleo-cell sap system. These petite mutants possess mitochondria which have an inner and outer membrane (Yotsuyanagi, 1962) but are deficient in cytochrome oxidase, and cytochrome-b, as well as an energy transfer system (Schatz and Mason, 1974). In addition, their mtDNA is altered or absent (Linnane et al., 1972). Mitochondrial protein synthesis is lacking in these mutants (Faye et al., 1973). It follows that any protein present in their mitochondria must have been synthesised by cytoplasmic ribosomes. Proteins belonging to this category are the enzymes of the citric acid cycle, ferrochelatase, cytochrome c, cytochrome b, subunits of the mitochondrial F1 ATPase, mitochondrial elongation factors, and 3 subunits of cytochrome oxidase (Schatz and Mason, 1974).

These complementary lines of research have established:

i. Most mitochondrial proteins are coded for by nuclear genes and synthesised on cytoplasmic ribosomes.

ii. The mitochondrion synthesises a few essential polypeptides. Those so far identified are subunits of enzymes localised in the inner mitochondrial membrane. These enzymes also contain subunits synthesised by cytoplasmic ribosomes implying a close co-ordination of the two systems. Figure 1.1 summarises these results.

It has been frequently assumed that the polypeptides synthesised by the mitochondria are coded for by mtDNA. As yet, this has not been unequivocally proved.

Recent findings indicate that mtRNA from HeLa cells contains stretches of polyadenine (poly(A)), which is associated with mitochondrial polysomes and considered to be mitochondrial mRNA (Perlman et al., 1973). In addition these poly(A) containing mtRNA's have been resolved into at least eight distinct species, and each of these has been shown to hybridise with mtDNA, suggesting they are transcribed from mtDNA (Attardi et al., 1975).
Fig. 1.1 Part of a Mitochondrion showing Inner and Outer Membranes

- Nuclear DNA → Protein Synthesis
- Cytoplasmic Proteins
- Matrix Proteins
- ATPase complex
- Cytochrome Oxidase
- Cytochrome b
- Cristae
- Outer Inner membranes
- Mitochondrial DNA
- Mitochondrial Protein Synthesis
The protein synthetic machinery of the mitochondrion has frequently been compared to that of procaryotic organisms. It is surprising that the organelles apparently synthesise RNA containing poly(A), a class of RNA previously found only in nuclear and cytoplasmic mRNA and viral RNA of eucaryotic cells.

F. CONTROL OF MITOCHONDRIAL DEVELOPMENT

From the earlier discussion it is clear that mtDNA of animals, fungi and most likely plants, does not code for all the mt-RNA species and the mitochondrial proteins found in the functional organelle. Furthermore, it would appear the majority of mitochondrial proteins, specifically those of the outer membranes, matrix, and a substantial proportion of the proteins of the inner membrane are coded for by nuclear DNA, synthesised on cytoplasmic ribosomes, and subsequently integrated into the mitochondrial structure. Clearly, for the assembly of a functional organelle of such complexity, gene expression in the mitochondria and nucleus must be closely co-ordinated. There are suggestions that part of this co-ordination may operate through a feedback mechanism at the level of mitochondrial protein synthesis.

A system which has proved most suitable to the study of mitochondrial development is that of yeast cells undergoing respiratory adaptation. Yeasts grown anaerobically possess very rudimentary mitochondria, lacking in typical ultra-structure, cytochrome oxidase, and most respiratory chain intermediates (Watson et al., 1970). However, they contain mtDNA and possess the ability to synthesise proteins. When these yeast cells are aerated, derepression of the mitochondrial enzyme system occurs. Inclusion of cycloheximide or chloramphenicol in the growth medium during this derepression phase has shown that for normal development of the mitochondria, both cytoplasmic and mitochondrial protein synthesis is required.

Recently, Groot and Poyton (1975) have shown that part of this effect can be reproduced in vitro by mitochondria isolated from aerobically grown yeast. They showed that the synthesis of 2 of the 3 mitochondrially synthesised subunits of cytochrome oxidase is repressed when the isolated mitochondria are incubated under anaerobic conditions. It is suggested that these results show oxygen directly affects mitochondrial translation.

Opik (1973) has shown that if rice coleoptiles are grown under anaerobic conditions, the ultra-structure of the mitochondria, in contrast to those of yeasts, appears normal. However, cytochrome oxidase activity and the respiratory rate of the mitochondria is reduced. As in yeasts, this can be reversed by transfer to aerobic conditions.

Anomalies in rates of labelling of mitochondrially synthesised polypeptides (Weiss, 1973) have led to the suggestion that for continued protein synthesis by mitochondria, the complementary
cytoplasmically synthesised subunits of the protein must be present (Mahler, 1971). This was supported by the findings of Tzagoloff and Akai (1972) that a pre-incubation of yeast cells with chloramphenicol enhances subsequent mitochondrial protein synthesis when the inhibitor is replaced by cycloheximide. This may prove to be an analogous situation to that observed in chloroplasts. Here inhibition of a chloroplast synthesised polypeptide (large subunit of Fraction 1) follows as a result of inhibition of the cytoplasmically synthesised partner (small subunit Fraction 1) (Ellis, 1975).

These findings indicate some of the controls which may operate during mitochondrial biogenesis thus suggesting ways in which cytoplasmic and mitochondrial gene expression may be co-ordinated.
CHAPTER 2: MATERIALS AND METHODS

A. MATERIALS

1. PLANT MATERIAL

Mung beans (Phaseolus aureus) and peas (Pisum sativum var. Feltham First) were obtained from Suttons (Reading, England). Artichoke (Helianthus tuberosus var. Bunyards Round) were grown as a single clone of plants in the garden of the Botany Department of Edinburgh University. Mature, dormant tubers were harvested between November and February, and stored in damp sand in sealed polythene bags between 0°-4° C. The remaining plant material, namely turnip, cauliflower and potato, was purchased locally.

2. CHEMICALS AND RADIOISOTOPES

All chemicals were AnalaR grade, and all solvents were either AnalaR or chromatography grade.

Ammonium persulphate; glycine; 4-aminosalicyclic acid (PAS); sodium lauryl sulphate (SLS); trichloroacetic acid (TCA); octylphenoxypolyethoxy ethanol (Triton X-100) were obtained from British Drug Houses Ltd.

N,N'-methylenebisacrylamide and tri-isopropynaphthalene—sulphonic acid, sodium salt (TNS) were obtained from Eastman Organic Chemicals, Rochester, N.Y., U.S.A.

Comassie Brilliant Blue was obtained from G. T. Gurr.

Hydrogen peroxide (100 volumes) and Folin-Ciocalteau reagent were obtained from Hopkin and Williams Ltd.

N,N,N',N'-tetramethylenediamine (TEMED) was obtained from Koch-Light Laboratories.

Bovine serum albumin, Fraction V (BSA); 2-mercaptoethanol; cycloheximide; 2-amino-2-hydroxymethylpropane-1:3 diol (Tris, TRIZMA base) were obtained from Sigma Ltd.

Nutrient agar was obtained from Oxoid Ltd., London.

The following chemicals were kindly supplied gratis:
Actinomycin D from Merke, Sharpe and Dohme Ltd.
Lincomycin from Upjohn Ltd.
Chloramphenicol from Dr J. Ellis, University of Warwick.
[¹⁴C] leucine (specific activity 331mCi/mmole, radioactive concentration 50μCi/ml), [³H] uridine (specific activity 7Ci/mmole), [¹⁴C] amino acid mixture (specific activity 45mCi/milliatom carbon, radioactive concentration 50μCi/ml), L-[¹⁵S] methionine (specific activity 275Ci/mmole, radioactive concentration 0.8-5.0mCi/ml) were obtained from the Radiochemical Centre, Amersham, Bucks.

3. ENZYMES AND SUBSTRATES

Protease (Streptomyces griseus, Type V) and pancreatic ribo-nuclease A (Type 1A) were obtained from Sigma Ltd.

dADP (Grade 1), sodium acetate, malic acid, sodium succinate, and a complete range of amino acids were obtained from Sigma Ltd.

B. METHODS

1. GROWTH OF PLANT MATERIAL: Phaseolus aureus and Pisum sativum

Seeds were surface sterilised by soaking for ten minutes in absolute ethanol, rinsed with several changes of water, and then soaked for a further 10 minutes in a 10% (v/v) solution of sodium hypochlorite. The seeds were then rinsed thoroughly in tap water and imbibed overnight in running tap water.

The seeds were then planted in sterile moist vermiculite. The vermiculite (“Micafil” from Dupre Vermiculite, Tamworth Road, Hertford) was soaked in tap water for 12 hours prior to autoclaving at 15psi/15min.

Seed trays were placed in a well-ventilated dark growth room, maintained at a temperature of 25°C. The plants were watered on the second and the fourth day after planting and harvested on the sixth day when the shoots were about 4-6cm in height.

2. PREPARATION OF PLANT TISSUE FOR MITOCHONDRIAL ISOLATION

The roots and cotyledons of six-day-old mung bean seedlings were removed and the hypocotyls rinsed in water prior to immersion in 10% (v/v) sodium hypochlorite for three minutes. The hypocotyls were then extensively rinsed in sterile distilled water and then used directly as a source of mitochondria.

Peas were harvested in a similar manner, except that in this case the first leaves were discarded and mitochondria isolated from the etiolated epicotyl.
Artichoke and potato tubers, turnip roots and cauliflower inflorescences were thoroughly washed to remove soil and surfaces sterilised by immersion in 10% (v/v) sodium hypochlorite for 10 minutes followed by repeated rinsing in sterile distilled water. The tuber and root tissue was then peeled and diced into pieces measuring approximately 2 x 0.5 x 0.5cm prior to extraction of the mitochondria.

3. AGING OF THE ARTICHOKE TISSUE

When the artichoke tuber tissue was aged, the tissue was sliced into 1mm slices using a bacon slicer, which had been sterilised with ethanol, and then cut into pieces measuring approximately 0.5cm². The tissue was then rinsed in several changes of sterile distilled water and incubated in a shaking incubator maintained at 25°C ± 1°C as described in the results section.

4. ISOLATION OF MITOCHONDRIA

At all stages of mitochondria isolation, sterile media and glassware were used in order to minimise bacterial contamination of the final mitochondrial preparation. All solutions, except those of the sucrose gradients, were autoclaved at 15psi/15min, the solutions for the sucrose gradients being autoclaved at 5psi/20min. All plastic bottles and jugs used were sterilised by being filled with 10% (v/v) sodium hypochlorite for 20 minutes, followed by rinsing with sterile distilled water. All glassware used was heat sterilised in an oven at 100°C for 12 hours. Muslin was autoclaved for 15 minutes at 15psi.

Mitochondria were prepared by a modification of the method of Douce et al (1973).

Grinding Medium:

0.3M-mannitol
1mM-EDTA (disodium salt)
8mM-morphilino-propane-sulphonic acid
4mM-cysteine
0.1% bovine serum albumin adjusted to pH7.2 using 5N-KOH.

Wash Medium:

0.3M-mannitol
1mM-EDTA (disodium salt)
0.1% bovine serum albumin adjusted to pH7.2 using 5N-KOH.

Between 100g-1.0kg of tissue was homogenised for 4-6 seconds in at least two volumes of
sterile cold (24°C) grinding medium, using a Willems Polytron PT35 (Northern Media Supply Ltd., Hull, Yorks, U.K.). The homogenate was squeezed through four layers of absorbant muslin and the filtrate centrifuged at 1000 x g for 15 minutes at 2°C in an MSE Mistral 4L centrifuge. The supernatant was decanted and centrifuged at 10,000 x g for 15 minutes at 2°C in an MSE High Speed 18 centrifuge. The supernatants were discarded and the pellets gently resuspended in a total volume of about 30ml of ice cold wash medium with the aid of a glass-in-glass homogeniser, and centrifuged at 600 x g for 10 minutes. The supernatant was decanted and centrifuged at 10,000 x g for 15 minutes. The mitochondrial pellet was again resuspended in about 2.0ml wash medium. The mitochondria at this stage are referred to as “impure mitochondria”.

Further purification was carried out by layering the mitochondrial suspension on top of discontinuous sucrose gradients and centrifuging at 40,000 x g (r_{av} 9.1cm) in a Spinco SW25.1 rotor at 2°C. Several modifications of the sucrose gradients were tried in an effort to reduce contamination by cytoplasmic ribosomes. The gradients which were finally adopted and routinely used were prepared by layering sucrose solutions in the order 1.8M (2.0ml), 1.45M (6.0ml), 1.2M (6.0ml), 0.9M (6.0ml), 0.6M (5.0ml). All of these sucrose solutions contained 10mM-KHPO_4 phosphate buffer pH7.2 and 0.1%BSA. After centrifugation at 40,000 x g (r_{av} 9.1cm) for 1.5 hours, the mitochondria were carefully collected using a bent Pasteur pipette from a well-defined band at the 1.2M:1.45M interface. They were then diluted slowly with an ice cold solution of 10mM-Tris-pH7.2, 50mM-KCl until a sucrose concentration of 0.3M was achieved. The diluted mitochondria were centrifuged at 10,000 x g for 15 minutes and the resulting pellet, referred to as “pure mitochondria”, resuspended in a suitable medium. If the mitochondria were to be incubated with radioactive amino acid, they were resuspended in a medium similar in most respects to that in which they were to be incubated. This was, unless stated otherwise, 0.2M-mannitol; 12.5mM-Tris-pH7.2; 12.5mM-KCl; 5.0mM-KHPO_4 pH7.2; 15.0mM-MgCl_2.

5. INCORPORATION OF RADIOACTIVE AMINO ACIDS BY ISOLATED MITOCHONDRIA

i. Assay of amino acid incorporation.

For routine assay of amino acid incorporation by mitochondrial preparations, 100µl mitochondrial suspension (0.6-1.0mg of mitochondrial protein) was incubated in a final volume of 500µl which contained radioactive amino acid (0.5-50µCi) and a suitable incubation mixture as defined in Chapter 4B. The reactions were carried out in glass tubes measuring 5.0cm x 1.0cm in a water bath maintained at a constant temperature. The reaction was initiated by the addition of 100µl mitochondrial suspension to 400µl of the incubation mixture. The tubes were immediately shaken
and 50μl samples removed at intervals over a time course. The 50μl samples were dried down on to paper discs (Whatmans grade 3mm, 2.1cm diameter), supported on stainless steel pins, using a hair drier, and then quickly immersed in 500ml ice cold 10% (w/v) trichloroacetic acid (TCA) for at least 1 hour at 0°C. The wash procedure employed to remove unincorporated radioactive amino acid was essentially that of Mans and Novelli (1961). The filter paper discs were washed once in about 300ml of 5% (w/v) TCA at room temperature for 5 minutes, then for 10 minutes at 90°C in 300ml of 5% TCA and finally in 300ml of 5% TCA for a further 5 minutes at room temperature. The discs were dehydrated by washing first in 200ml of diethyl ether-ethanol (1:1 v/v) for 15 minutes at 37°C, and then in 200ml diethyl ether for 15 minutes, again at 37°C. The discs were removed and air dried. They were then placed in 10ml scintillation fluid (butyl PBD: 2-(4-t-butyl phenyl)-5-(4'-bi-phenyl)-1,3,4-oxidiazole in toluene, 4g/litre) and counted in an Intertechnique SL31 liquid scintillation counter at 6°C. If ^35S was the isotope to be counted, then an open channel with a window set at 330-990 was used. However, if an isotope other than ^35S was to be counted, e.g. ^14C, then the appropriately named channel was selected.

ii. Analysis of the labelled products of protein synthesis.

When highly-labelled preparations were required for electrophoretic analysis of the proteins synthesised by mitochondria, the level of radioactivity included in the incorporation medium was raised to 50μCi of [^35S] methionine per incubation. The mitochondrial preparations were denatured by the addition of sodium lauryl sulphate, such that the SLS:protein ratio was at least 2:1 (w/w). This was normally accomplished by the addition of double strength sample buffer containing:

- 6% SLS
- 6% β-mercaptoethanol
- 60mM-Tris-pH6.8
- 10% sucrose

The mixture was incubated at 70°C for 20 minutes to ensure complete solubalisation of the mitochondrial protein.

iii. Fractionation of proteins by polyacrylamide gel electrophoresis.

The method found to give the most satisfactory results was that based on Laemmli (1970) which used SLS as a protein solubaliser. This gel system has the added advantage of fractionating the proteins according to their size. An urea-acetic acid gel system was tried unsuccessfully, the
hydrophobic mitochondrial proteins remaining precipitated on the top of the gel. The following stock solutions were used to prepare the SLS gels:

1. Electrophoresis buffer
   50mM-glycine; 50mM-Tris; 0.1% SLS; 0.075% β-mercaptoethanol.
2. 4M-urea; 0.8% SLS.
3. 3.0M-Tris-HCl pH 8.5
4. 58% acrylamide (w/v); bisacrylamide 2% (w/v).
5. TEMED (N,N,N,N-tetramethylene diamine).
6. 0.14% (w/v) ammonium persulphate, freshly prepared.

Solutions 2, 3, 4 and 5 were kept as stock solutions at 2°C, solutions 4 and 5 were stored in dark bottles.

15% gels were prepared as described below. Lower percentage gels were made by suitable dilution of the stock acrylamide solution with distilled water.

The gels were polymerised at room temperature as follows, in strict sequence: 10ml stock acrylamide solution, 5ml 4M-urea stock solution, 5ml 3M-Tris stock solution and 20ml persulphate stock solution mixed in a round-bottomed quick-fit flask. The adaptor neck was sealed into the flask and the solution was degassed under reduced pressure for about 30 seconds. 23μl of TEMED was added and the contents of the flask gently swirled.

The solution was then pipetted using a 10ml pipette and rubber safety bulb into cylindrical perspex tubes, 6mm internal diameter and 9-10cm in length. Parafilm was wrapped over the base of the tubes to prevent the liquid running out prior to polymerisation. Water was carefully layered over the top of the gel using a finely-drawn out Pasteur pipette to ensure a flat top. The gels were allowed to polymerise at room temperature for 45 minutes and then the parafilm was removed and the gel tubes mounted vertically between two electrophoresis tanks. The cathode was in the top gel compartment, and the anode in the lower compartment. Electrophoresis buffer, 400-500ml, was added to each compartment. Air bubbles were removed, and the loading surfaces of the gels given several rinses with buffer. The gels were pre-run at room temperature for 30 minutes at 5mA/gel. The equivalent of 60-80μg of mitochondrial protein, pre-treated as described in Section 5ii of this chapter, was loaded on to each gel under a very low current to avoid diffusion. The gels were run at 2.5mA/gel for 30 minutes and then 5mA/gel for 4-8 hours, depending on whether all proteins were being considered or only the higher molecular weight proteins.

The gels were removed from the tubes by tap water pressure, stained for 5 hours in a 0.25% solution of ...
(w/v) commassie blue solution and then destained at 50\(^\circ\)C in destaining fluid. The commassie blue solution consisted of:

- 0.25% commassie blue
- 45% methanol
- 9.2% glacial acetic acid.

The destaining fluid consisted of:

- 5% methanol
- 7.5% glacial acetic acid.

The gels were scanned at 265nm in a Joyce Loebi U.V. scanner. The \(E_{265}\) was recorded on a Servoscribe potentiometric recorder geared to give scans twice the gel length.

Slab gel electrophoresis was performed according to the method of Laemmli (1970); and consisted of a 5% acrylamide (w/v) stacking gel and a 15% acrylamide (w/v) separating gel. The apparatus used was as described by Laemmli and Favre (1974). The protein sample to be electrophorised was pre-treated as described in Section BSii of this chapter, the sample was then loaded on to the gel and electrophorised at 10mA for 16 hours. The gel was then stained with commassie blue solution (0.25% w/v) for 2 hours, and then destained with destaining fluid, as described earlier in this section for tube gels.

The slab gels were dried down on to Whatman 3mm chromatography paper under reduced pressure and heat from a 230W heating lamp.

iv. Radioactive analysis of the gels.

The gels were frozen to the scanned length in an aluminium sheet trough on solid carbon dioxide. They were then sliced into 0.5mm slices using a Mickle gel slicer (Mickle Engineering Co., Gomshall, Surrey), 2 slices being placed in each scintillation vial. The slices were dissolved in 100 volume hydrogen peroxide; 0.6ml hydrogen peroxide was added to each vial, the top loosely screwed on, and placed in an oven at 70 \(^\circ\)C for 15 minutes. The tops were then quickly tightened, and the tray of vials returned to the 70\(^\circ\)C oven and incubated for 6 hours. After this time, 5.5ml scintillation fluid (2:1 mixture of toluene-Triton-X-100, 5g/litre butyl-PBD) was added, the vial vigorously shaken, then counted for 10 minutes in an Intertechnique SL31 liquid scintillation counter.

Slab gels were autoradiographed by the dried down gel being exposed to Kodak Blue Band X-ray film for 6-8 weeks. The films were then developed by standard procedures.
6. MEASUREMENT OF OXYGEN UPTAKE

The coupling of oxidation to phosphorylation was routinely used as a measure of the intactness of the mitochondrial preparations. Oxygen uptake was measured at room temperature (20-22°C) in a 2.8ml stirred cell using a Beckman Oxygen Electrode in conjunction with a Beckman 160 Physiological Gas Analyser. The reaction medium contained 0.2 M-mannitol; 5mM-MgCl₂; 10mM-KCl; 10mM-KH₂PO₄ ph7.2 and 0.5-2.0mg of mitochondrial protein.

7. PROTEIN ESTIMATION

The method used was based on that of Lowry et al (1951). The reagent mixture required was prepared each day by mixing 1ml of 1% (w/v) CuSO₄ 5H₂O with 1ml 2% (w/v) KNaC₄H₄O₄ 4H₂O (sodium potassium tartrate) and adding this to 100ml 2% (w/v) Na₂CO₃ in 0.1 M-NaOH.

An appropriate amount of sample to be estimated for protein content (about 40-100μg) was precipitated for 20 minutes by addition of cold TCA to a final concentration of 10% (w/v) in a conical centrifuge tube. It was then centrifuged for 10 minutes at top speed in a bench centrifuge in the cold room. The supernatant was carefully decanted, and the pellet resuspended with the aid of a Vortex mixer in 1.0ml 0.1M-NaOH. The pellet was allowed to stand for 30 minutes to come into solution, then 5ml of reagent mixture were added to each sample, and allowed to stand for 10 minutes. 0.5ml Folin-Ciocalteau reagent (prepared by diluting 2 parts of commercially available solution with one part 1.0M-NaOH) was added and the solutions immediately mixed. The samples were allowed to stand at room temperature for 30 minutes after which they were read on an EEL colorimeter with a 608 filter.

Bovine serum albumin was used as a standard in the range 0-120μg protein in order to construct a calibration curve.

8. ESTIMATION OF BACTERIAL AND FUNGAL CONTAMINATION OF THE MITOCHONDRIAL PREPARATION

As a routine measure, serial dilutions of the mitochondrial preparation, in sterile distilled water, were plated on to 2.8% (w/v) nutrient agar. The plates were incubated at 37°C for 48 hours at which time colony counts were made.

9. ANALYSIS OF MITOCHONDRIAL NUCLEIC ACIDS

i. Extraction of nucleic acids.

The mitochondrial pellet was resuspended in 5ml of buffer (10mM Tris-HCl-pH8.5;
50mM-KCl) and lysed by the addition of Triton-X-100 to 4% (v/v), then centrifuged at 10,000 x g for 5 minutes. The supernatant was shaken with an equal volume of detergent mixture (12% p-aniino salicylate, 2% tri-isopropyl-napthalene sulphonate, 50mM-KCl, 10mM-Tris-Cl pH8.5) followed by further shaking after the addition of an equal volume of a phenol mixture (phenol containing 5% redistilled m-cresol; 0.1% 8-hydroxyquinoline, saturated with 0.01M-Tris-Cl pH8.5) to effect deproteinisation. The upper aqueous layer was removed after centrifugation at 1200 x g for 10 minutes and re-extracted with an equal volume of phenol mixture. The nucleic acids were then precipitated from the final aqueous phase by addition of 2 volumes of cold ethanol, and stored at 0°C for 16 hours. The precipitate was washed twice by resuspension and centrifugation at 1200 x g for 10 minutes from 80% ethanol. The washed nucleic acid pellets were drained to remove excess ethanol, and dissolved in electrophoresis running buffer. The nucleic acid concentration was estimated from the absorption at 260nm in an SP800 spectrophotometer. It was assumed that 1mg of nucleic acid/ml had an $E_{260}$ of 20.

ii. Fractionation of RNA by polyacrylamide gel electrophoresis.

The method used was essentially that of Loening (1967), as modified by Leaver (1973). Before use, the acrylamide was recrystallised from chloroform, and the bisacrylamide from acetone. Polyacrylamide gels were prepared as follows from a stock solution of acrylamide containing 15% (w/v) acrylamide and 0.75% (w/v) bisacrylamide, in a dark bottle at 4°C.

In the preparation of 2.4% acrylamide gels, 5.0ml stock acrylamide solution, 6.25ml 5X concentrated E buffer and 19.75ml distilled water was pipetted into a round-bottomed flask. (E buffer contained: 36mM-Tris, 30mM-NaH$_2$PO$_4$, 1mM-EDTA (disodium salt), pH7.8, and was stored as a 5X concentrated stock at 4°C.)

The solution was degassed with a vacuum pump for 30 seconds, and 25μl TEMED followed by 0.25ml of freshly-prepared 10% (w/v) ammonium persulphate were added and mixed by gentle swirling. This solution was then pipetted using a 10ml pipette and rubber safety bulb, into cylindrical perspex tubes, 6mm internal diameter, and 9cm in length which were sealed at the base. Water was then carefully layered over the top of the gel using a finely-drawn-out Pasteur pipette, to give a flat surface. The gels were allowed to polymerise for 45 minutes at room temperature, and the seals from the bases of the gel tubes removed. The gel tubes were mounted vertically between electrophoresis tanks, and 400-500ml cold (2-4°C), E buffer containing 0.1% (w/v) SLS added to each compartment. Any air bubbles trapped in the base of the gel tubes were removed with a bent Pasteur pipette, and the loading surfaces of the gels given several rinses with E buffer. The anode was connected to the
lower tank, and the gels pre-run at 3mA/tube for 30 minutes. After pre-running the gels, 10-20μg of nucleic acid sample in electrophoresis buffer, made 10% with sucrose, was loaded on to the tops of the gels, and the gels run for 4-5 hours at 4°C and 3mA/gel.

After electrophoresis, the gels were removed from the tubes by air pressure applied via a rubber teat, and washed in distilled water for at least 30 minutes. This eluted any ultra violet-absorbing low molecular weight components which would interfere with the subsequent scanning of the gel. The gels were then transferred to a quartz cell and scanned in a Joyce Loebl U.V. scanner. The E was recorded with a Servoscribe potentiometric recorder geared to give scans twice the gel length. Approximately quantitative estimations of the relative amounts of the components were made by cutting out and weighing the areas under the peaks. Radioactive analysis of these gels was performed in a manner identical to that already described for 15% gels in Section 5iv of this chapter.

iii. Buoyant-density analysis of mitochondrial DNA.

Total mitochondrial nucleic acid samples containing between 1-2μg of DNA were taken up in 0.77mls of 15-mM-NaCl, 1.5mM sodium citrate pH7.2 and adjusted to a density of 1.720 g.cm⁻³ by the addition of solid caesium chloride. The samples together with marker Micrococcus lysodeikticus DNA with a density of 1.731g.cm⁻³ were centrifuged at 44,000rev/min at 25°C in a Beckman Model E Analytical Ultracentrifuge. Ultra-violet photos were taken after 20h and scanned with a Joyce Loebl microdensitometer.

10. ELECTRON MICROSCOPIC ANALYSIS OF MITOCHONDRIAL PREPARATIONS

The fixation procedure used was a modification of that of Wellburn and Wellburn (1972) for etioplasts. Solutions in this procedure are made up in 0.33M-phosphate buffer, pH7.5.

An equal volume of 5% (v/v) glutaraldehyde in 0.5M-sucrose, 0.33M-phosphate buffer, was added to the mitochondrial suspensions and left for 2 hours at 0°C. The mitochondria were then spun down at 3000 x g for 2 minutes at 0°C. The pellet was washed successively with 5ml of 0.5M-sucrose, 0.35M-sucrose and 0.2M-sucrose (in 0.33M-phosphate buffer) for 5 minutes each. 1ml of 2% (w/v) OsO₄ in 0.15M-sucrose-phosphate buffer was added to the pellet and left for 2 hours at room temperature. After removal of the osmium, 1ml 30% (v/v) acetone containing 0.1M-sucrose was added and left for 30 minutes.

The sample was then dehydrated with a series of acetone treatments, i.e. 30 minutes in 5ml each of 30%, 50%, 70% and 90% acetone. The sample was left in 100% acetone overnight; this was replaced with fresh 100% acetone 2 hours before embedding. Samples were embedded in Araldite.
overnight at room temperature and then left in a 70°C oven for 8 hours. Each pellet was subsequently cut and re-embedded in gelatine capsules. If micrographs were to be made of whole plant cells, the tissue was first diced into 0.5 cm cubes and then treated in a manner identical to that described for the isolated organelle.
CHAPTER 3: PRELIMINARY CHARACTERISATION OF THE ISOLATED MITOCHONDRIA

A. INTRODUCTION

The aim of the work described in this thesis was to examine the products of mitochondrial protein synthesis. It was envisaged that a major part of the study would involve measurement of the incorporation of radioactive amino acids by isolated, intact mitochondria, followed by purification and identification of the labelled mitochondrial proteins. The isolation of intact mitochondria, free from bacterial and cytoplasmic contamination, with their physiological integrity preserved, was therefore an important prerequisite to the study of protein synthesis by the organelle.

This section describes the criteria routinely employed to assess both the purity and respiratory competence of mitochondrial preparations. In addition, several modifications to the existing technique for mitochondrial isolation are compared.

Three methods were employed to judge the degree of cytoplasmic contamination of the mitochondrial preparations.

1. Electron microscopy of mitochondrial pellets obtained at various stages was used to monitor gross contamination by other subcellular organelles and membranous material.

2. The presence of contaminating cytoplasmic rRNA was estimated by gel electrophoresis.

3. Nuclear DNA was routinely monitored at all stages during the initial development of the isolation procedure, by buoyant density centrifugation.

Mung bean hypocotyl tissue was used as a source of mitochondria in the experiments described in this chapter unless otherwise stated.

B. PURITY OF STANDARD PREPARATIONS

1. ELECTRON MICROSCOPY

Plate 3.1 shows electron micrographs of mitochondria (a) in situ in the hypocotyl tissue (b) after the initial differential centrifugation steps, prior to sucrose gradient purification — referred to as "impure mitochondria"; (c) after purification on sucrose gradients, referred to as "pure mitochondria".

It can be seen that the "pure mitochondria" preparations contain a fairly homogenous population of mitochondria with little evidence of contamination by other subcellular structures.
Plate 3.1
Electron Micrographs of Mung Bean Mitochondria

Mitochondria were isolated by the procedures described previously (Chapter 2B4) and electron micrographs made of mitochondria as described in Chapter 2B10.

A - mitochondria in situ
B - “impure” mitochondrial preparation
C - “pure” mitochondrial preparation.

Approximate magnifications were 20,000, 30,000 and 30,000 times respectively.

Key:  V - vacuole
       cyt - cytoplasm
       mt - mitochondrion
       n - nucleus
       nl - nucleolus.
2. BUOYANT DENSITY CENTRIFUGATION OF DNA

Caesium chloride buoyant density analysis of total cellular DNA, Fig. 3.1(a) shows a single main band of nuclear DNA with a buoyant density of 1.697 g/cm$^3$. In Fig. 3.1(b), “impure mitochondria” as defined in paragraph B1 of this chapter shows an additional band of mitochondrial DNA with buoyant density of 1.707 g/cm$^3$. In purified mitochondria, Fig. 3.1(c), this band is quantitatively the most significant, the 1.697 g/cm$^3$ peak being reduced to less than 5%.

3. ESTIMATION OF MITOCHONDRIAL RIBOSOMAL RNA DURING PURIFICATION OF MITOCHONDRIA

i. Mitochondria purified by existing techniques.

Mitochondria were isolated by procedures described in Chapter 2B4. Nucleic acids were extracted from these mitochondria and fractionated by polyacrylamide gel electrophoresis. A comparison of the gel scans of nucleic acids extracted from whole tissue Fig. 3.2(a), impure mitochondria Fig. 3.2(b) and pure mitochondria Fig. 3.2(c) show a significant enrichment of mt-rRNA during the purification procedure. As can be seen in Table 3.1 it was not possible to detect mt-rRNA in the whole tissue nucleic acid extracts, while at the impure stage they constituted some 29% of the total, rising to 75% in the pure mitochondrial preparation.

<table>
<thead>
<tr>
<th>Source of nucleic acid</th>
<th>Mitochondrial rRNA as % of total rRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole tissue</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>Impure mitochondria</td>
<td>29%</td>
</tr>
<tr>
<td>Pure mitochondria</td>
<td>75%</td>
</tr>
</tbody>
</table>

Mitochondria were isolated as described in Chapter 2B4, and nucleic acids were extracted as described in Chapter 2B9i. RNA was fractionated by polyacrylamide gel electrophoresis and the mt-rRNA expressed as a percentage of total rRNA by the method described in Chapter 2B9i.

It was thought likely that the contaminating cytoplasmic ribosomes, were either bound to the outer mitochondrial membrane or were components of the “rough” endoplasmic reticulum which may co-sediment with the mitochondria.
Figure 3.1
Buoyant density centrifugation of DNA

Microdensitometer tracings of U.V. photographs of mitochondrial and total cellular DNA species from mung bean, obtained after analytical CsCl density gradient centrifugation as described in Chapter 2B9iii. Nucleic acids were extracted as described in Chapter 2B9i and mitochondria were isolated by the method described in Chapter 2B4. The marker Micrococcus lysodeikticus DNA had a density of 1.731 g/cm³.

(a) Total cellular DNA, the major band being nuclear DNA.
(b) Impure mitochondrial DNA.
(c) Purified mitochondrial DNA.
Figure 3.2

Polyacrylamide gel electrophoresis of nucleic acids from mung bean

Nucleic acids were extracted as described in Chapter 2B9i, and electrophorised on polyacrylamide gels as described in Chapter 2B9ii. Low molecular weight RNA species have run off the gels. RNA components are referred to their molecular weights in millions.

(a) Whole tissue.
(b) Impure mitochondria.
(c) Pure mitochondria.
ii. Mitochondria purified by modified techniques.

It is known that removal of magnesium ions tends to dissociate ribosomes and therefore EDTA (a magnesium ion chelator) was included in the sucrose gradient solutions. It was predicted that this would either encourage dissociation of contaminating cytoplasmic ribosomes, so reducing contamination with the small ribosomal subunit while leaving the large ribosomal subunit still attached to the membrane or, in addition, if the association of ribosomes with the membranes was a similar type of ionic bridge to that of large and small ribosomal subunit interaction, total removal of contaminating cytoplasmic ribosomes might be achieved. As can be seen from the data in Table 3.2 attempts to remove contaminating cytoplasmic ribosomes by addition of EDTA to part or all of the preparative sucrose gradients did not result in an increase in mitochondrial purity as judged by loss of contaminating cytoplasmic ribosomes. The level of contamination remained at about 25% in all cases.

**TABLE 3.2**
The effects of addition of EDTA to sucrose gradients

<table>
<thead>
<tr>
<th>Addition to gradient</th>
<th>Mitochondrial rRNA as % of total rRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>76%</td>
</tr>
<tr>
<td>1mM EDTA throughout gradient</td>
<td>77%</td>
</tr>
<tr>
<td>1mM EDTA in 0.6M and 0.9M sucrose layers</td>
<td>77%</td>
</tr>
<tr>
<td>10mM EDTA in 0.6M sucrose layer</td>
<td>75%</td>
</tr>
<tr>
<td>10mM EDTA in 0.6M and 0.9M sucrose layers</td>
<td>76%</td>
</tr>
</tbody>
</table>

Mitochondria were prepared as described in Chapter 2B4, with the exception that the above additions were made to the sucrose gradients. The nucleic acids from these mitochondria were extracted and electrophoresed as described in Table 3.1.

Results obtained with the sucrose gradients routinely used in this study were also compared with those described by Leaver and Harmey (1973). The gradients contained 10mM-Tris pH 7.5, 50mM-KCl and 1mM-EDTA and in addition were prepared by layering sucrose solutions with smaller differences in sucrose concentration in that region of the gradient where mitochondria sediment, with the object of improving resolution. The sucrose solutions layered, in order were:

1.9M (4.0ml); 1.8M (2.0ml); 1.65M (2.0ml); 1.5M (2.0ml); 1.35M (2.0ml); 1.2M (2.0ml); 0.9M (8.0ml). The impure mitochondrial suspension was layered on these gradients and centrifuged at 63,000 x g ($r_{av.}$ 9.1cm) for one or two hours. However, no significant difference in the level of
cytoplasmic rRNA contamination could be detected between this and the gradients from the existing technique. It was decided to accept this level of cytoplasmic ribosomal contamination, and use the gradients described in Chapter 2B4 for the routine purification of the mitochondria.

C. BACTERIAL CONTAMINATION

Bacterial counts were routinely carried out by plating aliquots of the mitochondrial preparation on complete nutrient agar, as described in Chapter 2B8. Bacterial contamination was normally less than $10^3$ bacteria/mg mitochondrial protein. If, for some reason, it was greater than $10^3$ bacteria/mg mitochondrial protein the results of the experiment were rejected and the experiment repeated.

D. PHYSIOLOGICAL INTEGRITY OF MITOCHONDRIAL PREPARATIONS

As a guide to the physiological integrity of the isolated mitochondria, their ability to carry out oxidative phosphorylation was routinely measured in an oxygen electrode, as described in Chapter 2B6.

Table 3.3 summarizes the respiratory characteristics of both impure and pure mitochondrial preparations from mung bean and artichoke.

<table>
<thead>
<tr>
<th>Table 3.3</th>
<th>Respiratory characteristics of isolated mitochondria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue</td>
<td>Condition of Mitochondria</td>
</tr>
<tr>
<td>Mung Bean</td>
<td>Impure</td>
</tr>
<tr>
<td></td>
<td>Pure</td>
</tr>
<tr>
<td>Artichoke</td>
<td>Impure</td>
</tr>
<tr>
<td></td>
<td>Pure</td>
</tr>
</tbody>
</table>

Oxygen uptake was measured as described in Chapter 2B6. State 3 is defined as the rate of oxygen uptake when both substrate and ADP have been added to the mitochondria, and State 4 by the rate of oxygen uptake that follows addition of 1$\mu$mol of ADP to the mitochondria under conditions when substrate, 20mM-malate in the above table, is not limiting. Respiratory control ratio is defined as State 3 divided by State 4. It was assumed that the mitochondrial medium was 250$\mu$M with respect to O$_2$. 

26
E. THE QUALITY OF THE MITOCHONDRIAL PREPARATIONS

The characteristics of the pure mitochondrial preparations described in this section were all highly reproducible. The respiration measurements and electron micrographs suggest that the mitochondria are intact and compare favourably with preparations described by other authors (Douce et al. 1973).

From the nucleic acid fractionation it would appear that mitochondrial ribosomal ribonucleic acids constitute the bulk of the nucleic acids. Such cytoplasmic ribosomes as are present constitute a proportion so constant that it seems possible that they are attached to the outer mitochondrial membrane and may be involved in the biosynthesis of the organelle.
CHAPTER 4: CHARACTERISATION OF AMINO ACID INCORPORATION INTO PROTEIN BY ISOLATED MITOCHONDRIA

A. INTRODUCTION

The claim by McLean (1958) that isolated mitochondria incorporated radioactive amino acids into protein aroused considerable controversy, and was for a long time regarded with scepticism. Therefore, before any examination of the product of mitochondrial protein synthesis could be undertaken, it had to be established beyond all reasonable doubt that the mitochondria were responsible for the incorporation. In this chapter results are presented which strongly suggest this incorporation is not an artifact.

Leaver (1975) has described a medium which was capable of supporting protein synthesis by isolated plant mitochondria. This medium was used as a basis for the work described in this and the subsequent chapters.

B. CHARACTERISATION OF THE CONDITIONS OF INCORPORATION OF RADIOACTIVE AMINO ACIDS

Figure 4.1 shows a time course of incorporation of radioactive amino acid when mitochondria, isolated from mung bean hypocotyl were incubated at 20°C in the following medium:

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannitol</td>
<td>200.0 mM</td>
</tr>
<tr>
<td>KCl</td>
<td>12.5 mM</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>15.0 mM</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>5.0 mM</td>
</tr>
<tr>
<td>Tris pH 7.2</td>
<td>12.5 mM</td>
</tr>
<tr>
<td>Malate</td>
<td>20.0 mM</td>
</tr>
<tr>
<td>ADP</td>
<td>1.0 mM</td>
</tr>
<tr>
<td>[¹⁴C] Leucine</td>
<td>0.5μCi (Specific Activity 342mCi/m mole)</td>
</tr>
</tbody>
</table>

In future this medium will be described as the mannitol based incubation medium.

The values given in Table 4.1 are derived from the same experiment and show the specific activity of the protein after a 1 hour incubation.
Mitochondria were isolated from artichoke tuber by the method described in Chapter 2B4. The mitochondria were incubated with 0.5μCi $[^{14}C]$ leucine in the mannitol based medium, and the time course of incorporation followed by the methods described in Chapter 2B5.

- $\bullet$ complete. i.e. 20mM malate + 1mM ADP.
- $\Delta$ minus malate + succinate. i.e. 10mM succinate + 1mM ADP.
- $\square$ minus malate + ADP.
- $\circ$ minus malate + acetate. i.e. 20mM acetate + 1mM ADP.
fig 4.1

![Graph showing cpm x 10^3 per mg protein vs time (min).]

- Three curves are plotted.
- The x-axis represents time in minutes (0 to 60).
- The y-axis represents cpm x 10^3 per mg protein (0 to 4).
- The upper curve is the highest, followed by the middle and then the lower curve.
TABLE 4.1
Requirements for the incorporation of \[^{14}C\] leucine by isolated mung bean mitochondria

<table>
<thead>
<tr>
<th>System</th>
<th>[(^{14}C)] leucine incorporated cpm/mg protein/60min</th>
<th>% Incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>4,050</td>
<td>100</td>
</tr>
<tr>
<td>Minus malate</td>
<td>84</td>
<td>2</td>
</tr>
<tr>
<td>Minus ADP</td>
<td>130</td>
<td>3</td>
</tr>
<tr>
<td>Minus malate + succinate (10mM)</td>
<td>2,840</td>
<td>70</td>
</tr>
<tr>
<td>Minus malate + acetate (20mM)</td>
<td>105</td>
<td>3</td>
</tr>
<tr>
<td>Complete (incubated in dark)</td>
<td>3,890</td>
<td>96</td>
</tr>
</tbody>
</table>

Mitochondria were isolated by the method described in Chapter 2B4 and incubated in the mannitol based incubation medium at 20°C. Incorporation of radioactive amino acid was followed by the method described in Chapter 2B5i. Results are expressed as percentages of the incorporation by the complete system.

The dependence of the incorporation on the presence of both oxidisable substrate and ADP was highly reproducible. It was considered consistent with mitochondrial protein synthesis. Certainly the incorporation could not be considered typical of protein synthesis by any contaminating micro-organisms. Omission of ADP and of malate were routinely included in experiments as controls.

The incorporation of \[^{35}S\] methionine (specific activity 280Ci/m mole) and a \[^{14}C\] amino acid mixture (specific activity 54mCi/m. atom carbon) were found to follow a similar time course to that of radioactive leucine. Again, the incorporation was dependent on both ADP and oxidisable substrate. Fig. 4.2 and Table 4.2 show this for the incorporation of \[^{35}S\] methionine by isolated mung bean mitochondria.

TABLE 4.2
Incorporation of \[^{35}S\] methionine by isolated mung bean mitochondria

<table>
<thead>
<tr>
<th>System</th>
<th>[^{35}S] methionine incorporated cpm/mg protein/60min</th>
<th>% Incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>60,240</td>
<td>100</td>
</tr>
<tr>
<td>Minus malate</td>
<td>2,670</td>
<td>4</td>
</tr>
<tr>
<td>Minus ADP</td>
<td>3,430</td>
<td>6</td>
</tr>
</tbody>
</table>

The details of the experimental procedures used are given in Table 4.1, except that 0.5μC \[^{35}S\] methionine was the radioactive amino acid used.
Figure 4.2

Time course of incorporation of $^{35}$S methionine into protein by isolated mung bean mitochondria

Experimental procedures are as given in Fig. 4.1 except that 0.5µCi $^{35}$S methionine was used as the source of radioactivity.

- •- complete, i.e. 20mM malate + 1mM ADP.
- -- minus malate.
- □-- minus ADP.
fig 4.2
C. THE EFFECT OF TEMPERATURE ON AMINO ACID INCORPORATION BY ISOLATED MITOCHONDRIA

Using the mannitol based incubation medium, optimisation of the temperature for incorporation was investigated. Fig. 4.3 shows the time course of incorporation of $[^{14}C]$ leucine by isolated mung bean mitochondria at 10°, 20°, and 30°C. Although the initial rate of amino acid incorporation was greatest in the 30°C incubation, the final level of incorporation was highest for the 20°C incubation. All subsequent incubations were carried out at 20°C.

D. THE EFFECT OF DIFFERENT AMINO ACIDS ON THE FINAL SPECIFIC ACTIVITY OF THE MITOCHONDRIAL PROTEIN

The ultimate aim of the study was to examine the products of mitochondrial protein synthesis. It was clearly desirable to maximise incorporation of radioactive amino acid into mitochondrial protein. $[^{35}S]$ methionine was the amino acid with the highest specific activity commercially available. It did indeed prove superior to $[^{14}C]$ leucine and to a $[^{14}C]$ amino acid mixture. Table 4.3 demonstrates this difference. In each incubation, 50μCi of the radioactive amino acid were incubated with isolated mung bean mitochondria in the mannitol based incubation medium.

<table>
<thead>
<tr>
<th>Source of radioactivity</th>
<th>Specific Activity</th>
<th>Radioactivity incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[^{35}S]$ methionine</td>
<td>280Ci/m mole</td>
<td>801,000</td>
</tr>
<tr>
<td>$[^{14}C]$ leucine</td>
<td>342Ci/m mole</td>
<td>96,000</td>
</tr>
<tr>
<td>$[^{14}C]$ amino acid mixture</td>
<td>54Ci/m atom carbon</td>
<td>40,000</td>
</tr>
</tbody>
</table>

The details of the experimental procedures used are given in Table 4.1 except that 50μCi of $[^{35}S]$ methionine, $[^{14}C]$ leucine and $[^{14}C]$ amino acid mixture were used as sources of radioactivity.

E. THE EFFECT OF ADDITION OF A NON-RADIOACTIVE AMINO ACID MIXTURE ON AMINO ACID INCORPORATION BY ISOLATED MITOCHONDRIA

It seemed probable that addition of a non-radioactive amino acid mixture, deficient in the amino acid which was radioactive could increase incorporation of labelled amino acid. Table 4.4
Figure 4.3

The effect of temperature on the time course of amino acid incorporation by isolated mung bean mitochondria

Experimental procedures are as given in Fig. 4.1 except that the mitochondria were incubated at different temperatures.

- • -  30°C.
- □ -  20°C.
- ▼ -  10°C.
fig 4.3
shows the results of adding such a mixture to an incorporation experiment using [\textsuperscript{35}S] methionine and another experiment using [\textsuperscript{14}C] leucine. The final concentration of each of the non-radioactive amino acids was 25\mu M.

\begin{table}[h]
\centering
\caption{Effect of addition of a non-radioactive amino acid mixture to the incorporation of [\textsuperscript{35}S] methionine and [\textsuperscript{14}C] leucine by isolated mung bean mitochondria}
\begin{tabular}{|c|c|c|c|}
\hline
System & Radioactive amino acid used & Radioactivity incorporated & % Complete system \\
\hline
Complete & [\textsuperscript{14}C] leucine & 4,015 & 100 \\
Complete+non-radioactive amino acid mixture & [\textsuperscript{14}C] leucine & 6,190 & 154 \\
Complete & [\textsuperscript{35}S] methionine & 32,250 & 100 \\
Complete + non-radioactive amino acid mixture & [\textsuperscript{35}S] methionine & 50,160 & 156 \\
\hline
\end{tabular}
\end{table}

The details of the experimental procedures used are given in Table 4.1, except that the incubations indicated included a non-radioactive amino acid mixture deficient in the amino acid which was radioactive. The final concentration of each amino acid was 25\mu M.

The addition of a non-radioactive amino acid mixture, deficient in the amino acid which was radioactive, clearly stimulated incorporation of radioactivity. It was subsequently routinely included in the “complete” incubation mixture.

F. COMPARISON OF THE ABILITY OF MITOCHONDRIA ISOLATED FROM DIFFERENT TISSUES TO INCORPORATE RADIOACTIVE AMINO ACIDS INTO PROTEIN

It was hoped that the proteins synthesised by mitochondria isolated from different tissues might be compared. In order to assess which tissues would be most suitable, mitochondria were isolated from the range of tissues listed below. In addition to their ability to incorporate radioactive amino acids their respiratory activity was measured.
### TABLE 4.5
Comparison of the ability of mitochondria isolated from different tissues to incorporate amino acid into protein

<table>
<thead>
<tr>
<th>Source of mitochondria</th>
<th>$[^3S] \text{methionine}$ incorporated cpm/mg protein/60min</th>
<th>Respiratory measurements</th>
<th>Respiratory Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mung bean hypocotyl</td>
<td>62,956</td>
<td>0.110</td>
<td>0.020</td>
</tr>
<tr>
<td>Pea epicotyl</td>
<td>30,012</td>
<td>0.065</td>
<td>0.014</td>
</tr>
<tr>
<td>Cauliflower inflorescence</td>
<td>24,918</td>
<td>0.109</td>
<td>0.026</td>
</tr>
<tr>
<td>Artichoke tuber</td>
<td>95,458</td>
<td>0.034</td>
<td>0.016</td>
</tr>
<tr>
<td>Artichoke tuber (aged for 12hr)</td>
<td>262,509</td>
<td>0.074</td>
<td>0.034</td>
</tr>
<tr>
<td>Turnip root</td>
<td>1,200</td>
<td>0.051</td>
<td>0.019</td>
</tr>
<tr>
<td>Potato tuber</td>
<td>583</td>
<td>0.012</td>
<td>0.012</td>
</tr>
</tbody>
</table>

The details of the experimental procedures used are given in Table 4.1 except that the named tissues were used as sources of mitochondria. The isolated mitochondria were incubated in the mannitol incubation medium with equal quantities of $[^3S] \text{methionine}$ (0.5μCi).

Mitochondria isolated from different tissues varied considerably in their ability to incorporate radioactive amino acids into protein and in their respiratory competence.

### G. DISCUSSION

The results presented in this chapter are consistent with mitochondrial protein synthesis. It was considered that neither bacteria nor cytoplasmic ribosomes could account for the incorporation of radioactive amino acids observed. These and other possibilities are discussed more fully in Chapter 8.
CHAPTER 5: THE EFFECTS OF INHIBITORS ON AMINO ACID INCORPORATION BY ISOLATED MITOCHONDRIA

A. INTRODUCTION

In this chapter the sensitivity of the system to various inhibitors is examined. The effect of all inhibitors on both the incorporation of radioactive amino acids and on respiration of the isolated mitochondria was measured.

It is now well established that several aspects of organellar protein synthesis resemble procaryotic protein synthesis rather than that found in the cytoplasm of eucaryotic cells. It was considered desirable to check the sensitivity of plant mitochondrial protein synthesis with a view to exploiting this difference in the in vivo situation, making possible a comparison between the in vitro and in vivo products of mitochondrial protein synthesis. In addition the dependence of protein synthesis upon oxidative phosphorylation was examined by comparing the effect of inhibitors and uncouplers of respiration upon the incorporation of radioactive amino acids by isolated mitochondria.

B. THE EFFECT OF INHIBITORS OF PROTEIN SYNTHESIS ON AMINO ACID INCORPORATION AND RESPIRATION BY ISOLATED MITOCHONDRIA

In all experiments with inhibitors, the mitochondria were pre-incubated with the inhibitor for five minutes. The initiation of the incorporation was accomplished by the addition of radioactive amino acid. Control incubations were similarly initiated. The respiratory measurements were also made after the mitochondria had first been pre-incubated with the inhibitor for 5 minutes.

Tables 5.1 and 5.2 show the effects of various inhibitors of protein synthesis. It is clear from these results that cycloheximide, at concentrations reported to inhibit cytoplasmic protein synthesis had little effect on incorporation of amino acids. The inhibition of respiration observed with this inhibitor has been reported by other workers (Wilson and Moore, 1973).

Substantial inhibition of incorporation occurs with D-threo chloramphenicol while the L-isomer had only a comparably slight effect at high concentrations.

Lincomycin has been reported to inhibit protein synthesis in isolated chloroplasts (Blair and Ellis, 1973). The results presented in Table 5.1 suggest little significant effect on isolated mitochondrial protein synthesis.

In Table 5.2 the effect on the system of a range of concentrations of fuscidic acid is shown.
TABLE 5.1
Effect of inhibitors of protein synthesis on the incorporation of $[^{35}\text{S}]$ methionine by isolated mung bean mitochondria

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Final Concentration</th>
<th>Radioactivity incorporated cpm/mg protein/60 min</th>
<th>% Incorporation</th>
<th>Effects on Respiration Respiratory Control</th>
<th>% Respiration</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>43,650</td>
<td>100</td>
<td>4.5</td>
<td>100</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>20µg/ml</td>
<td>41,904</td>
<td>96</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>50µg/ml</td>
<td>33,310</td>
<td>77</td>
<td>3.4</td>
<td>76</td>
</tr>
<tr>
<td>Lincomycin</td>
<td>10µg/ml</td>
<td>45,830</td>
<td>105</td>
<td>4.5</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>50µg/ml</td>
<td>38,704</td>
<td>89</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-threo CAP</td>
<td>0.1µM</td>
<td>4,365</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5µM</td>
<td>3,055</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0µM</td>
<td>2,182</td>
<td>5</td>
<td>3.2</td>
<td>71</td>
</tr>
<tr>
<td>L-threo CAP</td>
<td>0.1µM</td>
<td>32,737</td>
<td>75</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5µM</td>
<td>31,864</td>
<td>73</td>
<td>2.9</td>
<td>64</td>
</tr>
</tbody>
</table>

Mitochondria were isolated by the method described in Chapter 2B4. The mitochondria were pre-incubated for 5 minutes in the mannitol based incubation medium with the inhibitors listed in the table. The reaction was initiated by the addition of equal amounts of $[^{35}\text{S}]$ methionine (0.5uCi) to each incubation. The incorporation of radioactive amino acid was followed by the method described in Chapter 2B5i. Results are expressed as a percentage of the incorporation by the complete system. The respiratory measurements were made by the method described in Chapter 2B6. Respiratory control is defined in Chapter 3D. The results are expressed as percentages of the respiratory control of the complete uninhibited system.
## TABLE 5.2

Effect of fuscidic acid on incorporation of $[^35]S$ methionine by mitochondria isolated from artichoke

<table>
<thead>
<tr>
<th>Concentration of Fuscidic Acid (mM)</th>
<th>Radioactivity incorporated cpm/mg protein/60min</th>
<th>% Incorporation of Respiratory Control</th>
<th>% Respiration</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>137,833</td>
<td>100</td>
<td>1.95</td>
</tr>
<tr>
<td>0.005</td>
<td>132,319</td>
<td>96</td>
<td>1.75</td>
</tr>
<tr>
<td>0.050</td>
<td>130,941</td>
<td>95</td>
<td>1.72</td>
</tr>
<tr>
<td>0.50</td>
<td>9,648</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>1.00</td>
<td>6,800</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>5.00</td>
<td>6,891</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

The details of the experimental procedure used are given in Table 5.1. The results are expressed as percentages of the complete uninhibited system.
This inhibitor is reported to be an inhibitor of chain elongation in chloroplasts, mitochondria and micro-organisms (Ciferri and Tiboni, 1973).

It is clear from the results in Table 5.2 that fuscicid acid inhibits respiration and protein synthesis at between 50-500μM. Whether or not this inhibitor is affecting protein synthesis directly, or only as a result of inhibiting respiration, cannot be deduced from the results presented here.

C. EFFECTS OF INHIBITORS OF NUCLEIC ACID SYNTHESIS ON AMINO ACID INCORPORATION AND ON RESPIRATION BY ISOLATED MUNG BEAN MITOCHONDRIA

In Table 5.3 the effects of various inhibitors of nucleic acid synthesis on amino acid incorporation and respiration are shown. It is clear from the small effect observed with Actinomycin D, that continued RNA synthesis was not necessary for continued protein synthesis. In Chapter 7 it is shown that this level of Actinomycin D completely inhibits RNA synthesis. The inhibition observed with ethidium bromide would appear to be due to its effect on respiration rather than a direct effect on protein synthesis.

D. EFFECTS OF INHIBITORS AND UNCOUPLERS OF OXIDATIVE PHOSPHORYLATION ON AMINO ACID INCORPORATION AND ON RESPIRATION BY ISOLATED MUNG BEAN MITOCHONDRIA

The effects on amino acid incorporation and respiration of some inhibitors and uncouplers of oxidative phosphorylation are shown in Table 5.4. Dinitrophenol, the uncoupling agent, was effective in inhibiting protein synthesis. The rate of oxygen uptake of the mitochondria uncoupled by dinitrophenol was considerably reduced compared to that of the uninhibited mitochondria. Cyanide, an inhibitor of cytochrome oxidase, inhibited both respiration and protein synthesis.

Antimycin and Rotenone, potent inhibitors of respiration, also inhibited protein synthesis by the isolated mitochondria.

E. SENSITIVITY OF AMINO ACID INCORPORATION AND RESPIRATION TO RIBONUCLEASE

The results of an experiment in which ribonuclease was included with the mitochondria in the incubation mixture are shown in Table 5.5. It is clear from these results that ribonuclease had no effect on protein synthesis or on respiration.

The insensitivity of the system to ribonuclease was considered to indicate that the observed protein synthesis was not that of contaminating cytoplasmic ribosomes. This criterion has been used by others working with isolated organelles (Moore et al, 1971, and Ellis, 1975).
### TABLE 5.3

**Effect of inhibitors of nucleic acid synthesis on incorporation of [\(^{35}\)S] methionine by isolated mung bean mitochondria**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Final Concentration ( \mu g/ml )</th>
<th>Radioactivity incorporated ( \text{cpm/mg protein/60min} )</th>
<th>% Incorporation</th>
<th>Effects on Respiration</th>
<th>% Respiration</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>18,386</td>
<td>100</td>
<td>4.96</td>
<td>100</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>10</td>
<td>17,650</td>
<td>96</td>
<td>4.74</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>17,000</td>
<td>92</td>
<td>4.74</td>
<td>95</td>
</tr>
<tr>
<td>Ethidium Bromide</td>
<td>10</td>
<td>1,522</td>
<td>8</td>
<td>2.25</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>2,022</td>
<td>11</td>
<td>1.00</td>
<td>20</td>
</tr>
</tbody>
</table>

The details of the experimental procedure used are given in Table 5.1. The results are expressed as percentages of the complete uninhibited system.
<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Incorporation of $[^{35}S]$ methionine</th>
<th>% Incorporation</th>
<th>Respiratory</th>
<th>% Respiration</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>86,500</td>
<td>100</td>
<td>4.0</td>
<td>100</td>
</tr>
<tr>
<td>Di-nitrophenol</td>
<td>0.1 mM</td>
<td>13,058</td>
<td>15</td>
<td>1.0</td>
<td>25</td>
</tr>
<tr>
<td>Potassium cyanide</td>
<td>1.0 mM</td>
<td>9,328</td>
<td>11</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Antimycin</td>
<td>5 μg/ml</td>
<td>13,400</td>
<td>20</td>
<td>0.6</td>
<td>15</td>
</tr>
<tr>
<td>Rotenone</td>
<td>50 μg/ml</td>
<td>5,746</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The details of the experimental procedure used are given in Table 5.1. The results are expressed as percentages of the complete uninhibited system.
TABLE 5.5  
Incorporation of [\textsuperscript{35}S] methionine by isolated mung bean mitochondria in the presence of ribonuclease

<table>
<thead>
<tr>
<th>Concentration of ribonuclease ((\mu)g/ml)</th>
<th>Radioactivity incorporated cpm/mg protein/60min</th>
<th>% Incorporation</th>
<th>Effects on Respiration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Respiratory Control</td>
</tr>
<tr>
<td>0</td>
<td>38,780</td>
<td>100</td>
<td>4.2</td>
</tr>
<tr>
<td>20</td>
<td>41,690</td>
<td>107</td>
<td>100</td>
</tr>
<tr>
<td>40</td>
<td>38,220</td>
<td>99</td>
<td>4.1</td>
</tr>
</tbody>
</table>

The details of the experimental procedure used are given in Table 5.1. The results are expressed as percentages of the complete uninhibited system.
F. DISCUSSION

It is clear from the results presented in this chapter that the sensitivity of plant mitochondrial protein synthesis to inhibitors of protein synthesis is in broad agreement with that established for mitochondria of other organisms. It is also apparent that, in the short-term at least, continued RNA synthesis is not necessary for mitochondrial protein synthesis but, under the conditions described, oxidative phosphorylation is required.

A more detailed discussion of the results in this chapter is given in Chapter 8.
CHAPTER 6: CHARACTERISATION OF THE PRODUCTS OF MITOCHONDRIAL PROTEIN SYNTHESIS

A. INTRODUCTION

In the previous two chapters, evidence was presented that the observed incorporation of radioactive amino acids by isolated mitochondria, was indeed mitochondrial protein synthesis. This chapter considers the characterisation of the products of mitochondrial protein synthesis.

The number and approximate molecular weights of the polypeptides synthesised by mitochondria isolated from artichoke was estimated. A comparison was made of the polypeptides synthesised by mitochondria isolated from mung bean hypocotyl and fresh and aged artichoke tuber.

Preliminary experiments were performed using artichoke tuber tissue in which cytoplasmic protein synthesis was selectively inhibited by cycloheximide, to compare the mitochondrial proteins synthesised in vivo with those proteins made by isolated mitochondria in vitro.

Experiments were also performed to determine the localisation of the mitochondrially synthesised protein. The effect of organic solvent extraction and of alkali on mitochondrial proteins was also investigated to see if it would allow further fractionation of the radioactive products. Lastly the effects of anaerobiosis on the polypeptides synthesised by isolated mitochondria was examined.

B. ESTIMATION OF THE NUMBER OF POLYPEPTIDES SYNTHESISED BY ISOLATED ARTICHOKE TUBER MITOCHONDRIA

Isolated artichoke mitochondria were incubated in the mannitol based medium with 50μCi [35S] methionine for 1 hour. The products of protein synthesis were then analysed by electrophoresis in SLS-polyacrylamide tube gels. When stained with Comassie blue, 20 or more protein bands became visible, (Fig. 6.1); when the gels were sliced and the radioactivity measured at least 7 labelled polypeptides were discernible.

Towards the end of this investigation fractionation and visualisation of the labelled polypeptides by SLS-polyacrylamide electrophoresis performed on a slab gel and subsequent autoradiography allowed a greater resolution of the mitochondrial translation products (Fig. 6.2). At least 17 labelled polypeptides were detected by this method.

C. MOLECULAR WEIGHTS OF THE POLYPEPTIDES SYNTHESISED BY ISOLATED ARTICHOKE MITOCHONDRIA

The apparent molecular weights of the major polypeptides synthesised by isolated
Figure 6.1
Analysis of the products of protein synthesis by mitochondria isolated from artichoke tuber on SLS-polyacrylamide tube gels.

Artichoke mitochondria were incubated with 50μC of [35S]-methionine for 1 hour at 20°C in the mannitol based medium. The mitochondrial sample was diluted with sample buffer prior to loading on 15% polyacrylamide tube gels, as described in Chapter 2B5iii. The samples were electrophorised for:

A - 4 hours
B - 6 hours and
C - 8 hours

Details of the subsequent staining, scanning, and radioactive analyses of gels is given in Chapter 2B5iv. The solid line represents the absorbance of 265nm and the histogram shows the radioactivity in each 1mm gel fraction.
fig 6.1

A

B

C

cpm \times 10^2

e_{265}\text{nm} \ (\text{arbitrary units})


cpm \times 10^2

e_{265}\text{nm} \ (\text{arbitrary units})


cpm \times 10^2

e_{265}\text{nm} \ (\text{arbitrary units})

Electrophoretic mobility (cm)
Figure 6.2

Analysis of the products of protein synthesis by mitochondria isolated from artichoke tuber on SLS-polyacrylamide slab gels.

The details are the same as for Fig. 6.1 except that slab gel electrophoresis was used, as described in Chapter 2B5iii. A photograph of the stained gel (B) and the autoradiograph of this gel (A) is shown opposite. The molecular weights of both the 9 major radioactivity labelled polypeptides and the 7 most prominent polypeptides stained by commassie blue are shown.
Fig 6.2

mol. wts. \( \times 10^3 \)

<table>
<thead>
<tr>
<th>mol. wts.</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>96.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>57.5</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td>57.5</td>
<td>b</td>
<td></td>
</tr>
<tr>
<td>44.5</td>
<td>c</td>
<td></td>
</tr>
<tr>
<td>25.5</td>
<td>d</td>
<td></td>
</tr>
<tr>
<td>21.5</td>
<td>e</td>
<td></td>
</tr>
<tr>
<td>17.5</td>
<td>f</td>
<td></td>
</tr>
<tr>
<td>16.5</td>
<td>g</td>
<td></td>
</tr>
<tr>
<td>10.5</td>
<td>h</td>
<td></td>
</tr>
</tbody>
</table>

electrophoresis
mitochondria were estimated from gels calibrated with standards of known molecular weights by the method described by Weber and Osborn (1969). Fig. 6.3 shows a plot of electrophoretic mobility of the standard polypeptides against the log of their molecular weights on 10% and 15% tube gels and a 15% slab gel.

Using this standard calibration the molecular weights of the labelled mitochondrial polypeptides were estimated and are shown in Table 6.1.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Approximate molecular weights</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10% tube gel</td>
</tr>
<tr>
<td>a</td>
<td>55,000</td>
</tr>
<tr>
<td>b</td>
<td>37,000</td>
</tr>
<tr>
<td>c</td>
<td>29,000</td>
</tr>
<tr>
<td>d</td>
<td>21,000</td>
</tr>
<tr>
<td>e</td>
<td>18,000</td>
</tr>
<tr>
<td>f</td>
<td>12,000</td>
</tr>
<tr>
<td>g</td>
<td></td>
</tr>
<tr>
<td>h</td>
<td></td>
</tr>
<tr>
<td>i</td>
<td></td>
</tr>
</tbody>
</table>

Artichoke tuber mitochondria were isolated and incubated with $[^{35}S]$ methionine as described in Fig. 6.1. The radioactive mitochondrial protein was electrophorised in parallel with markers of known molecular weights. From the relative mobilities of the radioactive peaks to these standards, their molecular weights were estimated.

There is reasonable agreement between the 3 sets of values. However it must be realised that this method of estimating the molecular weights of the polypeptides is approximate, particularly for the lower molecular weight species. Only peak a appears coincident with a major polypeptide stained by Commmassie blue (Fig. 6.2).

D. INVESTIGATION OF A “TAILING” PHENOMENON

During the early stages of this investigation, a regular feature of the radioactive profiles of the gels was a tail of counts at the start of the gel. For example see Fig. 6.4B. The tail at the top of the gel was presumably due to insoluble radioactive polypeptides remaining aggregated there. As already stated in Chapter 2B5ii the protein sample, prior to electrophoresis was diluted with half a volume of
Figure 6.3
Calibration plot to estimate the molecular weights of the labelled polypeptides synthesised by isolated artichoke mitochondria on:

A - 10% Acrylamide tube gel
B - 15% Acrylamide tube gel
C - 15% Acrylamide slab gel.

The molecular weights were calculated by running in parallel proteins of known molecular weights:
1. bovine serum albumin (68,000).
2. large subunit of Fraction 1 (55,500).
3. ovine albumin (43,000).
4. glucose-3-phosphate dehydrogenase (37,000).
5. chymotrypsinogen (24,500).
6. lysozyme (14,500).
fig. 6.3

A

B

C

log Mol. Wt.

Mobility

log Mol. Wt.

Mobility

log Mol. Wt.

Mobility
sample buffer. An additional step which was later omitted, was the incubation of the diluted mitochondrial protein sample at 70°C for 20 minutes. This was considered to facilitate maximum binding of SLS to polypeptides, thereby completely dissociating polypeptide subunits from each other.

It seemed possible that increasing the concentration of SLS could reduce this tail. However, increasing the final concentration of SLS to 5% had no effect on the size of the "tail" nor upon the radioactive profile observed.

It was thought that omission of the heat treatment might reduce the "tail". To test this, half of a radioactive mitochondrial sample was incubated at 70°C for 20 minutes while the other half was not. The samples were then electrophorised and the radioactivity in the gel was estimated. Fig. 6.4 shows the results of such an experiment. It was clear from these results that the pre-heating treatment was responsible for the presence of the insoluble radioactive material at the top of the gel. The fractionation of the unlabelled polypeptides stained by commassie blue was not affected by the omission of the heat treatment.

E. THE EFFECT OF PROTEASE ON THE PRODUCTS OF PROTEIN SYNTHESIS BY MITOCHONDRIA ISOLATED FROM ARTICHOKE TUBER

Confirmation that the radioactive peaks are indeed polypeptides is illustrated by the result shown in Fig. 6.5. A labelled mitochondrial preparation was divided into two, one half was incubated with 10µg of protease (Streptomyces griseus, type V) at 37°C for 4 hours, and then electrophorised in parallel with the untreated control sample. The radioactively labelled peaks were completely sensitive to digestion by protease (Fig. 6.5B) whereas in the control incubation (Fig. 6.5A) no degradation of the labelled peaks was evident, implying that the preparation was not markedly contaminated by endogenous proteases.

F. COMPARISON OF THE PRODUCTS OF PROTEIN SYNTHESIS BY MITOCHONDRIA ISOLATED FROM DIFFERENT TISSUES

1. A COMPARISON OF THE PRODUCTS OF PROTEIN SYNTHESIS BY MITOCHONDRIA ISOLATED FROM MUNG BEAN AND ARTICHOKE TUBER.

Fig. 6.6 shows a comparison of the products of protein synthesis by mitochondria isolated from mung bean hypocotyl and artichoke tuber. It can be seen that both the number and electrophoretic mobilities of the major labelled polypeptides are similar. This can be seen more clearly in Fig. 6.7.
Figure 6.4

The effect of heat treatment prior to gel electrophoresis on the products of protein synthesis by mitochondria isolated from artichoke tuber

Details of the experimental procedures used are as given for Fig. 6.1 except that in A the protein sample was not heated before electrophoresis while in B the sample was heated at 70°C for 20 minutes prior to loading on the gel.
**Fig 6.4**

- **A**
  - Graph with c.p.m. x 10^2 on the y-axis and electrophoretic mobility (cm) on the x-axis.
  - Peaks labeled from 'a' to 'i'.

- **B**
  - Graph with c.p.m. x 10^4 on the y-axis and electrophoretic mobility (cm) on the x-axis.
  - Peaks labeled from 'a' to 'i'.
Figure 6.5

The effect of protease on the products of protein synthesis by isolated artichoke tuber mitochondria

Details of experimental procedures used are as given for Fig. 6.1 except that two identical preparations of the labelled mitochondrial protein were incubated at 37°C for 4h, in the case of sample A there were no additions, while sample B contained 10μg of protease. The samples were loaded on to the gels after the addition of sample buffer and subjected to electrophoresis for 6h.
fig 6.5

A

Electrophoretic mobility (cm)

B
Figure 6.6

Comparison of proteins synthesised by mitochondria isolated from artichoke and mung bean

Experimental procedures used are given in Fig. 6.1. Polyacrylamide gel electrophoresis of the products of protein synthesis by mitochondria isolated from artichoke (A and C), and mung bean (B and D). Gels A and B were electrophorised for 6h, and gels C and D for 8h.
Figure 6.7

Comparison of the polypeptides synthesised by mitochondria isolated from artichoke and mung bean

Experimental procedures are given in Fig. 6.1. Polyacrylamide slab gel electrophoresis was performed as described in Chapter 2B5iii. Photographs of the gel of mitochondrial polypeptides fractionated by this method are shown opposite.

(A) mitochondria isolated from artichoke
(D) mitochondria isolated from mung bean.

The polypeptides synthesised by the mitochondria were detected by autoradiography of the gel

(B) autoradiograph of the polypeptides synthesised by artichoke mitochondria
(C) autoradiograph of the polypeptides synthesised by mung bean mitochondria.
which is a comparison of the labelled product from artichoke and mung bean fractionated on a slab gel and autoradiographed.

2. COMPARISON OF THE PRODUCTS OF PROTEIN SYNTHESIS BY MITOCHONDRIA ISOLATED FROM FRESH AND AGED ARTICHOKE TUBER.

Earlier results (Table 4.5, Chapter 4) had suggested that mitochondria isolated from artichoke tuber discs aged for 12 hours were more active than mitochondria isolated from fresh tuber tissue in the incorporation of radioactive amino acid into protein. As it has been suggested (Sakano and Asahi, 1971) that there is active biogenesis of mitochondria during ageing of tissue discs, it was of interest to compare the in vitro products of protein synthesis by mitochondria isolated from fresh and aged tissue. In addition, this tissue was suitable for an examination of the in vivo products of mitochondrial protein synthesis by labelling intact cells in the presence of cycloheximide to inhibit any contribution by cytoplasmic protein synthesis.

Fig. 6.8 shows a comparison of the products of protein synthesis of mitochondria isolated from fresh and 12 hours aged artichoke tuber. The radioactive profiles of the two gels are very similar, the electrophoretic mobilities of the peaks being virtually the same. There are, however, slight differences, e.g. peak i in gel B of Fig. 6.8 is broader than the corresponding peak in gel A of the same figure. Also peak e is more prominent in gel A than gel B. The range of polypeptides synthesised by mitochondria isolated from fresh and aged artichoke tuber appear to be very similar, although the relative sizes of individual peaks may differ.

G. THE IN VIVO PRODUCTS OF MITOCHONDRIAL PROTEIN SYNTHESIS

It was clearly desirable to demonstrate that the pattern of in vitro radioactive polypeptide products of mitochondrial protein synthesis was similar to that synthesised by the organelle in vivo. In comparison with a number of other tissues artichoke tubers were considered most suitable for in vivo labelling because it had been reported that biogenesis of mitochondria occurred in tuber tissue during the ageing process in distilled water (Sakano and Asahi, 1971) and in addition, the tissue could be obtained in near sterile condition.

It has previously been shown that mitochondria isolated from artichoke tuber tissue aged for 12 hours incorporated amino acid into protein at a much greater rate than mitochondria isolated from fresh tissue (Chapter 4F). The procedure adopted for the in vivo labelling of the products of mitochondrial protein synthesis was to age artichoke tuber tissue for 12 hours prior to the addition of cycloheximide and radioactive amino acid. The rationale being that the cycloheximide would
Polyacrylamide gel electrophoresis of the products of protein synthesis by mitochondria isolated from (A) fresh artichoke tuber and (B) artichoke tuber aged for 12h. Experimental procedures used are given in Fig. 6.1.
fig 68

A

B

Electrophoretic mobility (cm)
inhibit cytoplasmic protein synthesis, while leaving, at least in the short-term, mitochondrial protein synthesis unaffected. The in vivo labelled mitochondria were then isolated by the normal method (Chapter 2B4) and the constituent polypeptides analysed by SLS-polyacrylamide gel electrophoresis.

To examine the suitability of this approach for the investigation of in vivo mitochondrial protein synthesis the following experiment was performed. Two flasks containing sterile diced artichoke tuber, were aged in the manner described in Chapter 2B3. It was thought that the inclusion of D-threo-chloramphenicol (CAP) at a low concentration, in one flask, would serve two purposes. Firstly it would prevent growth of any bacteria present, and secondly, considering the results reported by Tzagoloff and Akai (1972) it appeared possible that ageing the tissue in CAP might enhance the clarity of results. Tzagoloff and Akai found that in order to obtain a clear result from in vivo labelling of yeast mitochondria, a period of growth in the presence of CAP prior to addition of cycloheximide and radioactive label, improved the resolution of the radioactively labelled polypeptides. However, it was realised that in the plant system, CAP may be irreversibly absorbed thus confusing the results. To overcome these possible complications a flask containing 100g of artichoke discs was aged in sterile distilled water (flask 1), while in a second flask 100g of discs were aged in sterile distilled water containing 50µg/ml CAP (flask 2). After 12 hours both batches of discs were aged for a further period of 1 hour in three changes of sterile distilled water in an attempt to wash out the residual chloramphenicol in the second batch of tissue.

Both batches of discs were then transferred to solutions of cycloheximide (20µg/ml). These solutions were replaced after 20 minutes by equal volumes of cycloheximide at the same concentration. Forty-five minutes after the introduction of cycloheximide 250µCi [35S]-methionine was added to each flask and the discs were then aged for a further 6 hours.

After this labelling period the discs were removed from the flasks and the mitochondria isolated in the usual way. Fig. 6.9 compares the radioactive profiles of the mitochondrial polypeptides isolated from tissue aged in sterile distilled water (gel C) or in 50µg/ml CAP (gel B) prior to the addition of cycloheximide, with the in vitro labelled products obtained from mitochondria extracted from fresh tissue (gel A).

The radioactive profiles of gels A and C are similar. The most prominent radioactive peaks are common to both gels. There are minor differences, e.g., peak c is much more prominent in vivo than in the in vitro label. Peak d is more prominent in the in vitro label while the relative proportions of peaks g and i differ noticeably.

As can be seen from gel B, CAP at 50µg/ml inhibited incorporation of [35S] methionine. This is contrary to the predictions suggested from the work of Leaver and Edelman (1965). However,
Artichoke tuber discs were aged for 12h as described in Chapter 2B3. The mitochondria were then labelled in vivo with $[^35]S$ methionine in the presence of cycloheximide (20μg/ml) before being isolated (gels B and C) or the mitochondria were isolated from fresh artichoke tuber and labelled in vitro (gel A). The mitochondria were then fractionated by SLS-polyacrylamide gel electrophoresis, and the radioactivity estimated as described for Fig. 6.1.

A mitochondria labelled in vitro
B mitochondria labelled in vivo after being aged in 50μg/ml CAP
C mitochondria labelled in vivo after being aged in sterile distilled water.
fig 6.9

A

B

C

Electrophoretic mobility (cm)

cpm x 10^3

cpm x 10^5

cpm x 10^4

E_{v30} (arbitrary units)

E_{v30} (arbitrary units)

E_{v30} (arbitrary units)
considering the results reported earlier in this investigation (Table 5.1) mitochondrial protein synthesis in vitro was inhibited by this level of CAP.

In gel B peak i is completely absent. As peak i is present in gel C of the same figure it is clear that this peak cannot be free methionine.

There have been suggestions that this lowest molecular weight peak (peak i) may not be a polypeptide, but could be phospholipid (Küntzel 1975). This seems unlikely considering that there are no sulphur containing lipids, the peak is sensitive to protease (Fig. 6.5), and the results presented in Fig. 6.8B, which shows that after labelling mitochondria under conditions when lipid metabolism would not be expected to be inhibited, peak i is absent.

H. LOCATION OF PROTEINS SYNTHESISED BY MITOCHONDRIA ISOLATED FROM MUNG BEAN

In view of earlier reports that the products of mitochondrial protein synthesis in ascomycetes and animals were components of the inner mitochondrial membrane (Beattie et al, 1967) it was considered of interest to examine the location of the protein synthesised by plant mitochondria. The following experiment describes a fractionation of the mitochondria into a membrane and soluble fraction. The method is based on that used by Eaglesham and Ellis (1974) for fractionation of the soluble and membrane components of chloroplasts.

An in vitro labelled mitochondrial preparation was frozen and thawed twice. It was then diluted tenfold with cold 2.5mM-Tris-glycine pH 8.5, and centrifuged at 100,000 x g for 2 hours in an MSE 3 x 3ml swing-out rotor. The supernatant was carefully removed and reduced to a suitable volume by dialysis against a saturated solution of polyethylene glycol 4000. The membrane pellet was washed by resuspension in 10ml of 2.5mM-Tris-glycine pH 8.5 and then centrifuged at 20,000 x g for 20 minutes in an MSE 18 centifuge. The pellet was resuspended as before and the wash cycle repeated twice. Finally the pellet was resuspended in a suitable SLS-containing buffer for electrophoresis. The recoveries from such an experiment using mung bean hypocotyl as a source of mitochondria, are shown in Table 6.2. Fig. 6.10 shows gels of the various fractions obtained from the same experiment.

In agreement with Beattie et al (1967) the majority (98%) of radioactive polypeptides synthesised by the mitochondria are associated with the membrane fraction. The small peak of counts (molecular weight 57,000) observed in the soluble fraction was a reproducible feature, and also appeared in similar fractionations of radioactive artichoke mitochondria.
Figure 6.10

SLS-polyacrylamide gel electrophoresis of the membrane and soluble polypeptides fractionated from in vitro labelled mitochondria

Mitochondria were isolated from mung bean hypocotyl and labelled with $[^{35}\text{S}]$ methionine as described in Chapter 2B. Part of the radioactive sample was fractionated into membrane and soluble fractions as described in Chapter 6H.

A unfractionated mung bean mitochondria
B membrane fraction
C soluble fraction.
TABLE 6.2
Fractionation of in vitro labelled mung bean mitochondria into membrane and soluble fractions

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg)</th>
<th>Counts/Minute</th>
<th>Specific Activity (cpm/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole mitochondria</td>
<td>0.93</td>
<td>323,347</td>
<td>347,684</td>
</tr>
<tr>
<td>Membrane fraction</td>
<td>0.45</td>
<td>203,280</td>
<td>451,733</td>
</tr>
<tr>
<td>Soluble fraction</td>
<td>0.11</td>
<td>4,400</td>
<td>40,000</td>
</tr>
</tbody>
</table>

Mitochondria were extracted from mung bean hypocotyl and incubated with $[^35]S$ methionine for 60 minutes under standard conditions. 0.93mg of the mitochondrial protein was fractionated into membrane and soluble components as described in Chapter 6H.

I. EXTRACTION WITH ORGANIC SOLVENTS OF RADIOACTIVE PROTEIN FROM MITOCHONDRIA

Several reports suggested the mitochondrially synthesised proteins were preferentially extracted with organic solvents (Küntzel et al. 1975). This had obvious relevance to the purification and subsequent identification of these proteins, the ultimate aim of this study. The next experiment describes the results of an experiment in which in vitro labelled mitochondria were extracted with organic solvents. The extraction described here is based on that used by Tzagoloff and Akai (1972).

A radioactive preparation of artichoke mitochondria was extracted with 1ml of 90% methanol for 10 minutes at 25°C. The solution was then centrifuged at 8000 x g for 6 minutes and the supernatant carefully removed (Extract I). The pellet was resuspended in 10ml of a 2:1 (V/V) chloroform-methanol mixture containing 10mM-HCl and incubated at 50°C for 30 minutes. The solution was then centrifuged at 8000 x g for 6 minutes and the supernatant carefully removed (Extract II). The pellet, referred to as the residue, was solubilised in the appropriate buffer for electrophoresis. Both extracts were dried down under nitrogen and then redissolved in SLS-buffer for electrophoresis.

Table 6.3 shows the distribution of labelled protein in the resulting extracts. The polyacrylamide gel fractionation of Extract I showed no protein by commassie blue stain or radioactivity. Fig. 6.11 shows gels of Extract II and the residue. It is clear from a comparison of the radioactive profiles of gels A, B and C presented in Fig. 6.11 that the bulk of the radioactivity remained with the residue. However, virtually all of peak i (mol. wt. 10,500) was removed in Extract II.
Mitochondria from artichoke tuber were isolated and incubated with \[^{35}\text{S}]\text{methionine}\) in vitro as described in Fig. 6.1. The labelled mitochondria were extracted with 90% methanol, and then with a 2:1 (v/v) chloroform-methanol mixture. The residue and extract were electrophorised and estimated for radioactivity as described in Fig. 6.1.

A unextracted artichoke mitochondria
B residue from chloroform-methanol extract
C chloroform-methanol extract.
TABLE 6.3
Extraction with organic solvents of radioactive protein isolated from in vitro labelled artichoke tuber mitochondria

<table>
<thead>
<tr>
<th>Source</th>
<th>Protein (mg)</th>
<th>% Total Protein</th>
<th>Counts/Minute</th>
<th>% Total Counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract I</td>
<td>0.02</td>
<td>8</td>
<td>1,000</td>
<td>1</td>
</tr>
<tr>
<td>Extract II</td>
<td>0.08</td>
<td>32</td>
<td>30,500</td>
<td>19</td>
</tr>
<tr>
<td>Residue</td>
<td>0.13</td>
<td>52</td>
<td>110,600</td>
<td>73</td>
</tr>
<tr>
<td>Recovery</td>
<td>0.13</td>
<td>92</td>
<td></td>
<td>93</td>
</tr>
</tbody>
</table>

Mitochondria were isolated from artichoke tuber and incubated with [35S] methionine for 60 minutes under standard conditions. The labelled mitochondria (0.25 mg protein, 150,000 cpm) was then extracted with organic solvents as described in the text.

J. THE EFFECT OF TREATING THE LABELLED MITOCHONDRIAL POLYPEPTIDE PRODUCTS WITH SODIUM HYDROXIDE

Tzagoloff et al (1973) reported an unusual effect of sodium hydroxide on the protein products synthesised by yeast mitochondria. Namely that there was a conversion of the higher molecular weight polypeptide products to a small polypeptide. The following experiment describes the results of a similar treatment of labelled mitochondrial protein isolated from artichoke.

Part of a radioactive mitochondrial preparation was made 0.16 M with respect to NaOH, and incubated at 25°C for 15 minutes prior to electrophoresis. The radioactive profile of the gel of protein treated in this manner is shown in Fig. 6.12 gel B. Comparing this gel with the untreated sample shown in gel A of the same figure, it is clear that 0.16 M–NaOH has little obvious effect on the molecular weights of the polypeptides synthesised by the mitochondria. Certainly there is not the dramatic effect reported by Tzagoloff. Similar results to those described above, were obtained in another experiment using mung bean as the source of mitochondria.

K. EFFECT OF ANAEROBISIS ON PROTEIN SYNTHESIS BY ISOLATED MITOCHONDRIA

A recent report from Groot and Poyton (1975) claimed to show that oxygen was directly involved in the control of mitochondrial protein synthesis. They showed that two of the three sub-units of cytochrome oxidase synthesised by isolated yeast mitochondria were not made under anaerobic conditions while the other sub-unit continued to be synthesised under these conditions. It was therefore considered of interest to examine, in a more general sense, the effect of anaerobisis on
Mitochondria were isolated from artichoke tuber and incubated with \([^{35}\text{S}]\) methionine as described in Fig. 6.1. The radioactive mitochondrial preparation was adjusted to 0.16M with NaOH and incubated at 25°C for 15 minutes. A half volume of sample buffer was added, the sample electrophorised, and the gels estimated for radioactivity as described for Fig. 6.1.

A untreated artichoke mitochondria
B artichoke mitochondria treated with 0.16M-NaOH.
fig 6.12

A

Electrophoretic mobility (cm)

B

E265nm (arbitrary units)
protein synthesis by isolated plant mitochondria. To this end, the following modifications were made to the usual incubation regime.

Firstly, the incubations which were to be anaerobic had liquid paraffin carefully layered over the incubation mixture, prior to the addition of the mitochondria.

Secondly, an energy generating system was included in all but one control incubation. It was argued that in the absence of oxygen, oxidative phosphorylation could not occur and therefore an alternative energy source would be required. The final concentrations of the components of the energy generating system were:

- 1mM-ATP
- 1mM-GTP
- 8mM-creatine phosphate
- 100μg/ml creatine phosphokinase

This was in addition to the usual 1mM-ADP, 20mM-malate.

Thirdly, in all incubations radioactive methionine was used to initiate the incorporation. This was done 10 minutes after the mitochondria had been added to the incubation medium. This delay was designed to allow the mitochondria to consume the dissolved oxygen in the anaerobic incubation mixture. It was calculated from the oxygen electrode trace that the incubation medium would be anaerobic after 7 minutes. Sixty minutes after the initiation of the reaction the radioactive methionine incorporated into protein was measured. The results are shown in Table 6.4.

<table>
<thead>
<tr>
<th>Addition</th>
<th>State</th>
<th>Counts incorporated (cpm/mg protein/60min)</th>
<th>% Incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Aerobic</td>
<td>29,720</td>
<td>100</td>
</tr>
<tr>
<td>Energy generating system</td>
<td>Aerobic</td>
<td>19,318</td>
<td>65</td>
</tr>
<tr>
<td>None</td>
<td>Anaerobic</td>
<td>14,260</td>
<td>48</td>
</tr>
<tr>
<td>Energy generating system</td>
<td>Anaerobic</td>
<td>46,108</td>
<td>140</td>
</tr>
</tbody>
</table>

TABLE 6.4
Effect of anaerobiosis and of addition of an energy generating system on the incorporation of [³⁵S]-methionine into protein by isolated mitochondria

Mitochondria were isolated from artichoke tuber as described in Chapter 2B4. The mitochondria were incubated in the mannitol based incubation medium with the additions detailed above. The energy generating system was as described in Chapter 6K, and the tubes were made anaerobic by the layering of liquid paraffin over the incubation mixture in the tubes.
The addition of the energy generating system under aerobic conditions depressed incorporation by 35% relative to the control, however, in anaerobic conditions, the addition of the energy generating system stimulated the incorporation by 40%. Under anaerobic conditions, amino acid incorporation was not completely inhibited, implying the presence of a pool of ATP or that glycolysis was occurring.

The radioactive profile of the gels from the same experiment described above are shown in Fig. 6.13. Considering this figure, it is apparent that if there are differences in the polypeptides synthesised under aerobic and anaerobic conditions, they are subtle differences rather than obvious ones.

Comparing gels A and B, Fig. 6.13, peak d would appear to have the "shoulder" on its leading edge missing under anaerobic conditions. In a repeat of this experiment the tube gels appeared somewhat similar, though not identical to those presented in Fig. 6.13. Peak d appeared of less significance in the anaerobic incubation. In addition a very small peak, between b and d, appeared relatively smaller in the anaerobic incubation. This is not really apparent in Fig. 6.13.

The resolution of the techniques used here were considered insufficient to answer conclusively the question of whether differences exist between the proteins synthesised by plant mitochondria under aerobic and anaerobic conditions. However, they are suggestive that further experimentation in this field would be worthwhile when it is possible to increase the resolving power of existing polypeptide fractionation techniques.

L. THE EFFECT OF DIFFERENT INCUBATION MEDIA ON THE PRODUCTS OF ISOLATED MITOCHONDRIAL PROTEIN SYNTHESIS

In view of the dramatic effect of potassium chloride on amino acid incorporation into protein by isolated chloroplasts reported by Ellis and his co-workers (1975) considerable time was spent early on in this investigation, evaluating its effectiveness as an osmoticum in the isolated mitochondrial system.

The characteristics of the incorporation of radioactive amino acids by isolated mitochondria incubated in the standard medium described in Chapter 4B, except that mannitol was omitted, and the concentration of KCl was increased to 112mM, were basically similar to that observed for the mannitol based incubation medium. The incorporation was dependent on the addition of both an oxidisable substrate, and ADP. However, the final level of incorporation was about half that observed when mannitol was the osmoticum. When the protein products of isolated mitochondria incubated in this medium were examined by polyacrylamide gel electrophoresis and estimated for radioactivity as
Mitochondria were isolated from artichoke tuber as described in Chapter 2B4. The mitochondria were incubated in the mannitol based medium which had been modified in two ways. Firstly, the mitochondria incubated in “B” were covered by a layer of liquid paraffin in order to make this incubation anaerobic. Secondly, to both incubations, an energy generating system was added, as detailed in Chapter 6K. At the end of the 60-minute incubation, the mitochondrial proteins were fractionated and estimated for radioactivity as described for Fig. 6.1.

A aerobic
B anaerobic.
described in Chapter 2B5, the radioactive peaks were much less distinct than had been observed with the mannitol based incubation medium.

Several possible causes of the indistinct nature of the radioactive peaks were suggested and tested. For example indiscriminate binding of unincorporated radioactive amino acid to mitochondrial protein, or endogenous protease activity digesting the newly synthesised polypeptides. However, none of these explanations proved correct. With hindsight, considering the success in obtaining meaningful results with mannitol as the osmoticum, it would appear that inaccurate translation was responsible for the poor results obtained with KCl as the osmoticum.

M. DISCUSSION

The results presented in this chapter suggest that plant mitochondria synthesise at least 8 distinct polypeptides, ranging in molecular weight from about 10,500 to 57,000. These polypeptides are hydrophobic, and are mainly associated with the mitochondrial membrane. A more detailed discussion of the properties of the polypeptides synthesised by plant mitochondria will be given in Chapter 8.
CHAPTER 7: RNA SYNTHESIS BY ISOLATED MITOCHONDRIA

A INTRODUCTION

It seemed worthwhile using the techniques already developed for in vitro mitochondrial protein synthesis to examine mitochondrial RNA synthesis. It was hoped that the products of RNA synthesis might resemble mitochondrial rRNA and/or indicate that the mitochondria could synthesise mRNA.

B. CHARACTERISTICS OF MITOCHONDRIAL RNA SYNTHESIS

Mitochondria were prepared by standard procedures (Chapter 2B4) and incubated in the mannitol based incubation medium as described previously (Chapter 4B) with the addition of $^3$H-uridine (specific activity 7Ci/m mole). The method of estimating the incorporation of uridine into RNA was similar to that described for assaying the incorporation of radioactive amino acid into protein (Chapter 2B5i) except that the second TCA wash at 70°C was omitted, and the scintillation counter was set to count tritium.

A time course of incorporation of uridine is shown in Fig 7.1. It is similar in shape to that obtained for the incorporation of radioactive amino acid (Fig. 4.1). The incorporation is clearly not bacterial being sensitive to the omission of malate and ADP. Table 7.1 shows in addition to these two controls, the sensitivity of the incorporation to Actinomycin D. This sensitivity to Actinomycin D contrasts with the lack of effect of this inhibitor on protein synthesis already reported (Chapter 5C).

<table>
<thead>
<tr>
<th>System</th>
<th>$[^3]$H Uridine Incorporated</th>
<th>% Incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>93,010</td>
<td>100</td>
</tr>
<tr>
<td>Minus malate</td>
<td>4,280</td>
<td>5</td>
</tr>
<tr>
<td>Minus ADP</td>
<td>6,160</td>
<td>7</td>
</tr>
<tr>
<td>Complete + Actinomycin D (20μg/ml)</td>
<td>5,750</td>
<td>6</td>
</tr>
</tbody>
</table>

Mitochondria were isolated from artichoke tuber and incubated in the mannitol based medium, as described for Table 5.1, except that $[^3]$H uridine was used as the source of radioactivity.
Figure 7.1

Time course of incorporation of [³H]-uridine into nucleic acid by isolated artichoke mitochondria

Mitochondria were isolated from artichoke tuber by the method described in Chapter 2B4. The mitochondria were incubated with 100μCi [³H]-uridine in the mannitol medium and the time course of incorporation followed by the methods described in Chapter 7B.

- ○ - complete, i.e. 20mM malate + 1mM ADP
- △ - omission of ADP, i.e. 20mM malate
- □ - omission of malate, i.e. 1mM ADP.
fig 7.1
C. THE PRODUCTS OF RNA SYNTHESIS

Three samples of artichoke mitochondria containing 1 mg protein, were incubated with 100\mu Cl\(^{3}H\) uridine for one hour, under standard conditions. The samples were then combined, and the RNA extracted and fractionated by gel electrophoresis as described in Chapter 2B9. The gels were scanned at \(E_{260}\) and estimated for radioactivity.

Fig. 7.2 shows the radioactivity and OD\(_{260}\) profiles from the experiment.

The RNA synthesised by the isolated mitochondria was polydisperse in size, ranging from about \(1 \times 10^6\) to \(2.5 \times 10^6\). There were no obvious peaks of rRNA or their precursors. However, this experiment demonstrates that isolated mitochondria contain the enzymes which convert uridine to UTP and are capable of utilising this nucleotide as a substrate for RNA synthesis. Further experimentation using a variety of incubation media and additional substrates (e.g. ATP, GTP and CTP) is obviously required in order to evaluate the capabilities of isolated mitochondria to synthesise specific RNA molecules.
Figure 7.2

Products of RNA synthesis by mitochondria isolated from artichoke

Mitochondria were isolated from artichoke tuber and incubated in the mannitol medium with 100μCi [3H]-uridine. The RNA as extracted from the labelled mitochondria and fractionated by polyacrylamide gel electrophoresis. The radioactivity in the gels was estimated as described in Chapter 2B9.
fig 7.2

Electrophoretic mobility (cm)
CHAPTER 8: DISCUSSION

The importance of mitochondrial protein synthesis in the development of a functional orangelle has been known for some time. The biogenesis and assembly of a respiratorily competent mitochondrion results from the joint expression of two distinct genetic systems, mitochondrial and nuclear-cytoplasmic. Although the means by which the expression of these two genetic systems is mediated, is as yet uncertain, recent studies have revealed the importance of the co-ordinated functioning of mitochondrial and cytoplasmic protein synthesis in mitochondrial biogenesis. Estimates from work on ascomycete and animal mitochondria (Schatz and Mason, 1974) suggest that 8-10 polypeptides, which form part of three enzyme complexes of the inner mitochondrial membrane — cytochrome oxidase, cytochrome b, and oligomycin-sensitive ATPase are the only products of mitochondrial protein synthesis so far identified. The majority of the mitochondrial proteins are apparently specified by nuclear genes and synthesised on cytoplasmic ribosomes. Since mitochondrial DNA from higher plants is larger than that found in animal and ascomycete mitochondria it is of interest to determine whether or not plant mitochondria are capable of synthesising additional proteins to those synthesised by other organisms. These results should determine if there has been conservation, during evolutionary modification of the mitochondria, of the ability to synthesise several key proteins necessary for the correct assembly of the relatively large number of cytoplasmically synthesised mitochondrial proteins into a functional mitochondrion. This, in turn, may indicate a key and possibly controlling role for these mitochondrially synthesised proteins in the biogenesis of active mitochondria.

The results presented in this thesis provide a basis for the further characterisation of those proteins synthesised by plant mitochondria. The results can be conveniently discussed in two major sections. Firstly, there is the characterisation of the conditions for amino acid incorporation into protein by isolated plant mitochondria, including a consideration of the sensitivities of this to inhibitors of protein synthesis, nucleic acid synthesis and respiration. Secondly, there is the characterisation of products of isolated mitochondrial protein synthesis and a comparison of these with the proteins synthesised in vivo.

However, before these major facets of the work are discussed, some consideration will be given to the quality of the isolated mitochondrial preparation. The two contaminants of the isolated mitochondria considered to be most important in this study were that of bacteria and cytoplasmic ribosomes. A significant contribution by either of these would seriously affect interpretation of the results, and invalidate any conclusions drawn from them. While strenuous efforts were made to remove both of these contaminating factors, neither was completely eliminated (see Chapter 3E). Part
of the aim of the first section of the results was to discount both of these contaminants as possible sources of significant incorporation of radioactive amino acids.

The results presented in Table 4.1 and Fig 4.1 suggest that bacteria are not responsible for the incorporation of radioactive amino acids into protein. It would be highly unlikely that 1mM-ADP would have such a stimulating effect on bacterial protein synthesis. The inability of acetate to replace malate as an energy source and the time course of incorporation (Fig 4.1) argue against the protein synthesis being bacterial. The time course shown in Fig. 4.1 is in good agreement with the results reported by the other workers in the field of isolated mitochondrial protein synthesis (see Schatz and Mason, 1974).

The complete insensitivity of incorporation to the inclusion of RNAase in the incubation medium (Table 5.5) provides strong evidence that the observed incorporation is not due to contaminating cytoplasmic ribosomes. This is confirmed by the lack of response of the incorporation to inhibitors of cytoplasmic ribosome function in contrast to the sensitivity of the system to certain inhibitors of procaryotic protein synthesis (Table 5.1). The electron micrographs (Plate 3.1) of the purified mitochondrial preparation used in the in vitro incorporation studies shows no evidence of contaminating bacteria or plastids. The absence of any effect of light on the incorporation also suggests that plastids do not contribute to the observed incorporation (Table 4.1).

Hochberg et al (1972) has suggested that mitochondrial protein synthesis was an artifact, resulting from the binding of radioactive amino acids to lipo-protein complexes of the mitochondrial membranes. The results presented in this thesis would argue against such an interpretation, e.g. the stimulation of incorporation observed when a non-radioactive amino acid mixture is added to the incubation (Table 4.4) is incompatible with a binding phenomenon.

Considering the above arguments, contaminating bacteria, cytoplasmic ribosomes, or plastids cannot be considered to contribute significantly to the observed incorporation of radioactive amino acids into protein.

Table 4.1 and the work of Leaver (1975) shows the in vitro incorporation of radioactive amino acids is dependent upon a supply of oxidisable substrate, ADP, inorganic phosphate and “coupled” mitochondria in a simple buffered medium.

Considering the time course of amino acid incorporation (Fig. 4.1) it can be seen that it is linear for the first 20-30 minutes and then gradually plateaus between 40-60 minutes. This raises the questions, what initially limits the rate of incorporation, and, indeed, why should the rate of incorporation decrease in this manner?

The relatively simple time course of incorporation belies the complexity of mitochondrial protein synthesis. In vitro radioactive amino acid incorporation into mitochondrial protein involves
not only the formation of the peptide bond but also the transport of amino acids across the mitochondrial membrane and other energy dependent processes, which under normal conditions are closely coupled with ATP synthesis by oxidative phosphorylation. Each step is potentially rate limiting, from uptake of amino acids to any of the termination steps of protein synthesis, or oxidation of exogenous substrate to phosphorylation of ADP. Similarly the decreasing rate of incorporation could be due to the failure of any one of these functions. It should also be borne in mind that any limitations imposed by experimental conditions such as ionic or osmotic composition of the medium, on any of these associated processes may drastically affect protein synthesis itself.

Isolated mitochondria are usually labelled in a medium containing an osmotic stabiliser, e.g. sucrose, a buffer around pH7.5, magnesium ions, a mono-valent cation, inorganic phosphate, ATP or an ATP generating system. This basic assay mixture is frequently elaborated on, particularly the last item, namely that of the energy source. Energy sources which have been used in addition to the oxidisable substrate and ADP reported here and elsewhere (Roodyn, 1965) include phosphoenol pyruvate and pyruvate kinase (Ibrahim et al, 1973). More recently Mokel and Beattie (1975) have claimed that glutamate and actractylate (an ATP translocase inhibitor) can provide an improved energy source. The inhibition of incorporation under aerobic conditions by the addition of an energy generating system (Table 6.4) confirms the results of Leaver (1975) and are consistent with the report of McCoy and Doeg (1972) who found that addition of phosphoenol pyruvate inhibited in vitro amino acid incorporation by rat liver mitochondria.

The substitution of potassium chloride as the osmoticum in this investigation produced disappointing results. Blair and Ellis (1973), using a relatively simple incubation medium of 0.2M-KCl, MgCl₂ and buffer, successfully characterised the products of isolated chloroplast protein synthesis. They considered the KCl to serve a dual function — that of an osmoticum and as a cofactor for protein synthesis. Potassium chloride is known to effect swelling of isolated mitochondria (Opik, 1968) and it has been used as an osmoticum in the study of mitochondrial protein synthesis by mitochondria isolated from rat liver (Ibrahim et al, 1973).

The rate of incorporation of radioactive leucine by isolated mung bean mitochondria was calculated to be 150-180pmoles/mg protein/hour. This calculation ignores any endogenous pool of free amino acid which would tend to cause an under-estimate of the true rate. However, this rate compares favourably with those claimed by other authors, which vary from around 100pmoles/mg protein/hr (Ibrahim et al, 1973) to about 200pmoles/mg protein/hr (Linnane et al, 1968). These differences in rate are not surprising, considering that the mitochondria were isolated from different tissues by different methods before being assayed in different incubation media. The importance of the choice of tissue was illustrated in Table 4.5. The protein synthetic and respiratory capacities of
mitochondria isolated from the range of plant tissues, varied considerably. The increased protein synthesis activity of the mitochondria isolated from aged artichoke tissue had been suggested by the work of Asahi and his colleagues (Asahi et al, 1966, 1969). A somewhat similar situation has been shown to occur in rat liver, where mitochondria isolated from regenerating liver are reported to be 2-3 times more active than mitochondria from non-dividing liver (Malkin, 1970).

The effects of a wide range of inhibitors on mitochondrial protein synthesis have been extensively reported. Inhibitors of protein synthesis are generally considered to fall into two categories. The first specifically inhibit synthesis on 80S ribosomes found in the cytoplasm of eucaryotes, while the second inhibits synthesis on 70S procaryotic ribosomes. Both mitochondria and chloroplast protein synthesis are inhibited by several antibiotics belonging to the second category (Boulter et al, 1972). Amino acid incorporation by both artichoke and mung bean mitochondria exhibit characteristic sensitivities towards inhibitors of organelar protein synthesis (Table 5.1). The incorporation of radioactive amino acids was shown to be insensitive to inhibition by cycloheximide (an 80S type inhibitor) and sensitive to chloramphenicol (a 70S type inhibitor). However, there are exceptions to this general rule, e.g. lincosmycin. This compound, a potent inhibitor of chloroplast protein synthesis and 70S ribosomes (Ellis, 1970) caused no significant inhibition of plant mitochondrial protein synthesis (Table 5.1). This has been reported elsewhere (Leaver, 1975) and may be a real insensitivity of the mitochondrial protein synthetic machinery, or merely a reflection of impermeability of the plant mitochondrial membrane to this inhibitor. This possibility has been suggested by Kroon and De Vries (1971).

Inhibitors and uncouplers of respiration (Table 5.4) inhibit amino acid incorporation, their effect apparently being directly related to their effect on respiration.

Isolated mitochondrial protein synthesis was not inhibited by Actinomycin D (an inhibitor of RNA synthesis). This suggests that over the period investigated, continued RNA synthesis was not required for protein synthesis, which presumably occurs upon pre-existing mRNA.

Mitochondria isolated from artichoke tuber were shown to be able to incorporate [³H]uridine into RNA. This incorporation was dependent upon oxidative phosphorylation, and was inhibited by Actinomycin D (Table 7.1).

The results discussed so far are in broad agreement with those presented elsewhere. They were considered to provide a solid foundation for the next phase of the investigation, namely an examination of the products of isolated mitochondrial protein synthesis.

Two basically similar methods were used to fractionate the labelled polypeptides synthesised by the mitochondria. These were, firstly, tube gel SLS-polyacrylamide gel electrophoresis, followed by estimation of the radioactivity in the sliced gel, and, secondly, slab gel SLS-polyacrylamide gel
electrophoresis followed by autoradiography of the dried down gel. This latter procedure, which was only used at a later stage of the work described in this thesis, gave a much higher degree of resolution of the labelled polypeptide translation products.

It was possible to detect 7 or 8 labelled polypeptides by tube gel electrophoresis (Figs. 6.4 and 6.8) while the improved resolution obtained from the slab gels permitted the detection of at least 17 polypeptide species (Fig. 6.2). It would be premature to conclude that all the polypeptides detected represent unique gene products. Polypeptide aggregation, specific and/or non-specific cleavage products cannot be ruled out, particularly for the minor products.

The molecular weights of the polypeptides synthesised by artichoke tuber mitochondria are listed in Table 6.1 and range from 57 to 10 x 10^3. There are no other estimates of the range of the protein products synthesised by higher plant mitochondria in the literature. The great majority of the research on mitochondrial protein synthesis has been focussed on a few organisms, principally yeast and to a lesser extent, Neurospora. There are reports of mitochondrial protein synthesis in mammalian cells (Coote and Work, 1971; Mockel and Beattie, 1975; Ledermann and Attardi, 1973). Table 8.1 lists the number and molecular weight range of the polypeptides synthesised by mitochondria from a variety of organisms which fulfill the criteria of mitochondrial translation products. The estimates presented, with the exception of Hawley and Greenawalt (1975) and Douglas and Butow (1976) were all obtained by tube gel electrophoresis on SLS gels followed by estimation of the radioactively labelled polypeptides in the sliced gel. This approach can only be thought of as giving a minimal estimate, and by the use of the greater resolving power of slab gel

<table>
<thead>
<tr>
<th>Organism</th>
<th>Minimum No. of Proteins</th>
<th>Range of Molecular Weights</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast</td>
<td>5</td>
<td>45,000-7,800</td>
<td>Tzagoloff and Akai (1972)</td>
</tr>
<tr>
<td>Yeast</td>
<td>7</td>
<td>44,000-13,000</td>
<td>Weislogel and Butow (1971)</td>
</tr>
<tr>
<td>Yeast</td>
<td>5</td>
<td>42,000-12,000</td>
<td>Poyton and Groot (1975)</td>
</tr>
<tr>
<td>Yeast</td>
<td>10</td>
<td>94,000-11,000</td>
<td>Schatz and Groot (1972)</td>
</tr>
<tr>
<td>Yeast</td>
<td>21</td>
<td>43,000-9,000</td>
<td>Douglas and Butow (1976)</td>
</tr>
<tr>
<td>Neurospora</td>
<td>12</td>
<td>80,000-7,500</td>
<td>Hawley and Greenawalt (1975)</td>
</tr>
<tr>
<td>Rat</td>
<td>5</td>
<td>38,000-10,000</td>
<td>Mockel and Beattie (1975)</td>
</tr>
<tr>
<td>Hela</td>
<td>10</td>
<td>42,000-11,000</td>
<td>Constantino and Attardi (1975)</td>
</tr>
<tr>
<td>Artichoke</td>
<td>7-8</td>
<td>57,000-10,500</td>
<td>This thesis</td>
</tr>
</tbody>
</table>

and Butow (1976) were all obtained by tube gel electrophoresis on SLS gels followed by estimation of the radioactively labelled polypeptides in the sliced gel. This approach can only be thought of as giving a minimal estimate, and by the use of the greater resolving power of slab gel
electrophoresis followed by autoradiography, it is possible that these estimates will be revised upwards (e.g. Hawley and Greenawalt, 1975; Douglas and Butow, 1976).

However, bearing in mind the limitation of these methods, the estimate of the range of molecular weights of the polypeptides synthesised by plant mitochondria given in this thesis are in broad agreement with those quoted for other organisms (Table 8.1).

Within the limits of resolution of the analytical methods used, the number and molecular weights of the proteins synthesised by mitochondria isolated from artichoke tuber and mung bean appear similar (Fig. 6.6 and 6.7). The clarity of the radioactive peaks in the gels of the polypeptide products synthesised by artichoke mitochondria was invariably superior to that obtained for mitochondria isolated from mung bean (cf. Fig. 6.6A and B). It is of interest to note the close resemblance of the radioactive profiles of polypeptides synthesised by plant mitochondria presented in this thesis with those reported by Tzagoloff and Akai (1972) for yeasts.

The polypeptides synthesised by mitochondria isolated from both fresh and aged (12hr) artichoke tuber appear almost identical (Fig. 6.8). This result taken in conjunction with the much higher specific activity of the labelled mitochondrial protein obtained from in vitro labelling of mitochondria from the aged tissue (Table 4.5) would seem to confirm the observation that mitochondrial biogenesis is occurring during ageing of storage tissue slices (Asahi and Majima, 1969).

A comparison of the products of in vitro and in vivo protein synthesis by artichoke mitochondria (Fig. 6.9A and C) shows that they are basically very similar with only minor differences in the distribution of radioactivity incorporated into the various polypeptides. Thus peak c (molecular weight 34,000) is diminished in vitro, while peak d (molecular weight 31,500) is diminished in vivo. This is not unexpected as the constraints on the system in vivo and in vitro are almost certainly different and could easily lead to a difference in the labelled polypeptide profile. In addition, the polypeptides synthesised in vitro were synthesised over a period of 1 hour while those in vivo were synthesised over a period of 6 hours.

To confirm unequivocally the identity of the in vitro and in vivo products it would be necessary to carry out at least tryptic peptide analysis of comparable polypeptides.

The close similarity of the products of in vitro and in vivo translation by the artichoke mitochondria gives confidence as to the validity of using the approach of in vitro labelling of mitochondria as a means of studying the products of mitochondrial protein synthesis. A similar positive comparison has been used by other authors (Poyton and Groot, 1975) to assess the fidelity with which the in vitro system reproduces the in vivo situation.

It is apparent from a review of the literature that the most productive approach to the investigation of mitochondrial protein synthesis has been in vivo labelling of the organelle in the
presence of an inhibitor of cytoplasmic protein synthesis. For example, all but one (Mockel and Beattie, 1975) of the results reported in Table 8.1 used this method. In vitro studies have frequently yielded spurious results and as a consequence this method has come in for fierce criticism. For example, Schatz and Mason (1974) state:

"This system was cumbersome, and at times outright malicious."

It was realised that both the analytical methods used in this investigation fractionated the mitochondrial proteins on the same basis, namely that of size. It was obviously desirable to find another method for fractionating the protein on a different criterion, e.g. charge. A polyacrylamide gel electrophoretic system using a urea-acetic acid buffer was tried. However, all the radioactivity and most of the mitochondrial proteins remained precipitated at the top of the gel. This was considered to be a reflection of the hydrophobicity of the mitochondrial proteins, a problem which has been widely reported (Beattie, 1971). Even extreme solvents, e.g. phenol-acetic acid have proved unsatisfactory for dissolving mitochondrial proteins (Coote and Work, 1971). The detergent SLS has been shown to be the only system suitable for solubilising mitochondrial proteins.

Another criticism which could be levelled at the characterisation of the products of mitochondrial protein synthesis was that only one amino acid, methionine, was used to label the polypeptides. Should any of the polypeptides synthesised by the mitochondrion not contain methionine, these proteins would not be detected. However, when the isolated mitochondria were labelled with radioactive leucine or a radioactive amino acid mixture, the specific activity of the protein proved too low for the products to be satisfactorily analysed by the methods used here. The increased availability of labelled amino acids with higher specific activities, coupled with more sensitive means of detection of labelled polypeptides, should allow this criticism to be answered in the near future.

It is now generally accepted that less than 10% of total mitochondrial protein is synthesised by the organelle (Schatz and Mason, 1974). The vast majority of the structural and enzyme mitochondrial protein is synthesised on cytoplasmic ribosomes and imported into the mitochondria during its biogenesis. In other systems the mitochondrially synthesised polypeptides have been shown to be components of the inner mitochondrial membrane. Ultimately it will be important to relate the polypeptides synthesised by higher plant mitochondria to known components of the mitochondrial membrane.

Preliminary results (Table 6.2 and Fig. 6.10) show that more than 97% of the mitochondrially synthesised polypeptides are associated with the mitochondrial membrane system, while the 3% remaining in the soluble fraction was most probably accounted for as a minor contamination by the 57,000 molecular weight component—peak a.
The solubility in organic solvents of the polypeptides synthesised by the mitochondria has also been widely reported and is an important preliminary step to their eventual purification (Tzagoloff et al., 1973). The proportion of the polypeptides synthesised by the mitochondria of yeast that are soluble in neutral or acidic chloroform is around 50% (Tzagoloff and Akai, 1972). The figure reported here for plants of about 20% is significantly lower than this (Table 6.3). However, the finding that the lowest molecular weight polypeptide (molecular weight 10,500) is soluble in acidic chloroform-methanol is in good agreement with both Künstzel et al. (1975) and Tzagoloff and Akai (1972) who have used this property to purify the low molecular weight species (Tzagoloff et al., 1973).

The products of protein synthesis of mitochondria so far identified are shown in Table 8.2. The three protein complexes, cytochrome oxidase, oligomycin-sensitive ATPase, and cytochrome b, have a dual origin, some of the component polypeptides being synthesised by cytoplasmic ribosomes, some by mitochondrial ribosomes (see Fig. 1.1). Proteins consisting of several subunits frequently pose problems during their purification, i.e. when is a particular polypeptide a genuine subunit or merely a tightly associated contaminant? This is highlighted by the ATPase complex and may be the reason for the discrepancy between the results reported for yeasts and Neurospora (Jackel and Sebald, 1975).

The reported amino acid analysis of the polypeptides synthesised by the mitochondria is shown in Table 8.3. It is of interest to note that methionine is present in all of them. For comparative purposes, the amino acid analysis of the cytoplasmically synthesised subunits of cytochrome oxidase is included in Table 8.3. The predominance of hydrophobic amino acids in the polypeptides synthesised by the mitochondria is apparent.

Assuming that it will be confirmed that the plant mt-rRNAs and mt-tRNAs are coded for by mtDNA, and taking into account the estimated number and molecular weights of the polypeptides synthesised by plant mitochondria (and assuming that these also are coded for by mtDNA) it is possible to calculate the total coding capacity required for plant mtDNA (Table 8.4). Such a calculation leaves 5/6 of the coding capacity not accounted for. It is possible that a small proportion of the residual space on mtDNA could accommodate the cistrons for as yet unidentified mitochondrial translation products and/or for rRNA and tRNA sequences removed during maturation. Obviously, the plant mtDNA is large enough to code for all the known products of mitochondrial transcription and translation, with a vast excess of DNA without any known function. A proportion of this could presumably have regulatory functions. Some may also be reiterated and therefore redundant DNA (Bernardi and Drunel, 1974).

One approach to the problem of the function of mtDNA has been to transcribe isolated
fig 8.1

NUCLEUS

mRNA

80S cytoplasmic ribosomes

CYCLOHEXIMIDE

MITOCHONDRION

Inner membrane

ML RNA POLYMERASE

mtDNA

mt mRNA

70S mitochondrial ribosomes

mt-tRNA

PROTEIN

soluble proteins

CHLORAMPHENICOL

MT RNA POLYMERASE

mtDNA

mt mRNA

PROTEIN

insoluble membrane proteins

insoluble membrane proteins
### TABLE 8.2

The Polypeptides Identified as being synthesised by mitochondria

<table>
<thead>
<tr>
<th>Organism</th>
<th>Protein</th>
<th>Number</th>
<th>Molecular Weight x 10^3</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast</td>
<td>Cytochrome Oxidase</td>
<td>3 out of 7</td>
<td>42, 34.5, 23</td>
<td>Mason and Schatz (1973)</td>
</tr>
<tr>
<td><em>Neurospora</em></td>
<td>Cytochrome Oxidase</td>
<td>3 out of 7</td>
<td>40, 30, 20</td>
<td>Sebald <em>et al</em> (1973)</td>
</tr>
<tr>
<td><em>Neurospora</em></td>
<td>Cytochrome b</td>
<td>1 out of 2</td>
<td>30</td>
<td>Weiss and Ziganke (1974)</td>
</tr>
<tr>
<td><em>Locust</em></td>
<td>Cytochrome b</td>
<td>1 out of 2</td>
<td>30</td>
<td>Weiss (1973)</td>
</tr>
<tr>
<td>Yeast</td>
<td>Oligomycin-sensitive ATPase</td>
<td>4 out of 9</td>
<td>29, 22, 12, 7.5</td>
<td>Tzagoloff and Meagher (1972)</td>
</tr>
<tr>
<td><em>Neurospora</em></td>
<td>Oligomycin-sensitive ATPase</td>
<td>2 out of 14</td>
<td>19, 11</td>
<td>Jackel and Sebald (1975)</td>
</tr>
</tbody>
</table>
### TABLE 8.3

Amino acid composition of cytochrome oxidase from *Neurospora* (a) and subunit 9 of oligomycin-sensitive ATPase from yeast (b)

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Cytochrome Oxidase Components</th>
<th>Oligomycin Sensitive-ATPase component 9</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>6.45</td>
<td>8.55</td>
</tr>
<tr>
<td>Threonine</td>
<td>4.80</td>
<td>4.15</td>
</tr>
<tr>
<td>Serine</td>
<td>10.09</td>
<td>9.36</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>4.28</td>
<td>9.80</td>
</tr>
<tr>
<td>Proline</td>
<td>6.84</td>
<td>7.33</td>
</tr>
<tr>
<td>Glycine</td>
<td>10.20</td>
<td>6.81</td>
</tr>
<tr>
<td>Alanine</td>
<td>7.53</td>
<td>4.90</td>
</tr>
<tr>
<td>Valine</td>
<td>7.14</td>
<td>7.71</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.27</td>
<td>2.05</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>8.72</td>
<td>9.16</td>
</tr>
<tr>
<td>Leucine</td>
<td>13.37</td>
<td>11.68</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>4.18</td>
<td>4.89</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>8.25</td>
<td>5.38</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.51</td>
<td>2.50</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.56</td>
<td>2.64</td>
</tr>
<tr>
<td>Arginine</td>
<td>2.81</td>
<td>3.07</td>
</tr>
</tbody>
</table>

(a) From Sebald *et al* (1973); (b) from Tzagoloff (1973). Components 1, 2 and 3 of cytochrome oxidase, and component 9 of the oligomycin-sensitive ATPase, have been shown to be synthesised by mitochondria. n.d. — not determined.
<table>
<thead>
<tr>
<th>Mitochondrial Product</th>
<th>Molecular Weight</th>
<th>Molecular Weight of double stranded DNA required for coding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large rRNA</td>
<td>$1.15 \times 10^6$</td>
<td>$2.3 \times 10^6$</td>
</tr>
<tr>
<td>Small rRNA</td>
<td>$0.78 \times 10^6$</td>
<td>$1.56 \times 10^6$</td>
</tr>
<tr>
<td>5S-rRNA</td>
<td>$0.035 \times 10^6$</td>
<td>$0.07 \times 10^6$</td>
</tr>
<tr>
<td>20tRNAs</td>
<td>$0.56 \times 10^6$</td>
<td>$1.12 \times 10^6$</td>
</tr>
<tr>
<td>Mitochondrially Synthesised Polypeptides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a</td>
<td>$5.7 \times 10^6$</td>
<td>$1.06 \times 10^6$</td>
</tr>
<tr>
<td>b</td>
<td>$4.2 \times 10^6$</td>
<td>$0.78 \times 10^6$</td>
</tr>
<tr>
<td>c</td>
<td>$3.4 \times 10^6$</td>
<td>$0.63 \times 10^6$</td>
</tr>
<tr>
<td>d</td>
<td>$3.1 \times 10^6$</td>
<td>$0.57 \times 10^6$</td>
</tr>
<tr>
<td>e</td>
<td>$2.3 \times 10^6$</td>
<td>$0.43 \times 10^6$</td>
</tr>
<tr>
<td>f</td>
<td>$1.9 \times 10^6$</td>
<td>$0.35 \times 10^6$</td>
</tr>
<tr>
<td>g</td>
<td>$1.8 \times 10^6$</td>
<td>$0.33 \times 10^6$</td>
</tr>
<tr>
<td>h</td>
<td>$1.6 \times 10^6$</td>
<td>$0.30 \times 10^6$</td>
</tr>
<tr>
<td>i</td>
<td>$1.0 \times 10^6$</td>
<td>$0.19 \times 10^6$</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>$9.69 \times 10^6$</td>
</tr>
</tbody>
</table>

Available ca $60 \times 10^6$

ca 1/6 utilised?
mtDNA in vitro using E. coli RNA polymerase followed by transcription of the products in an E. coli S30 protein synthesising system (Scragg and Thomas, 1975). While this method holds considerable promise, results obtained from such studies have as yet yielded equivocal results.

The work presented in Chapter 7 of this thesis showed isolated plant mitochondria could synthesise RNA, by a process dependent upon oxidative phosphorylation. Further refinements of this system may eventually allow the identification of discrete and recognisable molecules.

It is informative to compare the mitochondrion with the chloroplast. Both are regarded as semiautonomous in that they contain circular DNA, the means of replication and transcription of this DNA, and a protein synthesising system (see Fig. 8.1). Protein synthesis in the chloroplast and mitochondria resembles that of procaryotes, for example in the use of N-formyl methionine for initiation and the differential sensitivity to chloramphenicol and cycloheximide (Ellis, 1975). The chloroplast has been shown both in vivo and in vitro to synthesise a limited number of polypeptides, only one of which has been well characterised. This is the large subunit of Fraction 1 protein, which is the major soluble chloroplast protein. It has two related enzymic activities, one is a ribulose bisphosphate carboxylase activity and the other a ribulose bisphosphate oxygenase activity. Chan and Wildman (1972) have shown that the large subunit of Fraction 1 protein is encoded in chloroplast DNA, while the small subunit of Fraction 1 is encoded in nuclear DNA. The remaining 5 or so polypeptides synthesised in the chloroplast are associated with the chloroplast membrane system and are as yet unidentified. The synthesis of a soluble polypeptide in the chloroplast contrasts with the exclusively membrane location of the polypeptides synthesised by the mitochondrion.

The biogenesis of mitochondria and chloroplasts therefore results from the joint expression of distinct genetic systems. The results presented in this thesis provide very preliminary evidence that there has been conservation, during the evolutionary modification of mitochondria, of their ability to synthesise several key proteins of the mitochondrial membrane system. There is mounting evidence that these proteins, which represent less than 10% of the total mitochondrial protein, are necessary for the correct assembly of a much greater number of cytoplasmically synthesised mitochondrial proteins, and thus play a key role in the control of the biogenesis of the active mitochondrion (Schatz and Mason, 1974).

In vitro studies on yeast mitochondria by Groot and Poyton (1975) suggested that oxygen regulates the synthesis of two of the three mitochondrially synthesised subunits of cytochrome oxidase, directly at the level of translation. Parallel experiments with plant mitochondria reported in this thesis (Fig. 6.13) were inconclusive, owing to the limitations of the analytical methods employed. However, the results obtained did look worthy of further investigation.

Studies of petite mutants of yeast have indicated that cytoplasmic protein synthesis is not
tightly coupled to mitochondrial protein synthesis (Schatz and Mason, 1974). For example, petite mutants which lack the mitochondrial protein synthetic machinery, still contain organelles recognisable as mitochondria, albeit with severely impaired respiratory functions. However, the converse does not hold; under conditions in which cytoplasmic protein synthesis has been inhibited, mitochondrial protein synthesis does not continue for a long period. This has been suggested as evidence that the products of cytoplasmic protein synthesis act as activitators for mitochondrial proteins synthesis. The observation that mitochondrial protein synthesis can be stimulated by a pre-incubation in chloramphenicol, during which time it is proposed the pools of these cytoplasmically synthesised "activator" proteins accumulate, appears to agree with this theory (Tzagoloff and Akai, 1972; Tzagoloff et al, 1973).

The work described in this thesis provides a basis for future identification of the polypeptides synthesised by higher plant mitochondria. The in vivo experiments described in Chapter 6G provide a model system for the study of the co-ordination of the synthesis and assembly of mitochondrial proteins during the developmental changes in mitochondrial activity which are known to occur in higher plants. An understanding of this should advance our knowledge of their biogenesis and turnover, and aid our understanding of the way in which their main metabolic function of respiration is geared to the development of the plant cell.
REFERENCES


