Studies on the relationship between mitochondrial structure and functioning in *Amoeba proteus*.

Thesis submitted for the degree of Doctor of Philosophy by Robert A. Smith.

December, 1977.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title page</td>
<td>i</td>
</tr>
<tr>
<td>Table of contents</td>
<td>ii</td>
</tr>
<tr>
<td>Abstract</td>
<td>v</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>vi</td>
</tr>
<tr>
<td>Abbreviations and nomenclature</td>
<td>vii</td>
</tr>
<tr>
<td>Diagram of mitochondrial characters</td>
<td>viii</td>
</tr>
<tr>
<td><strong>1. GENERAL INTRODUCTION</strong></td>
<td></td>
</tr>
<tr>
<td>1.1. Basic structure and chemical composition</td>
<td>1</td>
</tr>
<tr>
<td>1.2. Mitochondrial size and numbers</td>
<td>2</td>
</tr>
<tr>
<td>1.3. Mitochondrial autonomy</td>
<td>4</td>
</tr>
<tr>
<td>1.4. Energy-production by mitochondria</td>
<td>5</td>
</tr>
<tr>
<td>1.5. Structural changes correlated to function 'in vitro'.</td>
<td>7</td>
</tr>
<tr>
<td>1.6. Mitochondrial changes observed 'in vivo'</td>
<td>9</td>
</tr>
<tr>
<td>1.7. Different structural forms existing simultaneously within cells and tissues.</td>
<td>11</td>
</tr>
<tr>
<td><strong>2. MATERIALS AND METHODS</strong></td>
<td></td>
</tr>
<tr>
<td>2.1. Culturing</td>
<td>13</td>
</tr>
<tr>
<td>2.2. Cell treatments</td>
<td>14</td>
</tr>
<tr>
<td>2.3. Cell synchronisation</td>
<td>15</td>
</tr>
<tr>
<td>2.4. Anaerobic culturing</td>
<td>16</td>
</tr>
<tr>
<td>2.5. Micrurgical operations</td>
<td>19</td>
</tr>
<tr>
<td>2.6. Preparation of cells for the Electron microscope</td>
<td>20</td>
</tr>
<tr>
<td>2.7. Cytochemical method for demonstrating cytochrome oxidase</td>
<td>23</td>
</tr>
<tr>
<td>2.8. E.M. autoradiography</td>
<td>25</td>
</tr>
<tr>
<td>2.9. Collection of data from the E.M.</td>
<td>27</td>
</tr>
<tr>
<td><strong>3. STRUCTURAL CHARACTERISTICS OF MITOCHONDRIAL TYPES IN</strong></td>
<td></td>
</tr>
<tr>
<td><strong>A. PROTEUS AND THEIR PRESENCE THROUGHOUT THE CELL CYCLE</strong></td>
<td></td>
</tr>
<tr>
<td>3.1. Introduction</td>
<td>29</td>
</tr>
<tr>
<td>3.2. Parameters used to quantify mitochondrial types</td>
<td>31</td>
</tr>
</tbody>
</table>
3.2.1. Gross shape 33
3.2.2. Appearance of cristae 35
3.3. Relative proportions throughout the cell cycle 37
3.4. Discussion 38

4. INFLUENCE OF GROWTH TEMPERATURE AND STARVATION ON THE MITOCHONDRIAL TYPES
4.1. Introduction 46
4.2.1. Effect of temperature on the cell cycle 49
   4.2.2.1. Ultrastructural changes associated with growth temperature 52
   4.2.2.2. Reversion of temperatures to 20°C 59
4.3.1. Effect of starvation on ultrastructure 60
4.3.2. Observations on refed cells 66
   Discussion 68

5. ACTION OF SUBSTANCES WHICH UNCOUPLE OXIDATIVE PHOSPHORYLATION
5.1. Introduction 74
5.2. Effects of uncouplers on cell morphology and viability 77
   5.2.1. Effects of DNP 80
   5.2.2. Effects of PCP 88
   5.2.3. Effects of CCCP 88
5.3. Ultrastructural changes associated with uncouplers 89
   5.3.1. DNP treatments 89
   5.3.2. PCP treatments 96
   5.3.3. CCCP treatments 97
   5.3.3.2. Reversibility of structural changes 101
5.4. Discussion 101

6. EFFECTS OF FACTORS ACTING ON ELECTRON TRANSPORT
6.1. Introduction 109
   6.1.1. Anaerobiosis 109
   6.1.2. Metabolic inhibitors 111
6.2.1. Effects of anaerobiosis on the whole cell 112
6.2.2. Effects of anaerobiosis on mitochondria 115
6.3. Exposure to KCN
   6.3.1. Viability studies 123
   6.3.2. Effects on ultrastructure 125
6.4. Exposure to Antimycin A 129
6.5. Discussion 129

7. THE USE OF CYTOCHEMISTRY, AUTORADIOGRAPHY AND MICRURGY IN STUDYING MITOCHONDRIAL TYPES

7.1. DAB cytochemistry to demonstrate CO activity 134
   7.1.1. Introduction 134
   7.1.2.1. DAB staining of untreated cells 137
   7.1.2.2. DAB staining of anaerobic cells 141
   7.1.2.3. DAB staining of MNU-treated cells 141

7.2. Studies involving mitochondrial DNA
   7.2.1. Introduction 141
   7.2.2. Incorporation of labelled thymidine by A. proteus 143

7.3. Micrurgical investigations
   7.3.1. Introduction 149
   7.3.2. Observations on cells injected with nucleotides, phosphate and succinate 150

7.4. Discussion of the results of the three experimental approaches. 154

8. GENERAL DISCUSSION AND CONCLUSIONS 160

BIBLIOGRAPHY 169
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Abstract

FACULTY OF SCIENCE

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Doctor of Philosophy

'Studies on the relationship between mitochondrial structure and functioning in Amoeba proteus.'

By Robert Anthony Smith.

The mitochondrial polymorphism existing within A. proteus upon aldehyde fixation was investigated using electron microscopy, related cytochemical techniques and autoradiography. Its significance was ascertained in relation to whole cell activity.

Type I, II and Int configurations were quantified by analysis of overall shape, cristal form and matrix density. Structural changes occurred following treatments which affected mitochondrial functioning and the work aimed to relate these with altered function. The relative proportions of the different mitochondrial types varied in different aged cells and could be experimentally changed when cells were cultured under stress conditions of lowered and raised growth temperatures and prolonged starvation. Control levels were again approached once the adverse culturing was terminated.

Hydrogen or nitrogen anaerobic culturing caused the elimination of Type I forms, with the generation of vacuolated Type II—Int organelles. Identical cristal vacuolation was observed in potassium cyanide-treated amoebae. On re-aeration Type I forms returned; the reappearance correlated with a reversion to normal whole cell activities.

Prolonged anaerobiosis induced cristal degradation and a concommitant appearance of matrical filamentous inclusions. Similar inclusions resulted after exposure to metabolic inhibitors (KCN) and uncoupling agents (DNP and PCP); these were considered indicative of impaired respiratory function.

The rapidity with which uncouplers and metabolic inhibitors induced structural changes supported the conclusion that mitochondrial structure was associated with organelle metabolic functioning. For CCCP treatment, structural changes were shown to be completely reversible.

Initial results from microinjection experiments using nucleotides and substrate reinforced the conclusions that structural forms represented different organelle functional states.

Reduced cytochrome oxidase staining ability when methyl-nitrosourethane or anaerobic treatments preceded the DAB reaction indicated the technique's potential in toxicological studies. The present results yield additional ultrastructural information for the established A. proteus cell model.
Acknowledgements

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The sympathy and comfort of many friends, particularly Agatha, Martyn, Ross and Eric, has been invaluable during the long months of writing this manuscript; as has Julie's efficient typing.

Finally I would like to dedicate this thesis to my mother and my close family who have encouraged me through many years of study; not fully understanding the results but always realising the reasons for my strivings.
ABBREVIATIONS and NOMENCLATURE

1. Cellular Structures

CV - Contractile vacuole
ch - cytoplasmic helix
DB - DNA-containing body
ER - Endoplasmic reticulum
f - cytoplasmic filamentous bundle
GB - Golgi body
HSB - Heavy spheroid body
M<sub>1</sub> - Type I mitochondrion
M<sub>int</sub> - Type Int mitochondrion
M<sub>II</sub> - Type II mitochondrion
mf - matrical filaments within the mitochondrion
N - Nucleus
nh - Nuclear helix
V - Vacuole

2. Chemicals

ADP - Adenosine diphosphate
ATP - Adenosine triphosphate
Ant A - Antimycin A
CCCP - mChloro carbonyl cyanide phenylhydrazone
DNP - 2, 4 dinitrophenol
DAB - Diaminobenzidine
KCN - Potassium cyanide
PCP - Pentachlorophenol
mit DNA - Mitochondrial deoxyribose nucleic acid
Diagramatic representation to show the mitochondrial forms of Amoeba proteus and to demonstrate the characters used in their classification.

Type I.  

Type II.

(a.) The length:width (L:W) ratio or index.  
(b.) The width and complexity of the cristae.  
(c.) The density of the matrix.

The relative proportions of the two types, as well as the presence of Type Int transitional forms were also studied.
Chapter One

General Introduction

Over recent years there has been a growing interest in relating the structure of the various cellular components to the different functions which they perform. Too often in the past isolated mitochondria have been studied by biochemists from a functional viewpoint with little regard to their morphology, so that it was difficult to perceive the reaction of the organelle within the living cell. Similarly the early ultrastructural studies tended to be divorced from any functional approach, changes in mitochondrial morphology being recorded but with no corresponding functional data. Three years ago, however, two important reviews of the knowledge of mitochondria were published bringing together the literature extensive on mitochondrial structure with the equally voluminous reports of functional properties (Lloyd, 1974; Munn, 1974).

In considering together the relationship between mitochondrial structure and function 'in situ' a system conducive to experimental manipulation is sought. Such a cell model is found with Amoeba proteus; the advantages of which have already been well argued (Ord, 1970). Amoebae offer a means of studying cell components at particular regions of the cell cycle, with controlled culture conditions and under various chemical and physical treatments. The relatively large size of the cell permits micrurgical operations. In addition modern fixatives provide good preservation of the cell, allowing EM investigations to ascertain any changes at the ultrastructural level. With particular reference to mitochondria, A. proteus seemed worthy of further study as at least two distinct mitochondrial forms have been observed within the control cells (Flickinger, 1968a). It was the intention of this project therefore to extend the amoeba model in discerning what such mitochondrial differences were reflecting in the cell, so that similar changes in form resulting from chemical treatments might be correlated to any alterations in cell activity and functioning.

1.1. Basic Structure and Chemical Composition

Mitochondria with a basic structure similar to that first described by Palade (1952) have been observed within all aerobic, eukaryotic cells. This structure consists of an outer and inner
membrane separated by the peripheral space. The internal membrane is thrown into a series of foldings constituting the cristae, and in general the mitochondria of cells requiring the greatest sustained demands for energy transduction tend to have the largest cristal membrane surface. The inner membrane encloses the matrix, a semi-fluid state, where the enzymes of the tricarboxylic acid cycle; fatty acid oxidation; mitochondrial protein and nucleic acid synthesising enzymes are restricted.

Water constitutes the major component of mitochondria, while the bulk of the dry mass is represented by protein and lipid. Nucleotides and nucleic acids are also important factors. Most of the mitochondrial protein is enzymic in nature, or functions in associated carrier molecules such as the cytochromes, with the possible exception of a structural or core protein. Over 900 enzymes have been listed; the oxidoreductases, transferases and hydrolases accounting for approximately 75%; with lysases, isomerases and ligases also of importance. Some enzymes, such as cytochrome oxidase, have their sole cell localisation in the mitochondria while others e.g. isocitrate dehydrogenase, may exist as isoenzymes with one form mitochondrial whereas the other is present in the cytosol. Many of the enzymes are restricted in functional complexes i.e. succinic dehydrogenase. These complexes, involved in oxidative phosphorylation, are found bound to the inner membrane (Schwartzman and Greenwald, 1968), and it is considered that the position of these with respect to each other is of importance in energy transduction (Racker, 1970).

In beef heart mitochondria 26% of the dry mass is made up of lipid uniformly distributed throughout the membranes. Over 90% of the total lipid content is of phospholipids. Lipids serve as a permeability barrier preventing the unrestricted flow of solutes and aiding the movement of substrates across the membrane. Lipids are further required to maintain a suitable orientation of the various haem proteins functional in oxidative phosphorylation (Green and Fleischer, 1963).

Mitochondrial shape and size are reported to vary considerably depending on the organism or cell type in which they are found. From the majority of the earlier studies cells were considered to contain numerous, small spherical and elongated mitochondrial forms with
lengths of up to 3\(\mu\). Reports of larger mitochondria were known: mitochondria in the large amoeba *Chaos chaos* reached 8\(\mu\) (Torch, 1955); in cells of ctenophore comb-plates profiles up to 8\(\mu\) were seen (Horridge, 1964); 7\(\mu\) organelles were present in human uterus mucosa cells (Merker et al., 1968); while in HeLa cells organelles of up to 30\(\mu\) with diameters of 15\(\mu\) have been recorded (Posakony et al., 1975). Giant profiles were generated in hepatocytes by the copper chelating agents Cuprizone and diethylthiocarbamate (Susuki, 1969; Asano and Wakabayashi, 1974), from which respiratory measurements have been made on single organelles (Succhy and Cooper, 1974). Alterations in nutritional and culture conditions may also produce enlarged profiles (Vartapetian et al., 1977).

Interest in recent years has focused on the construction of 3-dimensional models of mitochondria from serial sections. Such techniques have suggested that the numbers of organelles in certain cells is most probably lower than had previously been assumed from thin sectioning. In one strain of yeast a single mitochondrion was reported (Hoffman and Avers, 1973), although others suggested that diploid yeasts may contain over a hundred organelles (Grimes et al., 1974). A single organelle was constructed from serial thick sections of trypanosomes (Paulin, 1975), and below ten mitochondria/cell were estimated in the flagellate *Polytoma agilis* (Burton and Moore, 1974). The related species, *P. papillatum*, possessed one large chondriome prior to division but the two new daughter cells had a content of many small mitochondria (Gaffal and Kreutzer, 1977). Similar findings have been reported in *Euqlena gracilis* where up to forty mitochondria are present during division but as the cell neared the end of its cell cycle less than ten giant forms were seen, indicative of organelle fusion (Calvayrac et al., 1972; 1974; Osafune, 1973).

It was suggested that the presence of one or a few giant mitochondria per cell might be the general situation in eukaryotic cells. However, although more complex forms of a highly branched nature have been constructed within rat liver cells, not all the organelles were of this type and simple forms were also seen (Brandt et al., 1974). Using cytochemical staining the presence of many discrete mitochondria in the peripheral cytoplasm of HeLa cells had been
demonstrated (Posakony et al., 1975). With the alga *Gonyostomum semen* numerous separate organelles were indicated and it was proposed that the number of mitochondria may be related to cell volume, so that larger cells contain more mitochondria (Heywood, 1977). This would indeed seem to be the case for the mitochondrial cloud in *Xenopus* oocytes, where estimates of between 10,000 to 120,000 organelles have been proposed depending on the size of the egg cell (Billett and Adam, 1976). These workers however did not exclude the possibility that not all the organelles in the 'spaghetti-like' cloud were of a distinct nature.

In the large amoeba *Chaos chaos* estimates of up to 300,000 mitochondria have been put forward (Andresen, 1956) these being within the normal size range of 1-2μ (Pappas, 1959). Due to the relatively large size of *A. proteus*, 1.5 x 10^6 μ3 (Ord, 1968a) many separate mitochondria could be anticipated in *A. proteus* also.

1.3. Mitochondrial autonomy

Mitochondria appear to have a more independent existence within cells than other cytoplasmic organelles. By the use of enucleation experiments on amoebae, it was found that the mitochondria, although somewhat swollen after the removal of the cell's nucleus for five days, still retained functional integrity (Brachet, 1958). Mitochondrial profiles are still evident in enucleate halves of *A. proteus* after seven days, while Golgi complexes and endoplasmic reticulum have disappeared; the mitochondria begin to degenerate after this period (Flickinger, 1968b). This suggests some final control of their structure must originate from the nucleus for ultimate survival. Enucleation of *Thecamoeba* has shown that mit DNA may replicate and be transcribed without the presence of a cell nucleus (Perasso, 1973), but for complete mitochondrial biogenesis mRNA of a nuclear origin is required (Soslav and Nass, 1971) as mit DNA has a limited information content (Andre, 1971).

Mitochondria are known to contain mitoribosomes and tRNA's, these ribosomes functionally resemble bacterial rather than eukaryotic forms (For review see Borst and Grivell, 1971). This finding, together with other similarities between the organelles and bacteria, have led to endosymbiotic hypotheses for the origin of mitochondria within eukaryotic cells. Various schemes have been proposed
for the evolutionary transition of a free-living bacterial particle to a promitochondrial structure, including models based on prokaryotes such as Paracoccus (John and Whatley, 1975). The opposing proposition for the origin of mitochondria is that they arose as episomes which became bound by membrane structures (Raff and Mahler, 1972). Although the evolutionary origin of mitochondria is an interesting academic question, it is not within the scope of the present work.

1.4. The energy-production by mitochondria

The prime function of mitochondria is in the conservation of the redox energy generated during the oxidation of the food stuffs taken in by the cell, and its subsequent transformation into the high energy compound adenosine triphosphate (ATP) (Lardy and Ferguson, 1969; Van Dam and Meyer, 1971). The pairs of electrons derived from the intermediates of the TCA cycle flow down the successively lower energy levels of the multi-membered chain of electron-carrier enzymes, until they reduce the ultimate electron acceptor of respiration: molecular oxygen (Fig. 1.1).

Figure 1.1: Diagramatic representation of the mitochondrial electron transport chain

Pyruvate\[\rightarrow\text{FP}_5\] Succinate

Malate\[\rightarrow\text{FP}_2\]

Isocitrate\[\rightarrow\text{FP}_1\]

Glutamate\[\rightarrow\text{NAD}\rightarrow\text{Cyt}\rightarrow\text{Cyt}_1\rightarrow\text{Cyt}_c\rightarrow\text{Cyt}\rightarrow\text{O}_2\]

3-Hydroxyacyl Co A\[\rightarrow\text{FP}_3\]

Fatty acyl Co A\[\rightarrow\text{FP}_4\]

Glycerol Phosphate\[\rightarrow\text{FP}_0\]

ATP is formed at Sites I, II, III.

Rotenone, Antimycin A and cyanide inhibit the chain.
Energy from the electron flow is conserved as $\text{PO}_4^-$ bond energy in coupled mitochondria by the phosphorylation of ADP to ATP upon the expulsion of water.

The molecular mechanisms of oxidative phosphorylation are still not fully understood although all mechanisms so far envisaged state that the energy made available by the passage of reducing equivalents through the respiratory chain is primarily conserved in a form other than ATP. Whatever this primary form is, it is considered capable of driving a number of endergonic processes such as ATP synthesis, ion transport, and the energy-linked transhydrogenation between NAD and NADP. ATP synthesis and ion transport are reversible, i.e. the flow of reducing equivalents can cause ATP synthesis but ATP hydrolysis can also lead to a reversed flow of reducing equivalents.

Although numerous schemes have been proposed to account for the coupling between molecular respiration and phosphorylation, only three are worthy of serious consideration:

i) the chemical intermediate hypothesis (Slater, 1953);

ii) the chemiosmotic hypothesis (Mitchell, 1961; 1966); and

iii) a conformational theory (Green and Harris, 1969; Green, 1974).

The chemical intermediate theory proposed that electron transfer results in the formation of energy-rich intermediate compounds prior to the actual formation of ATP. This early explanation gained much support despite the fact that the postulated intermediates could not be identified.

In Mitchell's theory, however, it was suggested that such intermediates might not exist and instead a pH gradient and membrane potential were substituted as the coupling device. During respiration the oxidation chain was envisaged to develop a 'proton-motive-force' across the inner membrane as the protons removed from the substrate on the inside were translocated to the other side. A cyclic 'proton current' would be established if the protons were driven back through a reversible ATPase system and in doing so the dehydration of ADP and phosphate with the formation of ATP would be achieved. The chemiosmotic system does not however eliminate the need for postulating carrier molecules, as specific exchange-diffusion systems are required to regulate the internal pH without the collapse of the membrane potential. Both favourable (Greville, 1969) and unfavourable
(Slater, 1967) reactions to this scheme for oxidative phosphorylation have been presented.

Instead of a chemical intermediate or a membrane potential, conformational theories of energy transduction consider that the free chemical energy of oxidoreduction can be stored and manipulated through conformational strains of the enzyme complexes (Green, 1974). The release from these energised strains results in phosphorylation.

A mechanism encompassing all three main theories has been put forward (Ji, 1976); and doubtless others will be conceived before the precise events have been resolved. The most recent review of the mechanics of oxidative phosphorylation now accepts the chemiosmotic hypothesis as the basis for energy transduction (Boyer et al., 1977). This multiauthored review even contains a contribution from Slater who concedes Mitchell's theory to be correct. The final stages of the ATP synthesis reactions are still however, thought to involve other processes of possible conformational changes or chemical intermediates (See the Boyer and the Ernster sections of Boyer et al, 1977).

The recent findings from the laboratory of Griffiths also question whether the chemiosmotic theory satisfies all aspects of energy transduction. The observation that inhibition by antimycin A or rotenone does not stop net ATP synthesis by heart particles if dihydrolipoate is present has been taken as an indication that the terminal reactions of oxidative phosphorylation do indeed involve chemical intermediates (Griffiths, 1976; Griffiths & Hyams, 1977). Studies with a yeast mutant in addition to those on heart particles suggest that lipoic acid may act as an important cofactor or chemical intermediate (Griffiths et al., 1977). The whole question is opened to debate once more.

1.5. **Structural changes correlated to function in 'in vitro' studies**

Although the molecular mechanism involved in energy transduction remains unknown, certain workers have sought to relate reproducible configurational modifications of the inner mitochondrial membrane to the organelle's metabolic and respiratory state both in 'in vivo' and 'in vitro' studies (Williams et al., 1970). When phlorizin was used to block energy metabolism, isolated kidney mitochondria were seen to expand, while the addition of ATP caused a contracted form (Burgos et al., 1964). Using liver and heart preparations, reversible
ultrastructural transformations of the inner membrane have been
demonstrated (Hackenbrock 1966, 1968; Penniston et al., 1969;
Muscatello et al., 1972 a and b); these have been considered with
respect to the five respiratory states defined earlier on the basis
of oxygen consumption and light scattering measurements (Chance and
Williams, 1956).

Slight inconsistencies exist between the results from the
quick-sampling technique used for transmission EM (Hackenbrock,
1966; 1968) and those based on negative staining preparations which
had the advantage of overcoming the need for fixation (Muscatello
et al., 1972a). In general however oscillations between a condensed
or contracted and expanded or orthodox configuration were reported by
both sets of workers.

In respiratory State I, when both substrate and ADP concentra-
tions were low, oxygen consumption was also very low. The negatively
stained mitochondria appeared expanded. If ADP was added to these
preparations but the substrate concentration remained rate limiting,
oxygen consumption remained low, but State II mitochondria assumed
a condensed form. When substrate level was high in addition to the
ADP, State III respiration was stimulated, in which case the mitochon-
dria were as condensed forms. Both groups of workers obtained State
III from State IV preparations by the addition of ADP. State IV was
characterised by high substrate levels but with ADP as the limiting
factor, the mitochondria in this instance being of an expanded type.
Mitochondria returned to this expanded form once the ADP was
phosphorylated. This has led to the hypothesis that the condensed
form represents a state capable of undergoing oxidative phosphory-
lation and that the condensed to expanded transformation results as
a direct consequence of this process occurring. Preparations deprived
of oxygen assumed an expanded form according to Hackenbrock even
though ADP and substrate concentrations were high; Muscatello
however disagreed with this finding. These conformational states
are summarised in Table 1.1.
Table 1.1: Mitochondrial structural states 'in vitro' related to incubation conditions

<table>
<thead>
<tr>
<th>Respiratory State</th>
<th>Substrate Level</th>
<th>ADP Level</th>
<th>Oxygen Level</th>
<th>Mitochondrial appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Low</td>
<td>Low</td>
<td>&gt;0</td>
<td>Expanded</td>
</tr>
<tr>
<td>II</td>
<td>Low</td>
<td>High</td>
<td>&gt;0</td>
<td>Condensed</td>
</tr>
<tr>
<td>III</td>
<td>High</td>
<td>High</td>
<td>&gt;0</td>
<td>Very condensed</td>
</tr>
<tr>
<td>IV</td>
<td>High</td>
<td>Low</td>
<td>&gt;0</td>
<td>Expanded</td>
</tr>
<tr>
<td>V</td>
<td>Adequate</td>
<td>Adequate</td>
<td>Zero</td>
<td>Disputed as to whether expanded or condensed.</td>
</tr>
</tbody>
</table>

Studies of beef heart mitochondria, using competitive binding by inhibitors, indicated that the condensed configuration obtained in respiratory states II and III of the investigations of the Hackenbrock and Muscatello groups, resulted primarily from the binding and translocation of ADP (Weber, 1972; Scherer and Klingenberg, 1974). Earlier work had also considered the importance of ADP in regulating structural changes due to oxidative phosphorylation (Packer, 1960). Observations on isolated inner membrane particles, mitoplasts, prepared by critical-point drying have continued to show the importance of actual oxidative activity in inducing ultrastructural transformations (Andrews and Hackenbrock, 1975).

Evidence exists that the mitochondrial configurations become altered as a consequence of the water movements following the osmotic gradients set up by energy-dependent ion transport and accumulation by the organelle (Azzi and Azzone, 1966; Packer et al., 1968; Rottenberg and Solomon, 1969; Schmidt et al., 1977). An expanded orthodox form was generated by the uptake of calcium in the presence of phosphate, though it was claimed that the osmotic transformation in this case differed from the orthodox form resulting from respiration (Hackenbrock and Caplan, 1969; Hackenbrock, 1972). Ionic regulation and accumulation by mitochondria is certainly an important function of the organelle within the cell (Carafoli, 1975; Rose and Lowenstein, 1975).

1.6. Mitochondrial structural changes observed in 'in vivo' work

Whether the osmotic changes in mitochondrial ultrastructure...
occur 'in vitro' result from different oxidative phosphorylation states and the nucleotide binding associated with this function, or from energy-consuming events such as ion accumulation; mitochondrial structure is known to undergo alterations in many 'in vivo' systems when functional activity changes within the whole cell.

When rat liver tissue was incubated in the presence of succinate (Haydon et al., 1967) and intestinal strips in the presence of an amino acid mixture (Jasper and Bronk, 1968), treatments which possibly result in increased quantities of electrons passing down the electron transport chain, increased numbers of condensed types of mitochondria were recorded. Both these findings are consistent with the hypothesis of structural alterations being a direct consequence of respiratory functioning.

Recent work correlating mitochondrial structure with the ADP levels in unfertilised and fertilised sea urchin eggs has shown that for the transformation from a condensed to expanded form, which occurs at fertilisation, there must be a decrease in the ADP concentration (Innis et al., 1976). These authors were unable to state whether an increase in oxidative phosphorylation paralleled this event, although oxygen consumption was raised at fertilisation.

When cells are treated with uncoupling agents, mitochondria have been reported to undergo a contraction in form (Buffa et al., 1968). This is again suggestive of ADP levels and respiratory activity playing an important role in regulating mitochondrial structure.

Several reports of changes in matrix density and cristal form in the mitochondria of cells undergoing changes in activity level are known. In germ cell development of the female hamster, the mitochondria assumed an expanded type with highly variable cristae and a less dense matrix at stages of increased cell activity. At less active stages of oocyte development the matrix was of greater electron density (Weakley, 1975). On the other hand increased matrical density and changes in cristal form were evident in the parenchymal cells of the mouse mammary gland during mid-pregnancy and throughout lactation; this is indicative of ultrastructure being altered due to cell activity increases (Rosano and Jones, 1976).
During malignization of thyroid gland cells, there occurred a sharp decrease in the numbers of cristae within profiles and the matrix became more translucent (Dmitrieva, 1968). Mitochondria with a lighter matrix and of a swollen nature were generated in cells treated with fluoroacetate to decrease ATP levels (Buffa et al., 1977). This again supports the idea that altering cell and organelle activity alters mitochondrial form, i.e. fluoroacetate binds to enzyme complexes of the TCA cycle within the matrix.

In the differentiation of the trypanosome T. brucei, the mitochondria undergo alterations which have been correlated with the organism acquiring the ability to oxidase certain substrates (Brown et al., 1973). Variations associated with culture age are also reported for Tetrahymena pyriformis (Elliot and Bak, 1964). The ultrastructure of the mitochondria of Paramecium aurelia differ depending on whether axenic or monoxenic culturing has been employed (Prince, 1976). In this case a contracted and expanded form were reported.

1.7. Different ultrastructural forms existing simultaneously within cells and tissues

Within certain tissues and cells, different mitochondrial types have been reported to exist simultaneously. In some instances this difference rests mainly on gross shape but in others distinctions occur in the matrix density and cristal organisation. The latter examples are of greater interest in a study such as this one attempting to relate form to function.

In the femoral muscle of the cockroach three morphologically distinct mitochondrial forms were reported (Hagopian, 1967). Elongated forms were found wedged between the myofibrils; oval types associated with the sarcolemma; and Y-shaped profiles in close proximity to the Z-disc. Similarly with canary heart muscle not all mitochondrial types within a cell were alike, the gross form and the arrangement of the cristae being considered to be a sign of metabolic function (Slautterback, 1965).

Meristem cells of Zea mays possessed two mitochondrial forms, one of which was lost as the cells differentiated (Lund et al., 1958). Attempts were made in this study to correlate mitochondrial
form with the capacity to respire.

The ciliate *Euplotes minuta* was found to contain both expanded and denser contracted mitochondria within individual organisms (Jurand and Lipps, 1973). As with the earlier reports of at least two forms in *A. proteus* i.e. with matrix density and shape distinctions (Flickinger, 1968a), these authors demonstrated that this observation did not result from a fixation artefact, and speculated that the types might be in different physiological states or could perhaps be representing a juvenile and a mature form. The possibility of there being two different unrelated populations of organelles within the cell was also suggested.

The early light microscope work on amoebae demonstrated differences in mitochondrial structure in that elongated and spherical forms were evident (Torch, 1955). At the EM level using osmium tetroxide fixation gross shape was also used to distinguish different types of mitochondria in *A. proteus* (Mercer, 1959), although the internal differences in the complexity of the cristal organisation and matrical density were not realised until the double aldehyde fixative was employed (Flickinger, 1968a). Morphologically different mitochondria were also apparent in other species of Amoeba (Flickinger, 1974).

The aim of the present work was to consider whether any significance could be attached to the existence of the different mitochondrial types in *A. proteus*.

Variations in mitochondrial ultrastructure have been shown to arise in other amoebae during certain conditions, for example with *Chaos chaos* a complex 'zigzag' configuration has been noted during starvation (Pappas, 1959; Daniels and Breyer, 1968) which is also present during the mitotic stages of this amoeba (Daniels and Breyer, 1965). In view of these changes observed in other species related to alterations in cell and organelle function, experimental design focused on developing test conditions which might indeed be expected to at least alter function if not gross structure of the organelle. The project hoped that any detectable changes in organelle configuration would indicate what the fundamental differences between the various forms were representing within the normal control amoeba cell.
Cultures of *Amoebae proteus* used in the major part of this work were raised from cells of the strain *P. da X* 69 originally supplied by Dr. M.J. Ord. Additional cultures of *Amoebae proteus* P₁₁ and *A. discoides* D13 were kindly donated by Dr. S.J. Hawkins of Kings College, London in March 1977.

2.1. Culturing

2.1.1. Culture Medium

Cells were maintained by either wheat culturing or as *Tetrahymena*-fed cultures, following the basic methods of Griffin (1960). Culturing and handling of cells were carried out in a modified Chalkley's medium (Chalkley, 1930; Ord. 1970b); a concentrated stock solution of which contained the following salts in one litre of glass-distilled water:

<table>
<thead>
<tr>
<th>Salt</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>(1.37 x 10⁻³ M)</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>(4.76 x 10⁻⁵ M)</td>
</tr>
<tr>
<td>KCl</td>
<td>(2.68 x 10⁻⁵ M)</td>
</tr>
<tr>
<td>NaHPO₄ · 2H₂O</td>
<td>(2.79 x 10⁻⁶ M)</td>
</tr>
<tr>
<td>CaHPO₄</td>
<td>(7.36 x 10⁻⁶ M)</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>(4.92 x 10⁻⁶ M)</td>
</tr>
</tbody>
</table>

The medium used was prepared from 5 ml's of this stock solution made up to one litre with distilled water to give the final salt concentrations as shown above in parenthesis. The pH was adjusted to 5.9 - 6.0 with HCl before use.

2.1.2. Amoebae Stock Cultures

Stock cultures of amoebae were grown in plastic dishes (20 x 10 x 8 cm) as wheat cultures. Wheat cultures consisted of a balanced population of the ciliate *Colpidium sp.* and the smaller flagellate *Chilomonas sp.* These were associated with the mold *Dictyychus sterile* and bacteria which infest the boiled untreated wheat grains of the culture. Such cultures were subcultured at 4-5 week intervals by transferring an established wheat grain to a clean dish together with an aliquot of the ciliate/flagellate/mold suspension. Amoebae and new wheat grains were finally added to create a viable ecosystem.
Prior to use in any experimental procedure, the cells were removed from these stock cultures and maintained for at least 2 weeks by Tetrahymena-feeding.

2.1.3. Tetrahymena-feeding

*T. pyriformis* of strains ST or W, cultured in 100 ml. medical flats in sterile 2% proteose peptone, were harvested while still in the log phase of growth (i.e. within five days of innoculating the peptone). A sintered glass funnel of porosity 4 was used to filter-wash the tetrahymena to remove the excess peptone and waste materials before resuspension in Chalkley's medium. In order to supply at least 20 tetrahymena/amoeba/day 1-2 mls of peptone-free tetrahymena were added daily to amoebae cultured in Chalkley's to a depth of 1 cm in 75 mm diameter. Such mass cultures of amoebae were transferred to clean dishes three times a week by a tipping method to remove all waste materials and any older food organisms so producing healthy dividing cultures for use.

Normal cell culturing was controlled at 20 ± 1°C. Where other growth temperatures were required amoebae were transferred to pre-set incubators or waterbaths for supra-room temperatures. The 10 and 12°C environments were achieved by use of a small oven located within a 6°C cold-room. In all cases temperatures were monitored daily to ensure constancy.

2.2. Cell Treatments

The treatments of living cells with the various chemicals, incubation media and fixatives, used in the present study were affected in solid watchglasses by the following standard procedure:

The amoebae were transferred and allowed to reattach to the substratum before the Chalkley's medium was gently removed and immediately replaced by the test solution. This method of the addition of the solution was preferred to one where cells were directly pipetted into the test medium as favoured by others (Chatburn, 1977). It was considered that the undue energetic activity expended in re-attachment might result in additional stress affects at the onset of the treatment: thus any ultrastructural alterations observed upon fixation at the end of the
incubation period might not be due solely to the treatment itself. Similarly wherever possible the test solution was withdrawn at the end of the exposure period and replaced with either normal Chalkley's or fixative with minimal disturbance to the cells.

Except in the case of the incubation media for cytochemical staining, the chemicals under consideration were prepared by the dilution of stock solutions with normal Chalkley's to the final concentrations required and adjusting the pH.

Where cell viability was to be studied after treatment, the amoebae were cultured singly. Two means of single culturing were employed: either the watch glass technique generally used in Amoeba work, or the capillary tube method of Ord (1977).

In the watchglass technique cells are transferred by pipette to clean watchglasses three times a week and fed daily on tetrahymena. With the capillary method cells were transferred to clean sealed melting point capillary tubes (75 x 1mm) by a fine pipette. Dilute wheat food was introduced into the tubes to create a balanced system of Colpidia and Chilomonads. Such food was harvested from cultures two to three weeks old in order to maintain the correct proportions of the food organisms. The collected food was diluted with Chalkley's medium prior to its introduction into the tubes with the pH at 5.9. The capillary tube method avoids excess handling of treated cells once set-up, reducing the possibility of mechanical damage on manipulation - a necessary consideration if the chemical or treatment has caused alteration of the fragility of the cell membrane in any way.

2.3. Cell Synchronisation

Where experimental design necessitated the use of cells of a specific age, or when cells were to be starved, amoebae undergoing mitosis were selected from the mass cultures by use of a finely drawn-out pipette.

Division spheres are easily identified in *A. proteus* as the long pseudopods are retracted and replaced by a covering of short blunt pseudopods. These characteristic rounded forms lose attachment to the substratum. Sufficient cells of the same age may be
collected (+ 20 minutes) and grouped together in watchglasses until use, so giving synchronous cell samples. It is considered that metabolic processes are less disturbed by this method of synchronisation than in other techniques where temperature, nutritive or chemical shocks are involved (Mitchison, 1971).

2.4. Anaerobic Culturing

Anaerobic conditions were achieved in both hydrogen and nitrogen atmospheres by the use of two experimental set-ups.

1. Initially a microbial anaerobic culture jar (obtained from Baird and Tatlock) was used. This possessed a catalytic substance for use with an hydrogen atmosphere which functioned by reacting any traces of oxygen, remaining in the jar after the evacuation period, with the introduced hydrogen.

A redox indicator was included to check for any air leaks in the apparatus and to demonstrate that all detectable amounts of oxygen had been removed from the vessel prior to the introduction of the hydrogen or nitrogen. The indicator gel was made up as follows:

- 9% thioglycollic acid - 12 drops
- Phenol Red - 2 drops
- 2% Borax solution - 5 ml
- Methylene Blue (9.5 mg/100 ml) - 10 ml
- 2% melted agar - 10 ml

On boiling the above solution becomes colourless and was sealed in lengths of glass tubing until use in the apparatus. It is only in the presence of oxygen that the methylene blue is reoxidised resulting in the blue colouration, hence such a reaction in the anaerobic set-up indicated that evacuation had not been completed.

The commercial microbial culture jar was found to have two main disadvantages:

(a) Its large size meant a long period was required for the completion of evacuation and hence there was the danger of causing damage to the amoebae by subjecting them to prolonged pressure differences during this period.
(b) As the jar was constructed of stainless steel it was not possible to observe the cells during the incubations. Thus changes in cell morphology and behaviour could not be followed.

2. In order to overcome these difficulties a small experimental arrangement was designed consisting of well-greased sections of Quickfit (Fig. 2.1). This apparatus allowed one to follow the cell's state by use of an inverted microscope. The smaller air space allowed a more satisfactory evacuation of the apparatus. It also permitted further replenishment with the substituted gas as the incubation proceeded so that a relatively greater amount of hydrogen or nitrogen could be passed through the apparatus. The redox indicator and the catalytic substance, for when hydrogen was used, were retained.

Cultures were set up as follows:

In the initial group of experiments cells were placed in the Quickfit culture dish and allowed to settle so that the Chalkley's medium could be withdrawn to a depth of not more than 5 mm. Ordinary aerated Chalkley's was included at a pH of 5.9. An attempt to reduce the quantities of dissolved oxygen from the medium by boiling for five minutes raised the pH to over 7.0, indicating that the ionic composition of the medium had been disturbed. This step was therefore not pursued. In later experiments Chalkley's, through which 95% nitrogen had been bubbled for one hour prior to use, was included. This precaution however was not considered critical as the concentration of dissolved oxygen in the small quantity of medium should rapidly equilibrate with the gaseous phase above it and be removed on subsequent flushings with the oxygen-free atmosphere.

When a Quickfit culture dish was included in the apparatus the lid was firmly sealed to it by applying a layer of silicone greases. In later experiments the apparatus was further modified by permanently fixing the lid to a glass plate with Araldite, eliminating air leaks and improving cell visualisation. Amoebae were introduced into the chamber of this by pipette through inlet tube I.

With all other joints well greased, the evacuation of air
Fig 2.1. : The anaerobic-culturing set up.

\[ T_1 \] - Inlet tap 1 to which the vacuum pump or replacement gas supply was fitted.

\[ T_2 \] - Tap 2.

\[ I \] - Indicator gel.

\[ C \] - Catalyst

\[ A \] - Culture chamber for the amoebae.
commenced. The lead of a vacuum pump was attached to Tap 1 which was then opened (all other taps being closed at this stage). After five minutes tap 1 was closed, the pump lead removed and the hydrogen or nitrogen source connected. On reopening Tap 1 the replacement gas was sucked into the apparatus. Tap 2 was then opened so that further gas could be passed through the system. This process of flushing hydrogen or nitrogen through the set-up was repeated five times in the first hour after the removal of the air; and then at periodic intervals throughout the rest of the incubation ensuring that the atmosphere remained oxygen-free.

2.5 Micrurgical Operations

These operations were kindly performed by Dr. M.J. Ord using a Fonbrune micromanipulator at mag. 200 x and the oil chamber technique of Comandon and de Fonbrune (1939). Glass needles, hooks and micro-pipettes were constructed on a microforge.

2.5.1. Nuclear Transfers

These involved the insertion of nuclei of DNP-treated cells into host control anucleated cytoplasm. The reciprocal transfer could not be effected due to the fragility of the treated cell membrane. Hybrid cells were cultured singly in watchglasses.

2.5.2. Microinjection

In addition to the Fonbrune manipulator, an Agla Microsyringe Outfit (Wellcome Reagents Ltd.), which can deliver volumes as small as 10 ul., was employed. The whole system was oil filled for increased sensitivity. An attempt to standardise the amount of a reagent injected into each amoeba was made in that, by eye, the amount of reagent delivered on each occasion occupied the same length of pipette (Fig. 2.2). The presence of an oil plug prevented the reagent from leaking out into the medium prior to the injection of the cells, and also restricted entry of any fluid into the pipette by simple capillary action.
2.6. Preparation of cells for the Electron Microscope

The principles involved in the fixation, dehydration and embedding stages necessary for specimen preparation for the electron microscope have recently been clearly stated (Glauert, 1975).

2.6.1. Fixation

The primary fixative routinely used consisted of a glutaraldehyde-formaldehyde mixture as proposed by Karnovsky (1965). This would seem to be a most suitable fixative as the formaldehyde is considered to penetrate the cells rapidly, initiating the cross-linkage of the cell's protein moieties; while the dialdehyde penetrates less rapidly but subsequently stabilises and ramifies the protein cross-linking.

Williams et al. (1970) consider this fixative to be "eminently suitable for the preservation of mitochondrial configurational states present at the moment of fixation". The fixative was used as soon after preparation as possible and at least within a week.

25 ml of fixative were prepared as follows:

1 gm of paraformaldehyde was heated to 70°C in 7 ml of distilled water. Single drops of IN NaOH were added until the white flocculations disappeared. This solution was allowed to cool before the addition of 5 ml of 25% ultrapure glutaraldehyde (EM scope). The fixative was made up to 25 ml in a volumetric flask with 0.2M sodium cacodylate buffer. Extreme care should be followed in the preparation and subsequent use of this buffer in fixatives and washing solutions, all procedures being carried out in a fume cupboard in view of its potential hazardous effects (Weakley, 1977). 10 mg of CaCl_2 was added to the final solution as is customary in fixatives where cacodylate has been used as the buffer.
The fixative possessed final concentrations of 4% formaldehyde, 5% Glutaraldehyde and 0.1M cacodylate. The pH was adjusted to 7.1 prior to use and normally stored at 4°C to retard the degradation of the glutaraldehyde.

Cold unmodified fixative was added to the cells after the removal of as much Chalkley's or test solution from the amoebae as possible. The cells were left in the presence of the fixative for 45-60 minutes. Cells were then washed briefly for 5 minutes with 0.1M cacodylate buffer before post-fixation with 1% OsO₄ buffered with 0.1M cacodylate at pH 7.1 for one hour. Brevity of the washing stage is recommended to minimise any risk of lipid leeching (Busson-Mabillot, 1971) as the aldehyde fixatives do not stabilise these components. Indeed some workers (Ockleford, 1975) dispense with all washing stages, applying the aldehyde and osmium simultaneously. The secondary fixative was also replaced by cacodylate buffer prior to the cells being blocked in 2% agar for ease of further handling.

2.6.2. Agar blocking, dehydration and staining

The agar blocking of cells at this stage has many advantages in that the cells may be grouped together and oriented as desired to facilitate the sectioning and viewing of more than one cell on the E.M. at a time. It also reduces cell damage and loss due to handling, for once fixed the cells become sticky and have a tendency to adhere to the pipette on transfer to subsequent fluids if not blocked.

Agar blocking has been used by several authors in fixation techniques for small cell populations (Kimball & Perdue, 1962; Stone & Cameron, 1964; Flickinger, 1965; Hirsch & Fedurko, 1968). In the procedure developed in this laboratory the disadvantages inherent in some of these methods have been eliminated. Many of these workers employed a centrifugation step to pellet the cells before blocking. This is considered undesirable as it introduces the possibility of disrupting the internal organisation of the cell. The earlier techniques concentrated on trapping the cells in the middle of the agar block whereas our method ensures the amoebae are on the surface of the block, thus facilitating penetration by the dehydrating and embedding solutions.

Four to eight cells were pipetted onto an agar surface and arranged in groups by means of fine needles. In order to flatten the
amoebae to aid in sectioning, the cells were sometimes pipetted directly onto a glass surface rather than agar (although this increases the risk of the cells drying out). Excess fluid was withdrawn by means of a finer pipette so that when a drop of molten agar was placed onto the cells from the end of a glass rod, they did not disperse from the group. Once the agar had set, the block was carefully cut out and excess agar removed by manipulation with the two needles.

Such blocks were dehydrated in a graded series of ethanol alcohols (20, 35, 50, 70, 90, 95 and two changes of absolute) for 10 minutes each except that they were left in either the 50% or 70% alcohol for staining purposes for one hour. Initially 3% uranyl acetate was prepared in 70% alcohol and millipore-filtered before use. Stain precipitate however was found to be reduced by using only 2% uranyl acetate in 50% alcohol and so this was adopted in the later stages of the work. Little difference could be identified between the two stain concentrations.

2.6.3. Embedding

Wherever possible blocked cells were taken through the preparatory stages to Spurr resin (Spurr, 1969) on the same day that fixation had been performed. If cell treatments necessitated the cells being left overnight prior to this stage they were left in either 50% or 70% alcohol in preference to buffer or the lower alcohols (Spector, 1975). This was also to minimise lipid extraction from the material (Korn & Weisman, 1966). Spurr resin is a low viscosity epoxy resin and was prepared by thoroughly mixing the constituents together in a fume cupboard:

10 gm ERL 4206 (Vinyl cyclohexane dioxide)
6 gm DER 736 (diglycidyl ether of polypropylene glycol)
26 gm NSA (Nonenyl succinic anhydride)
0.4 gm SI (Dimethylaminoethanol)

Mixture was effected by means of a clean glass rod and the medium covered until use.

Before reaching the complete Spurr resin stage, the blocks were placed in a 1:1 mixture of Spurr and absolute alcohol for 30 minutes to aid in the impregnation of the cells by the polymer. For convenience the cells were left in Spurr resin overnight.
Finally the blocks were lifted into, and suitably arranged in, plastic BEE'Ci capsules and filled with fresh Spurr resin after any of the previous resin had been removed by pipette. Polymerisation was effected in a 70°C oven for two days.

2.6.3. **Sectioning**

Blocks were selected, trimmed and ultrathin sections with interference colours of silver-gold (600–900 Å) (Meek, 1970), were obtained on an LKB ultratome. Glass knives were constructed and used throughout the study.

Sections were stretched by xylene vapour while floating on the knife's watertrough and were mounted on grids. Normally 200-mesh copper grids were used, although nickel grids were also employed. Where serial sections were being studied, 2 x 1 mm copper grids covered with a film of Formavar were prepared and used. The grids were left to dry on a clean filter paper before storing in grid boxes until viewing.

Sections were examined on a Philips EM 3000 normally operated at 80kV.

2.7. **Cytochemical method for the demonstration of cytochrome oxidase**

The DAB reaction has proved useful in the ultrastructural localisation of peroxidative activities within many cells since its introduction by Graham-Karnovsky (1968). The following medium modified from that of Spector (1975) was employed in the present study to demonstrate cytochrome oxidase activity in the mitochondria:

\[ 10 \, \text{mg} \, \text{Na}_3\text{PO}_4 \, (0.15M) \, \text{buffer at pH 7.4 and containing} \, 0.15M \, \text{sucrose.} \]

\[ 10 \, \text{mg} \, \text{Diaminobenzidine hydrochloride (DAB) (B.D.H.)} \]

\[ 10 \, \text{mg} \, \text{Cytochrome c Type IV. (Sigma)} \]

\[ 10 \, \mu\text{g} \, \text{Catalase (Sigma)} \]

This was shaken vigorously to dissolve the DAB. All incubation media were always made up freshly and kept away from direct light in order to minimise auto-oxidation of the DAB. The catalase was added to remove endogenous peroxide, while the cytochrome c was included in an attempt to enhance staining. The pH was generally corrected to 7.4 as this is considered the optimum for cytochrome
oxidase activity, although the reaction has also been performed at lower pH's as recommended by some (Opik, 1975). In an attempt to increase structural preservation of the material without the subsequent loss of enzyme activity, the concentration of sucrose was varied in many incubations (Litwin, 1975; Posakony et al., 1975).

Incubations were performed on both live and prefixed cells. The prefixation step, using formaldehyde solutions, was included to stabilise structural integrity of the amoebae and also to aid in the penetration of the DAB across the cell membrane.

In a number of experiments 5% dimethyl sulphoxide (DMSO) was added to the medium as a further means of increasing penetration. This was particularly necessary when unfixed cells were incubated in the medium as it was desirable to obtain the shortest incubation time possible. DMSO, originally used as a cryogenic agent (Lovelock and Bishop, 1959), has more recently been developed in techniques requiring shortened incubation periods as it enhances permeability of membranes in animals, plants and micro-organisms (Reis, 1971; Ghajar & Harmon, 1968; Makita and Sandborn, 1971). DMSO is also beneficial in that it increases the solubility of the DAB in the incubation medium.

All incubations and subsequent rinses were performed at 20°C in a darkroom dimly illuminated by a yellow safety light. Even in these conditions a dark brown precipitate was occasionally noted in the incubations of more prolonged durations: presumably due to the auto-oxidation of the DAB. The incubation period extended from 20 minutes for some of the treatments of live cells to overnight for cells that had been given a relatively long period of prefixation.

Control incubations were included where either the cytochrome c and the catalase or the DAB were omitted from the complete medium. In other experiments KCN was added to the medium as an additional control.

After the required period of incubation the cells were thoroughly washed for thirty minutes in two rinses of the phosphate/sucrose buffer to remove any unreacted DAB. This is an important consideration as the DAB itself will undergo osmication with the OsO₄ and so must be removed to avoid erroneous results.

When fresh material or when cells with a brief prefixation had
been incubated, fixation with Karnovsky's fixative was carried out after the buffer rinses before the cells were handled further in the routine processes for E.M. Specimen preparation. Counter staining with uranyl acetate was not included.

2.8. E.M. Autoradiography

2.8.1. Labelling

Cells were selected from mass cultures as division spheres and incubated at the required age by replacing the Chalkley's medium with two drops of (CH₃)₂-Thymidine of specific activity 19 Ci/mM (purchased from Radiochemicals Centre, Amersham) at a concentration of 0.5 mCi/ml.

After the desired exposure period the cells were washed in three rinses of Chalkley's medium prior to fixation and E.M. preparation as described in section 2.6, except that staining with uranyl acetate was not included.

2.8.2. Application of the photographic emulsion

A modification of the loop technique of Caro and Van Tubergen (1962) was followed (Kanobdee, 1975).

Prior to the application of the Ilford L4 emulsion to the grids in the dark room, certain preparatory steps were performed. The sections from the ultratome were preferentially mounted on nickel grids, as in the initial experiments when copper grids had been used copper salts were deposited upon the sections in the final stages of the technique, making examination difficult. Two groups of five to ten grids were arranged on an alcohol-cleaned slide by means of securing the corner of each to a small strip of double sided sello-tape. The grids were transferred through all subsequent stages attached to the slide in this manner.

The L4 emulsion was transferred from the bottle to a clean beaker in the darkroom at a working distance of 4-5 feet from an amber safety light. The beaker was then placed in a water bath pre-heated to 45°C for 7-10 minutes, stirring occasionally. 10 ml of the liquified emulsion was then poured into a measuring cylinder and the volume made up to 25 ml with distilled water at the same temperature. The mixture was stirred gently so as to avoid the
introduction of excess air into the liquid. The dilute emulsion was transferred to a 32°C waterbath for five minutes to equilibrate and then left to cool at room temperature in a crystallizing dish.

A loop of silver wire was used which had been constructed of 0.6 mm diameter wire and had a loop diameter of approximately 4 cm secured into the end of a glass pipette by means of dental wax. The loop was dipped into the emulsion and withdrawn to form a thin monolayer film of emulsion across it. The loop was held vertically so that the excess emulsion would drain to the bottom and could then be absorbed onto a piece of mediwipe. After approximately 30 seconds - 1 minute, interference colours developed, beginning at the upper edge of the loop and moving downwards. When these had formed over the top half of the film, the loop was turned into the horizontal position and touched onto the surface of each slide containing grids.

The slides were dried vertically in a drying chamber before storage in a sealed black box containing crystals of silica gel as a drying agent. Exposure was at room temperature for 6 - 8 weeks.

2.8.3. Development

After the completion of the desired exposure period the slides were opened in the darkroom and transferred to Copland jars containing millipore-filtered D19 developer for 6 minutes at room temperature. This was followed by quickly rinsing the slides in three changes of distilled water prior to immersion in 20% sodium thiosulphate for 10 minutes. This step dissolved away the non-exposed silver bromide crystals of the emulsion leaving only the reduced silver grains in position.

The grids were given three rinses in distilled water for 10 minutes before proceeding to the next stage.

2.8.4. Gelatin removal

As the gel of the emulsion tends to obscure the ultrastructural detail in the final image, it was desirable to remove it prior to section staining. The slides containing the developed grids were therefore transferred to a Copland jar containing distilled water preheated to 37-40°C for 30 minutes. This step was immediately
followed by one where the slides were placed in 0.5M acetic acid for 15-30 minutes so that the softened gel was digested. The acetic acid was washed away with distilled water rinses and the slides dried so that the grids would be removed from the sellotape and stained individually.

2.8.5. Section staining for E.M. autoradiography

In view of the radioactive decay of uranium salts, cells for autoradiographic studies were not stained until after the development of the photographic emulsion. The procedure for the staining of these thin sections involved the use of Millonig's lead stain followed by a saturated solution of uranyl acetate.

Millonig (1961) suggested that lead hydroxide could be stabilised in solution by the addition of sodium-potassium tartrate. This reduced the levels of stain precipitate. For use with amoebae the best results have been obtained in this laboratory by the following method:

A stock solution was prepared with 20 gm NaOH and 1 gm K-Na-tartrate made up to 50 ml with distilled water. One ml of this was added to 5 ml of a 20% lead acetate solution in water. The resulting mixture was diluted five times with distilled water, shaken and filtered to remove the white precipitate which left a clear colourless solution stable at room temperature for several weeks. Storage was normally effected at 4°C.

Drops of the lead stain were pipetted onto a sheet of dental wax and the grids placed section-side downwards in these for 10 minutes. They were given a brief rinse in distilled water before transferring them to 7.5% uranyl acetate in 50% alcohol for 1 minute. The stained grids were washed in three rinses of water and dried in a covered petri dish ready for examination.

2.9. Collection of data from the Electron Microscope

In the consideration of the mitochondrial ultrastructure in the present study certain parameters were deemed as useful when characterising the organelles. At least two distinct types: a contracted Type I and an expanded Type II were evident in control amoebae, together with profiles intermediate in form, Type Int. These forms
were distinguished by differences in the matrical density, cristal organisation and gross shape. Means of quantifying these were considered.

Due to the non-randomness of mitochondrial distribution in amoebae, a consideration of a morphometric study was not pursued. The mitochondria tend to be more numerous at the cell periphery, in association with the contractile vacuole, and at regions close to the nucleus; other regions of the cytoplasm may be devoid of the organelle. As such stereological techniques were not considered a profitable approach for the present work.

For control and treated amoebae, estimates of the relative proportions of Type I and Type II were attempted to study whether the relative numbers of the two forms altered at different times in the cell cycle or under different experimental conditions. Such counts were routinely sampled directly from the microscope. Random areas of cells were scored and the counts from at least four separate sections for each cell pooled. Thus sampling error due to any non-random association of a particular mitochondrial type with specific cellular localisations was lessened. All individual experimental samples included some cells in which areas close to the nucleus and the contractile vacuole were studied.

Normally data was taken from 12-25 cells for each experimental treatment group to give the mean values presented in the text. For the different sections of the work, this gave large total samples. Thus in considering the various types throughout the cell cycle, over 220 cells were quantified and a total of over 30,000 profiles scored. Other cells were also viewed quantitatively in each case.

The various mitochondrial forms, together with an appraisal of the parameters investigated in classifying the mitochondria, and the significance of the changes in their relative proportions are discussed in greater detail in Chapter 3.
Chapter Three

The structural characteristics of the mitochondrial types of *A. proteus* and their presence throughout the cell cycle.

3.1. Introduction

Early work indicated that *A. proteus* contained a large number of small, discrete mitochondria (Mercer, 1959), which were concentrated in the hyaline layer adjacent to the external membrane, and in association with the contractile vacuole (Mast and Doyle, 1935). As with similar work on *Chaos* (Torch, 1955; Andresen, 1956), this was considered to indicate that the organelles were located in areas where high levels of ATP were required. In other systems, in addition to distinct regions within the cell requiring high ATP levels, specific points in the cell cycle have been reported, such as mitosis and during RNA and protein synthetic periods, where relatively greater energy demands existed (Amoore, 1963; Epel, 1963; Van't Hof, 1966; Webster and Van't Hof, 1969). The effect of such energetic differences on mitochondrial structure were not recorded.

The mitochondria of *A. proteus* are of interest because when Karnovsky's fixative, a glutaraldehyde-formaldehyde mixture, was used, different morphological forms were shown (Flickinger, 1968a). These types were classified as light, dark and intermediate forms depending on the density of the matrix and the organisation of the cristae. In other systems when different mitochondrial forms were observed in separate cells, either in different tissues or activity phases, it was questioned whether such variations represented genuine differences or not (due to the high osmolarity of aldehyde fixatives). This objection could not easily be applied to *A. proteus* where the coexistence of the two types in close proximity within the same cell indicates that the observation represents an actual chemical or physiological difference between the two types preserved by aldehyde fixation, rather than an artefact of fixation.

The absence of the dark form of mitochondria when osmic acid was used as the sole fixative required an explanation, and it was considered that perhaps the osmolarity difference might be of importance in this case. Furthermore, the presence of disrupted
vesicular structures in osmium-fixed cells, which had not been observed when an aldehyde prefixation had been carried out, needed clarifying. By fixing amoebae in sucrose-buffered osmium at the same osmotic strength as the Karnovsky's, Flickinger demonstrated that the osmolarity difference between Karnovsky's fixative and osmium tetroxide was not the crucial distinction between the fixation regimes. Since only one mitochondrial form was again preserved, as well as the vesicular structures, he concluded that the aldehydes maintained a chemical difference which was not caused primarily by osmotic factors. It was suggested that osmium tetroxide was a poor mitochondrial fixative and that the disrupted vesicd' possibly represented the missing mitochondrial form. The control dark and light mitochondria have since been classed as Type I and Type II forms respectively in a report, where changes in mitochondrial forms were recorded in A. proteus after treatment with certain carcinogens (Ord, 1976).

The double fixation method of Karnovsky's fixative followed by postfixation with osmium tetroxide has been used throughout the major parts of this study. The nomenclature of Ord i.e., Type I and II, together with the introduction of Type Int to distinguish the transitional intermediate forms has been adopted. With the acceptance of the obvious dangers of attempting to draw inferences concerning the dynamic relationships within the living cell from the static sections viewed on the EM; the initial aim of this project was to clearly distinguish and categorise the various mitochondria preserved from healthy, untreated cells of A. proteus. Flickinger had studied the mitochondria in some detail, presenting measurements of 0.5-1μ diameter by a 2μ long axis and mean cristal width of 660 Å for the dark mitochondria; and estimates of 0.7-1.5μ diameter and cristal widths of 500 Å for the light mitochondria. He reported that about 50% of the cells contained profiles of predominately Type I, about 25% had mainly Type II while the rest had the two types in roughly equal proportions. In general the minority type was also evident co-existing in the same cell, while 5-10% of the mitochondria possessed a matrix of intermediate density. Flickinger's work however was based on an undefined number of cells, of an unknown age and selected for fixation from a wide range of culture conditions. Such a control study was considered of little value as a basis in the
present work from which to recognise and accurately define the changes which could occur in the mitochondria when cells were exposed to stress situations or to chemical treatments.

The following basic study of the morphological characters, and the frequency of appearance of each control type was therefore performed for use in the subsequent work where conformational changes were induced. No estimate of organelle number per cell was attempted in the present study. Since it was observed that some differences in the relative frequencies of the different forms of mitochondria occurred as cells progressed through the cell cycle, which might possibly be related to changes in substrate levels or energy requirements, a cell cycle study was also undertaken so that the variabilities of form and frequency within the control cell could clearly be defined. As it was not feasible to include all times in the 48 hour cell cycle of *A. proteus*, special attention was given to points in the cycle where increased nuclear and cytoplasmic activity have been demonstrated (Ord, 1968b; 1973; Chatterjee and Bell, 1976; Minassian and Bell 1976a). Any temporal factors which might underlie the structural differences preserved upon aldehyde fixation would therefore be realised.

3.2. The structural parameters used to quantify mitochondrial types

The different mitochondrial types shown in Figs. 3.1, 3.2, 3.4 and 3.8 were routinely observed in control cells. At least two forms of a similar nature were also preserved in other species of amoebae (Fig. 3.7). These differences resulted when Karnovsky's fixative was employed, or when the primary fixation step involved formaldehyde or glutaraldehyde administered separately. Karnovsky's fixative gave the optimal structural preservation.

In estimating the basic structural parameters, care was taken to use cells fixed from many different experiments in which different batches of Karnovsky's had been prepared. It was hoped in this way to eliminate any subtle changes in form which might result from slight variations in the processes necessary for specimen preparation. The intensity of the matrix density was not quantified but estimates could be performed qualitatively. Cristal form and gross shape calculations were attempted.
Fig. 3.1.: Types I and II in close proximity to the cell's nucleus in a 13 hour cell. 
\[ \text{x 9,200} \]

Fig. 3.2.: Types I, II and INT preserved in a 1 hour old cell. 
\[ \text{x 9,200} \]

Fig. 3.3.: Type I mitochondria surrounding a contractile vacuole in a 24 hour cell. The large profile is approximately 5 - 10 \( \mu \text{m} \) long, although this is uncommon for Type I profiles in control cells. 
\[ \text{x 16,000} \]

Fig. 3.4.: Type I and Type II-Int forms in the cytoplasm near to the contractile vacuole of an 8 hour old cell. 
\[ \text{x 32,000} \]
Flickinger reported the occasional presence of fine filamentous inclusions within the matrix of both main mitochondrial types, though these were more noticeable in Type II profiles. Such filaments were not observed in healthy control amoebae in the present work, nor in other studies from this laboratory. Filamentous inclusions have been observed under certain conditions and are discussed fully in chapters 5 and 6.

Both mitochondrial types were found distributed throughout the cytoplasm. In some cells higher proportions of Type I were associated with the contractile vacuole (Fig. 3.3), while increased numbers of Type Int profiles were sometimes observed surrounding medium sized vacuoles. However, in general there was great variation from cell to cell as to which mitochondrial type predominated close to this organelle.

3.2.1. Gross shape considerations

Profile dimensions for the two main mitochondrial types were found to be of similar range of values when taken from cells of all the cycle ages studied and when measured at different localities within the individual cell. Data for the two main types is given below:

Table 3.1: Profile dimensions

<table>
<thead>
<tr>
<th></th>
<th>Type I</th>
<th>Type II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organelle long axis (μ)</td>
<td>1.65 ± 0.05</td>
<td>1.23 ± 0.03</td>
</tr>
<tr>
<td>short axis (μ)</td>
<td>0.6 ± 0.01</td>
<td>1.1 ± 0.02</td>
</tr>
<tr>
<td>Cristae width (Å)</td>
<td>400 - 1,000</td>
<td>400 - 600</td>
</tr>
<tr>
<td>Matrix density</td>
<td>Dark</td>
<td>Light</td>
</tr>
</tbody>
</table>

Occasionally Type I profiles had a long axis exceeding 3μ (Fig. 3.3), but these were not common in control amoebae and tended to be restricted in the cell to areas where high proportions of Type I mitochondria were observed.

From studies of a limited number of serial sections, and from a consideration of randomly sectioning plasticine models, it was discerned that the observed profiles were consistent with the
assumption that in three-dimensions Type II mitochondria are probably spherical forms, while Type I would be of a cylindrical nature. The random sections of Type I rods or cylinders thus resulted in both elliptical and circular profiles with a greater variation in actual profile dimensions than those from the sectioning of Type II spheres where only circular profiles were generated.

Rather than actual absolute measurements for each organelle, a consideration of the ratio of the long and short axes of the profiles was undertaken as a means of quantifying mitochondrial shape. The greatest organelle length was recorded for the long axis, while the average of three measurements was used to give the width or short axis. Using the calculated length to width (L:W) index, the spread of values for Type I mitochondria due to the random plane of sectioning was not as extreme as when absolute dimensions were considered. In order to eliminate some of the persisting variation which resulted from sectioning close to the diameter of the presumed cylindrical organelle and producing the smaller circular profiles, rather than sectioning near to the full length of the organelle which would produce the elliptical forms, the lowest 15% of calculated ratios were discounted from all estimates. In spite of this the calculated L:W index will still be an underestimate of gross organelle shape for Type I mitochondria as more cuts are possible nearer to the diameter of a cylindrical object than those approaching its length. However, in conjunction with the other criteria it is potentially useful in classifying the mitochondrial types.

Profiles from cells cultured at pH 4.0 as well as those of pH 5.9 grown cultures were measured to demonstrate that external pH changes did not affect mitochondrial structure within the cell. Mean L:W indexes were calculated for the different mitochondrial control types:
Table 3.2: Estimations of the L:W index for the control mitochondrial types

<table>
<thead>
<tr>
<th>Mitochondrial Type</th>
<th>Number of profiles measured</th>
<th>Matrix density</th>
<th>L:W index ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I pH 5.9</td>
<td>552</td>
<td>Dark</td>
<td>2.72 ± 0.05</td>
</tr>
<tr>
<td>Type I pH 4.0</td>
<td>216</td>
<td>Dark</td>
<td>2.62 ± 0.08</td>
</tr>
<tr>
<td>Type Int pH 5.9</td>
<td>370</td>
<td>Intermediate</td>
<td>1.95 ± 0.03</td>
</tr>
<tr>
<td>Type II pH 5.9</td>
<td>637</td>
<td>Light</td>
<td>1.39 ± 0.01</td>
</tr>
<tr>
<td>Type II pH 4.0</td>
<td>124</td>
<td>Light</td>
<td>1.43 ± 0.04</td>
</tr>
</tbody>
</table>

The Type Int form is seen therefore to have a L:W index lying between the values for the other two types.

3.2.2. Appearance of the cristae

Type I mitochondria appear to have the matrical space contracted, due both to the condensation of the whole organelle and to an expansion of the cristal membranes and space, when compared to Type II profiles. Means were sought of investigating whether cristal expansion was a real distinction or not. Stereological techniques using a regular point analysis overlying the mitochondrial profiles could be used in this study. However, in the present study it was preferred to use the more accurate, but more time-consuming, method based on the relative weights of the different mitochondrial compartments.

Individual profiles were cut from micrographs at a 40,000 x magnification and the paper weighed. The area represented by the cristae were then carefully removed and weighed separately. The proportion of the total weight represented by the cristae weight was taken as an estimate of the relative volume occupied by the cristae for comparison between Type I and Type II forms. This method assumes that the mean relative area of the cristae in a series of random profiles will be a consistent estimation of the relative volume throughout the entire organelle. The method further requires the supposition that the paper is of even thickness and weight throughout the entire micrograph profile. Without a detailed stereological study
the number of cristae per organelle could not be calculated and so no record of the numbers in the sections was attempted.

For the majority of profiles considered by this weighing method, a modification was introduced in that profile enlargement was achieved to a final magnification of 200,000 x. For this randomly selected profile images were traced by hand directly from the enlarged photographic plate. The replicas were processed in the same manner as the printed micrographs. This modification proved advantageous as the actual cutting could be effected more precisely and it also proved less expensive. It suffered the disadvantages however of potentially introducing slight inaccuracies in representing cristal arrangement due to the additional stage of making the tracing and also increasing the labour involved in the study.

Both profile tracings and micrographs were included in the total samples for Type I and Type II mitochondria. Comparisons of the two sampling procedures showed that measurements from micrographs have slightly higher values for the cristae proportions than the tracing method. 'In toto' 85 Type I and 82 Type II profiles were dissected and the relative area of the profile represented by the cristae estimated. The spread of values for Type II mitochondria was fractionally greater than for Type I; however the overlap region between the two populations was restricted and the sample means lay well apart (Fig. 3.5):—
Fig. 3.5: Frequency distribution of the relative cristal areas within Type I and Type II profiles.

Means for the two populations were:
- Type I: 0.453 ± 0.008
- Type II: 0.287 ± 0.008

i.e. mean relative area of profile occupied by cristae.

Insufficient EM plates of Type I int profiles have been collected for inclusion in the present work but a mean estimate lying between the two figures presented for Types I and II is anticipated.

3.3. Relative proportions of Type I and II mitochondria throughout the cell cycle.

Casual scanning of cells at different known age revealed more difference between cell age groups than could be expected from one individual cell to another within the age group. It was therefore necessary to study the cell cycle and its activities more closely.
to determine whether normal cell cycle changes had a specific affect on the proportions of the two main mitochondrial types. This investigation was especially useful in demonstrating whether the incidence of the mitochondrial types reflected age differences or differences associated with specific cell activities. Eight different ages were sampled from S-phase (when nuclear activity is high); two from the S-G2 transition period of the cycle; three from early in G2; one from mid-G2; and two from the end of the cycle. Mitotic cells were not scored for the relative proportions of the two types although mitochondria were examined in dividing cells. Estimations of the cycle phases correspond to the timings given previously for strain P (Ord, 1970a). Data was collected as described in Section 2.9.

Both Types I and II were present at all times in the cell cycle sampled (Fig. 3.1, 3.9 etc.). The relative proportions of Types I and II were however found to alter in a peak and dip manner (Table 3.3, Fig. 3.6). The highest peak of Type I occurred in 5 hour cells, while the lowest dip was seen at 32 hours. Other peaks of Type I were present at 13 hours, and 19-24 hours where Type II were less evident. Type II peaked also at 8 and 15 hours in addition to 32 hours. Towards the end of the cycle the mitochondria were represented roughly in equal proportions, although only two points were sampled and so peak and dip intervals may have been overlooked.

At certain ages in the cell, particularly during mitosis and in those cells observed between ½ to 1 hours (Fig. 3.8), a relatively high number of the profiles had an intermediate matrix density. The importance of assessing all the structural parameters in classifying the mitochondria as Type I or II was therefore realised in these samples. Higher proportions of Int forms were also evident in 13 hour cells. In general however Type I and II profiles were easily distinguished (Fig. 3.9) and the levels of Type Int forms less prevalent.

3.4. Discussion

The presence of at least two morphologically distinct mitochondrial types in such close association as was evident upon aldehyde
Table 3.3: Proportions of Type I mitochondria present at different phases of the cell cycle

<table>
<thead>
<tr>
<th>Cell Age (hours)</th>
<th>No. of cells Per age sample</th>
<th>No. profiles scored/age sample</th>
<th>% of profiles as Type I ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>12</td>
<td>1358</td>
<td>55.8 ± 8.3</td>
</tr>
<tr>
<td>1</td>
<td>13</td>
<td>1516</td>
<td>50.4 ± 3.9</td>
</tr>
<tr>
<td>2</td>
<td>13</td>
<td>2205</td>
<td>52.2 ± 2.9</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>1892</td>
<td>56.8 ± 2.5</td>
</tr>
<tr>
<td>5</td>
<td>13</td>
<td>2468</td>
<td>64.7 ± 2.9</td>
</tr>
<tr>
<td>6</td>
<td>11</td>
<td>1881</td>
<td>47.2 ± 4.1</td>
</tr>
<tr>
<td>8</td>
<td>14</td>
<td>2261</td>
<td>43.9 ± 3.6</td>
</tr>
<tr>
<td>10</td>
<td>15</td>
<td>2572</td>
<td>53.3 ± 4.1</td>
</tr>
<tr>
<td>13</td>
<td>17</td>
<td>2058</td>
<td>57.8 ± 2.4</td>
</tr>
<tr>
<td>15</td>
<td>14</td>
<td>2142</td>
<td>40.5 ± 3.0</td>
</tr>
<tr>
<td>17</td>
<td>14</td>
<td>3087</td>
<td>51.7 ± 5.3</td>
</tr>
<tr>
<td>19</td>
<td>17</td>
<td>2601</td>
<td>61.6 ± 1.6</td>
</tr>
<tr>
<td>23</td>
<td>18</td>
<td>3809</td>
<td>57.0 ± 2.0</td>
</tr>
<tr>
<td>32</td>
<td>10</td>
<td>1354</td>
<td>35.2 ± 4.2</td>
</tr>
<tr>
<td>45</td>
<td>15</td>
<td>2143</td>
<td>47.8 ± 3.0</td>
</tr>
<tr>
<td>52</td>
<td>13</td>
<td>1560</td>
<td>45.6 ± 2.9</td>
</tr>
</tbody>
</table>

Cells for each age sample were fixed from at least two separate experiments.
Fig. 3.6: Relative proportions of Type I and Type II mitochondria at different stages of the cell cycle.

The area above the curve represents the relative proportions of Type II mitochondria while the area below the curve represents the proportions of Type I.

Each point represents data collected from between 12-20 cells.
Fig. 3.7. : Different mitochondrial types associated with the contractile vacuole in *Amoeba discoides.*
\[ x \ 6,400 \]

Fig. 3.8. : Type Int forms from a \( \frac{1}{2} \) hour cell.
\[ x \ 16,000 \]

Fig. 3.9. : Types I and II in close proximity to the nucleus of an 8 hour old cell.
\[ x \ 16,000 \]
fixation in the present study indicates that some chemical or physiological difference exists between the forms within the cell. It is seen that simple criteria may be employed to distinguish these types:

(i) Differences in gross organelle dimensions which have been characterised by estimations of a L:W index.
(ii) The density of the matrix.
(iii) Determination of the relative proportion of the organelle which is occupied by the cristae.

The differences in matrix density and overall shape typical of Type I and II control profiles serve as baselines from which alterations induced by specific treatments can be perceived. L:W indexes for Type I and Type Int mitochondria probably represent underestimates of the extreme values due to the high frequency of small, more circular profiles generated when randomly sectioning a cylindrical object. The estimates however are of use as the Type I, II and Int indexes are clearly separated from each other (See Table 3.2). Estimates were not significantly different from pH 4.0 and 5.9 cells. For routine scanning of amoebae, when scoring the relative proportions of Types I and II it was not necessary to accurately estimate the L:W index. For this purpose only a rapid evaluation of shape was required as the matrix density usually sufficed to distinguish the types. It was only when the matrix character became unclear that the importance of accurate shape determinations were invoked.

The measurement of the relative proportion of the profile occupied by the cristae and inner membrane appeared to be a definable character. Its general use however was limited due mainly to the time required to collect the data. Tracing enlarged profiles from the negatives at least increased the accuracy of cutting and reduced the cost, but did introduce an extra potential source of error into the technique. Its use as a routine sampling procedure in determining mitochondrial types of experimental treatments is not recommended. The increased cristal complexity in Type I compared to Type II mitochondria can usually be assessed by eye although the present quantitative study served to confirm that a true distinction exists.

The three characters together allow one, with practice, to classify the forms directly from the microscope without need for
precise measurements.

The presence of types I and II at all stages sampled in the cell cycle eliminates the proposal that the types are associated solely with the age of the cell as form differences may be with other systems (Osafune, 1973). An independent organelle cycle either related to temporal, i.e. biogenetic, or functional, e.g. respiration events is favoured. If the differences reflect different stages of a biogenetic cycle for the organelle then one may assume that mitochondrial growth and division are not synchronised with the whole cell cycle as high levels of the different types are maintained throughout the cycle. If one type were to represent a possible divisional phase then one would have to conclude that approximately half the mitochondrial population at any time in the cycle was undergoing division. This seems unlikely but cannot be ruled out!

The possible criticism exists that some of the peaks and dips observed in the present study for the proportions of Type I relative to Type II mitochondria may have arisen due to the limited sampling method employed at each experimental age. However one may attach greater significance to these peak and dip patterns by considering the many other cellular processes which occur in a similar discontinuous manner i.e. being restricted to specific periods in the cell cycle. It is probable that greater energy demands are present during some phases (Webster and Van't Hof, 1969). In synchronous yeast cultures enzyme synthesis has been demonstrated either as a peak or dip pattern or in one step temporally restricted in the cell cycle (Mitchison, 1969). Various activities within amoebae have been shown to be associated with specific timings in the cell cycle. These have been taken as indications that different quantities of cell components were synthesised at different cycle times.

A summary of the timings in the cycle of peak activities compared with the mitochondrial peaks found in the present study is given below:
<table>
<thead>
<tr>
<th>Cell Process</th>
<th>Activity/Numerical Peaks (Cell Age, hours)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA synthesis</td>
<td>0.5-6; (large peak) 9-13 (secondary peak)</td>
<td>Ord, 1968b</td>
</tr>
<tr>
<td>RNA synthesis</td>
<td>5; 8-9; 13-17; 19-20; 26-27; 32-36.</td>
<td>Ord, 1973</td>
</tr>
<tr>
<td>Nuclear helices</td>
<td>5; 8; 15-16</td>
<td>Minassian and Bell 1976a</td>
</tr>
<tr>
<td>Protein synthesis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(i) leucine incorp.</td>
<td>19; 22</td>
<td>Chatterjee and Bell, 1976</td>
</tr>
<tr>
<td>(ii) lysine incorp.</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Mitochondria</td>
<td>4-5; 10-13; 19-24</td>
<td>Present study.</td>
</tr>
<tr>
<td>Type I</td>
<td>8; 15; 32</td>
<td>(Fig. 3.6).</td>
</tr>
<tr>
<td>Type II</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

It is evident that Type I mitochondrial proportions were relatively high between 0.5-6 hours during the period when 75% of the cell's nuclear DNA synthetic activity occurs. At 5 hours a peak in RNA synthesis has been reported in conjunction with peak numbers of nuclear helices believed to be composed of RNA and proteins. Type I mitochondria also peak at this age but then begin to decrease as the relative numbers of Type II increase at 8 hours. Between 9-13 hours a second small peak of DNA synthesis occurred and the relative numbers of Type I mitochondria rose correspondingly. At the end of the DNA synthetic period, and when RNA synthesis had reached a peak Type I dipped but then increased again from 17 hours onwards with relatively high numbers being maintained over the period involved in intense RNA and protein synthetic events (Ord, 1973; Chatterjee and Bell, 1976).

It seems the proportion of Type I mitochondria is high at the onset and during peaks of synthetic activity but dip towards the end of these periods when the mitochondria are represented at higher proportions of Type II. This is consistent with the proposal that the morphological differences might reflect different physiological or functional states related to either respiratory activity (Hackenbrock, 1972) or differences in ion accumulation (Packer et al., 1968).
The presence of Type Int mitochondria may represent transitions between the two distinct types suggesting rapid transformations between the different conformations. If this were the case the large variation in the relative numbers of the different types associated with particular cell locations such as the contractile vacuole would possibly be explained, if as is most likely, different phases of contractile vacuole cycle have been sampled. Different energy demands may exist as the contractile vacuole cycle progresses (Ahmad and Coulliard, 1975) and it may be predicted therefore that different mitochondrial types be represented if functional explanations for morphological variation are pertinent. Similarly the higher numbers of intermediate forms during mitosis and the first hour of the cycle may result from transitionary switches from one type to the other as energy levels alter.

The further sections of the present work were aimed at investigating whether such explanations could be invoked experimentally by changing the culture conditions or treating the cells with chemicals likely to affect mitochondrial functions.
Chapter Four

The influence of growth temperature and the effects of starvation upon the mitochondrial types in *A. proteus*.

4.1. Introduction

Means of effecting gross variations in cellular activity were sought to investigate further whether the peak and dip pattern observed for the proportions of the two main mitochondrial types throughout the cell cycle might be indicative of alterations in specific synthetic and metabolic functioning of the cell. Two different sets of culture conditions which have been demonstrated to affect cell activity in many organisms were used to study this: (i) variations in the growth temperature and (ii) the effects of progressive starvation.

(1) Temperature Variations

Although in general, organisms are able to tolerate wide variations in temperature away from the growth optimum for short periods, most physiological processes are affected by such changes (Schmidt-Nielson, 1975). In many organisms temperature has become a regulatory influence upon the life cycle and development of the animal. In *Hydra* there is a switch from asexual to sexual reproduction if the ambient temperature is lowered from 24°C to 15°C (Davison, 1976). All stages of development in multicellular organisms have long been known to undergo a regular acceleration with an increase in temperature (Lillie and Knowlton, 1897) although as cleavage proceeds there may be an increase in tolerance to temperature changes (Atlas, 1935). In the development of snake embryos, skeletal abnormalities result if the eggs are incubated at temperatures away from the optimum (Vinegar, 1974).

The cell cycle durations of both unicellular organisms, such as *T. pyriformis* (Mackenzie et al., 1966; Cleffman, 1967; Jauker et al., 1975) and of tissue culture cells (Sisken et al., 1965) are dependent on temperature: the cells requiring increasing periods of time to complete the cycle above and below the optimal range. In *T. pyriformis* cell size is increased by prolonged growth at low or high temperatures (Thormar, 1962); while repetitive treatments of
short durations of extreme temperatures, both by heat-shock  
(Sherbaum and Zeuthen, 1954) and cold-shock (Padilla and Cameron,  
1964), have been widely accepted as means of obtaining synchronous  
cell populations of the ciliate. Cold shock has also been used  
previously to obtain partial division synchrony in A. proteus  
(Dawson, 1937).

By varying the length of exposure to the sub-lethal temperature  
DNA replication as well as cell division may be synchronised (Zeuthen  
1971), suggesting that temperature can disturb cell activity at the  
level of macromolecular synthesis. Recent evidence has indicated  
that it is the activity of DNA polymerase that is inhibited (Ooka  
and Daillee, 1977).

RNA synthesis is also affected by alterations in culture  
temperature; in T. pyriformis there is a steady decline in C^{14}  
uridine uptake at high temperatures (Moner, 1965), with reduced  
amounts of RNA and protein present (Jauker et al., 1975). The in-
hibition of nucleotide pool formation and the breakdown of unstable  
RNA may partly account for this decrease (De Barros et al., 1973)  
in addition to the temperature changes interfering with the actual  
transcription and processing of the RNA molecules. In mouse L cells,  
protein synthesis is reduced by lowering the incubation temperature  
and it is suggested the rate of polypeptide initiation is preferen-
tially inhibited (Craig, 1975).

Other processes such as cell permeability have long been  
known to be affected by temperature alterations (McCutcheon et al.,  
1932). However, one of the major changes associated with temperature  
variation is the direct effect upon oxygen consumption. Alterations  
in the rate of oxygen utilisation have been demonstrated in many  
animals: in invertebrates such as Daphnia (Obreshkove et al., 1932)  
and in vertebrates, e.g. frogs (Vernon, 1894; Weathers, 1976) and in  
nakes (Zarrow and Pomerat, 1937). Oxygen uptake is also affected in  
isolated red blood cells from trout (Eddy, 1977) and in unicellular  
organisms such as giant amoeba Chaos chaos (Pace and Kimura, 1946).

By use of isolated organelles it has been shown that, depending  
on the mitochondrial substrate levels, oxygen consumption is affected  
by temperature changes (Newell and Pye, 1971) and that the efficiency
of oxidative phosphorylation is reduced by lower temperatures (Hannon, 1960; Kemp et al., 1969; Wodtke, 1973).

Mitochondrial configurational changes have previously been described in situ when slices of Morris hepatoma tissue were incubated at different temperatures (Galeotti et al., 1976) and also in skeletal muscle of cold-acclimated rats (Behrens and Himms-Hagen, 1977). The present study therefore set out to consider any changes in the mitochondrial structural types present when amoebae were cultured at different growth temperatures. Previous work has shown that temperature variations may affect many cellular processes in amoebae: locomotion (Mast and Prosser, 1932), plasmagel viscosity (Thornton, 1932); pinocytotic activity (De Terra and Rustad, 1959; Chapman-Andresen, 1962); contractile vacuole functioning (Ahmad and Coulliard, 1974); and mitotic events (Daniel and Chalkley, 1932). Many of these workers proposed that in addition to the kinetic considerations of temperature differences, the alterations which may result in energy metabolism at the different temperatures could have a direct effect on the specific functions.

(ii) Starvation Effects

A further culture state, that of increasing periods of starvation, was employed to investigate whether overall changes in cellular activity would produce alterations detectable at the level of the type of mitochondria preserved within the cell. Many processes are affected by starvation so that regimes of food deprivation have also been used to synchronise cell populations (Cameron and Jeter, 1970). As with temperature changes the absence of food intake has gross effects on the life cycle of A. proteus (Liesche, 1938) which probably result from alterations in metabolic functioning (Andresen, 1945). Other workers have shown that the cytoplasmic volume and normal cell behaviour are arrested by the absence of food from A. proteus (Mast and Hahnert, 1935; Heller and Kopac, 1955).

An earlier ultrastructural study of A. proteus (Cohen, 1957) reported that possible changes in mitochondrial morphology occurred when cells were deprived of food; and in Chaos starvation was seen to produce filamentous inclusions within mitochondrial profiles in addition to a change in the type of cristae preserved. (Daniels
and Breyer, 1968). The present work was intended to investigate further the effect of starvation upon the mitochondria of *A. proteus*, and to determine if such a state had a greater effect upon one configurational type than the other – a consideration not possible in the previous studies where aldehyde fixation had not been employed.

4.2.1. **Effect of growth temperature upon the cell cycle and morphology of *A. proteus***

Cell generation time was initially studied in an attempt to demonstrate whether overall cell activity was altered by changing the growth temperature. In order to determine this, cells were selected at division and maintained singly by the capillary method at the appropriate growth temperature. At least two separate sets of 20-25 cells were scored for cloning ability; the capillaries being checked daily to record the number of cells present.

For the temperature range of 15-24°C four division cycles were followed and used in the estimation of the individual cycle lengths from which the mean generation times for each temperature were calculated. At temperatures above 24°C, although cells progressed through three cell cycles without any high incidence of cell loss; the possible limitations of this culture method as predicted by Ord (1977) were evident. Thus cell death became apparent once eight or more cells were present in the tubes at these temperatures, due to possibly a reduced availability of oxygen and food organisms. The estimation of the cycle duration for cells cultured above 24°C was therefore based upon three division events. Only three divisions were recorded for cells below 15°C also.

Cells maintained at 6°C did not undergo division, confirming the earlier observation of Liesche (1938). Although division had been prevented, the cells remained viable, so that even after incubation at this temperature for periods of more than thirty days over 90% of the cells re-entered cycling when returned to room temperature (20°C). Of these 30% had completed two cell cycles within the first 72 hours back at 20°C. The mean generation time for cells returned to room temperature after incubation at 6°C is included in Table 4,1.
Table 4.1 Effect of temperature on the mean generation time in *A. proteus*

<table>
<thead>
<tr>
<th>Growth temperature</th>
<th>No. Cells</th>
<th>Cycle time ± S.E. (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>50</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>43</td>
<td>8.28 ± 0.25</td>
</tr>
<tr>
<td>15</td>
<td>90</td>
<td>7.45 ± 0.07</td>
</tr>
<tr>
<td>17.5</td>
<td>94</td>
<td>3.94 ± 0.09</td>
</tr>
<tr>
<td>20</td>
<td>147</td>
<td>2.46 ± 0.03</td>
</tr>
<tr>
<td>22</td>
<td>49</td>
<td>2.14 ± 0.03</td>
</tr>
<tr>
<td>24</td>
<td>95</td>
<td>3.10 ± 0.05</td>
</tr>
<tr>
<td>26.5</td>
<td>46</td>
<td>3.25 ± 0.09</td>
</tr>
<tr>
<td>29</td>
<td>38</td>
<td>3.12 ± 0.15</td>
</tr>
</tbody>
</table>

Return to 20° after 30 days at:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>No. Cells</th>
<th>Cycle time ± S.E. (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>24</td>
<td>2.54 ± 0.13</td>
</tr>
<tr>
<td>15</td>
<td>25</td>
<td>2.78 ± 0.08</td>
</tr>
</tbody>
</table>

Fig. 4.1 Effect of growth temperature on mean generation time for *A. proteus*.

<table>
<thead>
<tr>
<th>Cycle Length (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
</tr>
<tr>
<td>7</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>2</td>
</tr>
</tbody>
</table>

Temp. (°C)
together with the estimation for the other growth temperatures studied. These results are represented graphically in Fig. 4.1.

As the temperature was increased from 12-22°C the time required for the cell to complete its life cycle decreased four-fold. As with cells maintained at 6°C, the increase time required for the completion of the cell cycle did not include permanent changes in their cycling potential. Thus cells maintained at 15°C for thirty days, which acquire a generation time of 7.5 days, returned to cycling characteristic of 20°C cells when recultured at room temperature.

At temperatures above 22°C, although the first two cycles are completed in the majority of cells within a similar period to those of 22°C cells, the subsequent cycles became irregular with cells requiring longer periods to complete the cycle and also with increasing cell mortality within the clones. Thus at temperatures higher than the optimum of 22°C the mean generation time began to increase once more.

The overall morphology was seen to differ for cells at different regions of the temperature range. In order to study this further cells were maintained in solid watchglasses rather than in capillary tubes.

At 6°C the cells showed very little cytoplasmic streaming or locomotory ability and were commonly present as flattened forms in which the pseudopods had been retracted. Cell clumping was often observed, five to ten cells being present within a 'colony'. Such clumping was not evident in cultures maintained at 10°C to 12°C although these too remained as spherical rosette forms with only short pseudopods being present throughout the incubation periods.

A pseudopodal form which displayed significant locomotion was observed at all temperatures above 15°C, when a normal feeding form was also noted. Although these forms were both present in cultures up to 28°C, at the upper limit of the range the pseudopods were rather elongated and had a 'knotted' appearance. Cells at 28°C generally had a much looser attachment to the substratum.
4.2.2.1. **Ultrastructural changes associated with growth temperatures**

To investigate whether any alteration occurred in the ultrastructural appearance of cells cultured at temperatures different from those at which they are normally maintained, healthy stock cultures were transferred to the growth temperature under consideration and fed daily for five days prior to fixation and preparation for the E.M. Five days was considered to be a sufficient time interval for the cells to become acclimated to the new ambient temperature as this represents a period of more than twice the generation time of cells normally maintained at room temperature. Because the generation time differs at each temperature, cells of a fixed age were not used; the cell samples being of a mixed age. With cells grown at 6°C samples were also fixed after a period of thirty days to observe whether any changes had arisen during the quiescent period when no division was recorded.

In addition to any changes in gross structure resulting from growth at different temperatures, mitochondrial counts of the relative proportions of the two types were recorded as previously described. Data was collected from between 12—25 cells for each experimental point and in general at least two separate experiments for each temperature had been performed; this is included in Table 4.2 and graphically in Fig. 4.2.

The two main mitochondrial configurations were present at all temperatures studied; however the relative proportions of the two types did not remain constant throughout the temperature range. At lower temperatures the proportions of profiles of Type I mitochondria was much lower than in cells that had been cultured at 20-22°C. Above this temperature the numbers of Type I profiles again began to fall sharply so that at 28°C less than 20% were present as distinct Type I profiles. The peak of 61% Type I at 22°C corresponds to the temperature at which the generation time is at a minimum.

At 6°C the Type I profiles present were not as distinct for the characters of matrix density and cristal dimensions as at subsequent temperatures, although two forms were evident (Fig. 4.3). A small percentage of the Type II profiles were vacuolated although the majority of this type were of a normal appearance after five days of
Fig. 4.2: Proportions of Type I mitochondria in amoebae maintained at various growth temperatures (□), and after reversion from these temperatures to room temperature for 24 hours prior to fixing (●).
Table 4.2: Effect of growth at altered temperatures on the mitochondrial types present in *A. proteus*.

<table>
<thead>
<tr>
<th>Temp. (°C)</th>
<th>No. cells in sample</th>
<th>Total No. of profiles for each temperature group</th>
<th>% as Type I</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>18</td>
<td>2355</td>
<td>27.8 ± 4.0</td>
</tr>
<tr>
<td>10</td>
<td>12</td>
<td>3547</td>
<td>30.0 ± 2.3</td>
</tr>
<tr>
<td>12</td>
<td>15</td>
<td>2456</td>
<td>35.4 ± 2.3</td>
</tr>
<tr>
<td>15</td>
<td>25</td>
<td>4164</td>
<td>31.4 ± 2.1</td>
</tr>
<tr>
<td>17</td>
<td>16</td>
<td>2100</td>
<td>40.9 ± 3.5</td>
</tr>
<tr>
<td>20</td>
<td>221</td>
<td>33391</td>
<td>52.3 ± 2.1</td>
</tr>
<tr>
<td>22</td>
<td>15</td>
<td>2848</td>
<td>61.6 ± 1.3</td>
</tr>
<tr>
<td>24</td>
<td>14</td>
<td>2555</td>
<td>42.4 ± 2.3</td>
</tr>
<tr>
<td>26</td>
<td>23</td>
<td>2984</td>
<td>22.7 ± 2.2</td>
</tr>
<tr>
<td>28</td>
<td>15</td>
<td>3054</td>
<td>17.9 ± 2.2</td>
</tr>
<tr>
<td>6 (For 30 d.)</td>
<td>8</td>
<td>1210</td>
<td>30.5 ± 1.9</td>
</tr>
</tbody>
</table>

Table 4.3: Proportions of Type I forms after reversion from acclimated growth temperature to 20° for 24 hours.

<table>
<thead>
<tr>
<th>Acclimated Temp. (°C)</th>
<th>Sample No. of Amoeba</th>
<th>Profiles Scored for each group</th>
<th>% as Type I</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>12</td>
<td>2617</td>
<td>45.8 ± 1.7</td>
</tr>
<tr>
<td>10</td>
<td>12</td>
<td>3099</td>
<td>47.9 ± 7.4</td>
</tr>
<tr>
<td>20</td>
<td>221</td>
<td>33391</td>
<td>52.3 ± 2.1</td>
</tr>
<tr>
<td>22</td>
<td>14</td>
<td>3740</td>
<td>44.4 ± 1.5</td>
</tr>
<tr>
<td>28</td>
<td>15</td>
<td>2859</td>
<td>39.9 ± 2.0</td>
</tr>
</tbody>
</table>
Fig. 4.3. : Mitochondrial types within the cytoplasm of an amoeba maintained at 6°C for 5 days. DNA-containing bodies and a cytoplasmic helix may also be observed.

\[ x \text{ 25,000} \]

Fig. 4.4. : Cytoplasm of an amoeba maintained at 6°C for 5 days to show the predominate mitochondrial type (Type II). Flattened golgi bodies are also present.

\[ x \text{ 9,200} \]
culturing at 6°C (Fig. 4.4). More irregular profiles with an increased incidence of those possessing filamentous inclusions within the matrix as well as vacuolated forms were observed in cells cultured at 6°C for the thirty day period (Fig. 4.5).

The distinction of the mitochondrial types was more apparent at 10°C and higher temperatures (Fig. 4.6) although intermediate forms were also present as a minority type in cells, particularly at lower temperatures.

Other changes were also noted in the cytoplasm of cells cultured at 6°C. The Golgi complexes were present as an elongated flattened form (Fig. 4.4) rather than the more concave form seen in 20°C controls. They also appeared to be less numerous, though no quantitative study was performed. The number of ribosomes present as polysome arrays appeared to be increased with a corresponding decrease in the numbers attached to the E.R. An occasional cytoplasmic helix was observed in 6°C cells (Fig. 4.3) and these were much more evident in 6°C cells of the extended incubation period when they could be found in groups (Fig. 4.5). The nuclei of these cells had a normal appearance although there were possibly less nuclear helices present.

The morphology of the Golgi complexes was also affected by the lowered temperatures of 10-12°C where both an elongated and a completely circular form were noted instead of the more typical crescent shape (Fig. 4.7a + b). An alteration in Golgi morphology when amoebae were exposed to temperature extremes has previously been correlated with the reduction in active locomotion in these cells (Stockem and Korohoda, 1975).

The appearance of cells in the 15-24°C range showed little alteration except for the changes in the relative proportions of the mitochondrial types present (Fig. 4.6). The cytoplasm of cells cultured at 26-28°C did undergo changes in addition to the decrease in Type I mitochondria. These cells often had prominent amounts of cytoplasmic filamentous bundles, particularly at the cell periphery, which might possibly be correlated with the increase in locomotory activity of such cells. There was also an increase in the numbers of ribosomes present as a monosomal form (Ord, personal communication).
Fig. 4.5. : Cytoplasm of an amoeba cultured at 6°C for 30 days showing the presence of matrical inclusions within the mitochondria. A group of cytoplasmic helices is seen (such groups are not found in untreated amoebae maintained at room temperature).  
X 25,000

Fig. 4.6. : Mitochondrial types located near the nucleus of a cell maintained at 12°C for 5 days.  
X 9,200
Fig. 4.7. :

a) Golgi body alterations generated by 5 days of culturing at 10°C.
   \[ \times 25,000 \]

b) Control Golgi morphology in a cell maintained at 20°C.
   \[ \times 25,000 \]

Fig. 4.8. : Type II forms in the cytoplasm of an amoebae cultured at 28°C for 5 days.
   \[ \times 20,000 \]
with a decrease in the number of polysomal arrays observed at lower temperatures.

4.2.2. Reversion of growth temperature to 20°C

A further set of cells were prepared for the E.M. which had been grown at 6, 10, 12, 22 and 28°C for five days and then had been returned to 20°C for twenty four hours prior to fixation. It was hoped to determine whether the changes observed above were of a permanent nature or not, or whether, as with the generation times, the proportion of the mitochondrial types would return to values approaching those typical of 20°C cultured cells.

The mitochondria present in cells which had been returned to room temperature after five days maintenance at 6°C displayed a reversal to the types of profiles normally viewed in control cells. There was good distinction throughout these cells between type I and II profiles with the elimination of the higher proportions of intermediate forms observed in cells fixed while still maintained at 6°C. Further there was no evidence of the matrical inclusions reported above in cells grown for extended periods at this growth temperature. The most striking observation on these cells however was that the proportion of Type I mitochondria had returned to a level more typical of that for cells maintained at 20°C than at 6°C.

The other cytoplasmic changes associated with culturing at this growth temperature, particularly concerning the E.R. and Golgi complexes were also less evident. Thus the ribosomes were re-attached to the E.R. along with a polysomal pattern similar to that of healthy control cells. The Golgi apparatus appeared more numerous and included the concave form which was only rarely viewed in cells at 6°C where a flattened form predominated.

Similar reversions to the values approaching those more usual of 20°C cells were recorded for the proportions of mitochondrial types in cells returned to room temperature from growth at 10, 22 and 28°C. (Table 4.3 and Fig. 4.2). The distinction between the two types was in general good, except that the type II forms in some of the cells returned from 28°C did show a tendency to continued vacuolation. The greatest variation in the proportions of mitochondrial types was noted in cells which had reverted from growth at 10°C; for in this sample
a few cells still retained a higher number of Type II profiles than is normally encountered in control cells, whereas in a minority of other cells a higher number of Type I, some of which were of a very elongate nature, were present.

Other cytoplasmic organelles in reverted cells appeared identical to those of normal 20°C cells in structure. The only difference in morphology that was detectable apart from the changes in mitochondrial proportions was the persistence in cells reverted from 28°C of higher amounts of cytoplasmic filamentous bundles than is normal at 20°C but which had been observed in 28°C cells.

The results from these cells suggest that, as with the alteration in generation times which is produced by temperature changes the alterations at the ultrastructural level are also of a reversible nature.

4.3.1. An investigation of the effects of starvation upon the ultrastructure of A. proteus

Cells were taken from healthy stock cultures as division spheres and maintained singly in either solid watchglasses or by the capillary method. Watchglasses were changed daily to avoid bacterial contaminants becoming a significant food source as an early worker reported "a hungry amoeba eats any organism it can get hold of" (Schaeffer, 1916), capillary tubes were changed at more infrequent intervals.

As the period of starvation was extended the cells acquired a pseudopodal form which displayed poor attachment to the substratum. There was evidence of some cell loss, but in general even after two weeks culturing without Terahymena-Feeding about 70% cell survival was recorded and for shorter periods of food deprivation a higher incidence of survival was noted. Division was not observed in any of the cells and a visible decrease in cell volume was discernable.

After periods ranging from 2-13 days, cells were fixed and prepared for the electron microscope. Capillary-maintained cells were blown out into watchglasses and allowed to settle in fresh Chalkley's medium for at least an hour prior to fixation, in order to reduce the possibility that any of the fine structural alterations might otherwise be attributable to this different culture
environment. Ultrastructural differences between cells maintained by the two culture methods were not observed.

Profile counts were made on the mitochondrial types present in samples of 12-20 cells for each day of starvation and are recorded in Table 4.4 and graphically in Fig. 4.9. Cells in which the occasional vacuole containing yeast cells were observed were discounted. For the first five days of food deprivation, the two types were found to be present in similar proportions to those observed in cells from fed cultures. However after this time there was a gradual decline in the numbers of Type I mitochondria present; so that on subsequent days the relative numbers of Type I had fallen to between 20-30%. A linear regression analysis was performed on the data collected and from this it was calculated that the regression coefficient for the decline in Type I profiles as the days of starvation increased had a value of -2.

As the period of starvation increased, some of the Type II profiles were of a rather degenerate nature (Fig. 4.10). The matrix of type II forms from cells starved for greater than one week was often of a more electron dense nature (Fig. 4.11), though the cristae present as well as the gross shape indicated that these were Type II profiles and not an intermediate form. Daniels and Breyer (1968) found in their study of Chaos that increased starvation resulted in the presence of associations of microfilaments within the matrix; such filaments were not observed in the present study as starvation proceeded.

Starvation also had other effects on the cells. During the first 4-5 days of starvation there was an apparent increase in the amounts of lipid droplets within the cytoplasm (Fig. 4.12 and 4.13); but as the period of food deprivation was extended the numbers of these droplets was noticeably reduced. A possible reduction in the numbers of Golgi bodies was also suggested, but no quantitative work was attempted to investigate this. The number of vacuolar spaces increased with progressive starvation as did the numbers of autophagic vesicles. By day 6 and in cells from subsequent days, irregular membranous inclusions were evident (Fig. 4.14); as were large vacuoles containing a loosely-packed electron dense material.
Table 4.4: Effects of progressive starvation

<table>
<thead>
<tr>
<th>No. Days Starved</th>
<th>Cells in sample</th>
<th>Profiles Scored</th>
<th>% Type I ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>13</td>
<td>1658</td>
<td>43.7 ± 4.7</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>2003</td>
<td>37.1 ± 2.4</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>2014</td>
<td>47.4 ± 3.9</td>
</tr>
<tr>
<td>5</td>
<td>15</td>
<td>2008</td>
<td>46.2 ± 3.8</td>
</tr>
<tr>
<td>6</td>
<td>18</td>
<td>2148</td>
<td>34.8 ± 2.2</td>
</tr>
<tr>
<td>7</td>
<td>12</td>
<td>1998</td>
<td>30.1 ± 2.9</td>
</tr>
<tr>
<td>8</td>
<td>16</td>
<td>2231</td>
<td>30.1 ± 2.0</td>
</tr>
<tr>
<td>10</td>
<td>19</td>
<td>2648</td>
<td>20.9 ± 1.9</td>
</tr>
<tr>
<td>12</td>
<td>13</td>
<td>2251</td>
<td>34.9 ± 5.0</td>
</tr>
<tr>
<td>13</td>
<td>13</td>
<td>1940</td>
<td>19.5 ± 2.6</td>
</tr>
</tbody>
</table>

Fig. 4.9: Proportion of Type I mitochondria upon starvation

% Type I

\[ y = 48.4 - 1.97x. \]
Fig. 4.10. : A cell starved for 12 days showing an increase in the number of vacuolated Type II mitochondria.

x 12,000

Fig. 4.11. : Mitochondria of a cell maintained without feeding for 13 days to show the Type II-Int form.

x 12,000
Fig. 4.12. : Presence of increased numbers of lipid droplets in the cytoplasm of a 4-day starved amoeba.

x 9,200

Fig. 4.13. : Lipid droplets in the cytoplasm of another cell starved for 4 days.

x 9,200
Fig. 4.14. : 10 day starved cell illustrating the membranous bodies (mb) commonly seen in the cytoplasm of starved cells.

x 16,000

Fig. 4.15. : 10 day starved cell showing the presence of vacuoles containing an electron-dense material generated by prolonged starvation.

x 16,000
(Fig. 4.15). As culturing without food continued, the cells in the samples were often found to have depleted amounts of R.E.R. suggesting that the ribosomes had lost attachment to the E.R.

A simple morphometric method (Hally, 1964) was employed to consider if there was any change in the mitochondrial volume within the cytoplasm over the starvation period. This consisted of randomly placing a regular pattern of 340 points, in the form of a square lattice with 1 cm spacings, onto a series of micrographs of final magnification of 16,000 X. The relative area of a cell component was scored by counting the number of points 'n' lying over the component and dividing this by the total number of points lying over the section 'N'. Five counts were performed on each micrograph in an attempt to eliminate any sampling bias.

No significant difference was observed in the relative volume occupied by the mitochondria in the cytoplasm of starved cells compared to the values obtained from normal fed cells. In each sample the volume of mitochondria remained in the range of 4.5-7.0% of the total cytoplasmic volume. The validity of any estimation of organelle volumes by such stereological methods is, however, questionable. Such methods presume a totally random distribution of the organelle throughout the cytoplasm, and when this basic assumption cannot be made, the source of error in sampling is high. The spatial arrangement of organelles in A. proteus is not of a random nature, and this is particularly the case for mitochondria when one considers their association with specific regions of the cell such as the contractile vacuole.

4.3.2. Observations on refed cells

On the addition of food organisms to cells which have been subjected to an extended period of starvation, much variation in cell activity was noted. Because of the loose attachment to the substratum and very slow cytoplasmic streaming demonstrated, many cells had great difficulty in capturing *Tetrahymena* and as such generally remained floating with extended pseudopods. Attachment of the amoeba to the substratum has previously been demonstrated to be a prerequisite for feeding activity (Mast and Hahnert, 1935). Those that were successful in arresting and ingesting *tetrahymena* displayed a firmer attachment
and appeared capable of more efficient food-cup formation subsequently. During the first hour of re-feeding, the number of tetrahymena captured by an amoeba varied from 0-8 cells with a mean value of three organisms for cells that had been starved for 10 days. Control cells normally arrest between 6-12 organisms within an hour.

Of the 80% of the amoebae which remained after a starvation period of 10 days, approximately 75% were viable and displayed cloning ability, although in all cases there was an initial delay of 3-4 days before such viable cells re-entered cycling. This result is similar to that earlier observed by Liesche (1938) who concluded that cells must ingest a specific amount of food before re-entering division cycling.

In order to investigate the effects of refeeding at the ultrastructural level, samples of cells which had been subjected to starvation for 10 days were fixed 1, 4, 8 and 24 hours after the introduction of food organism to the culture dish. Because of the large variations in the ability to recommence feeding cells which appeared to have been successful at food capture were selected for fixation.

In the cells viewed from the 1-8 hours samples the proportions of mitochondria present as Type I remained low around the 20% level. The distinction between the configurational types was generally poor in the cells and many of the Type II profiles continued to be of a vacuolated form. These cells still only contained the occasional food vacuole indicating that food capture had not been very successful. The vacuoles containing dense material similar to those of Fig. 4.15 were still present in large numbers.

24 hours after the introduction of food to starved cells the percentage of Type I mitochondria had risen from the 20% value to about 40% although the distinction of the two types was still rather poor. These cells contained food vacuoles at various stages of digestion as well as a higher number of lipid droplets than is seen after this length of starvation. Other changes such as re-attachment of the ribosomes to the ER were indicated, suggesting the cells are returning to a more normal level of functioning.
Discussion

The observed effects on cell generation times and on whole cell morphology indicate that the external factors of temperature and the availability of food have a definite influence upon cellular activity in *A. proteus*. The study attempted to correlate any changes that were observed at the ultrastructural level with these overall alterations in cell functioning. For cells that had been maintained away from the optimal growth conditions either by the absence of food organisms or at unfavourably low temperatures there was in general a great reduction in cell mobility and an inhibition or cessation of cell cycling.

Within the cytoplasm of cells cultured at temperatures other than 20°C the present work demonstrated that several physiological processes were affected.

Numerical and morphological changes in the Golgi Bodies similar to those reported here at low growth temperatures have previously been attributed to the decreased role of the organelles in membrane formation and processing in cells where cell locomotion is reduced (Stockem and Korohoda, 1975). Certainly the cells maintained below 15°C displayed little or no locomotory ability in agreement with early work (Mast and Prosser, 1932).

The detachment of the ribosomes from the E.R. with a subsequent increase in the polysomal arrays at low temperatures and in starved cells suggests that protein synthesis is affected possibly by an inhibition of the overall translation rate or by inhibiting specific steps in the process such as peptide chain elongation or the release of the completed protein molecule from the ribosome.

The increased occurrence of cytoplasmic helices at lower growth temperatures may also reflect changes in the translational processes in the cell, if these structures do indeed represent intermediate stages between the nuclear helices and the cytoplasmic polysomal arrays as has been proposed (Minassian and Bell, 1976). Increased numbers of monomeric ribosomes in cells cultured at higher growth temperatures may indicate a changed pattern of protein synthesis in these cells where polysomal arrays may be kinetically unstable.
Alterations in protein synthesis rates have previously been reported in mouse L cells cultured at different growth temperatures (Craig, 1975). In these cells there was a decrease in the polysomal arrays in the temperature range 36-10°C but a subsequent increase in the numbers at temperatures approaching 0°C. Craig concluded that temperature changes might result in different processes being rate limiting at different regions of the temperature range studied.

The alteration in the cells' generation times showed that the nuclear-cytoplasmic factors concerned in controlling division are disturbed in some way by the temperature changes. In the present study the structure of the nucleus, including the form of the nuclear helices, remained of normal appearance throughout the temperature range employed. It is probable however that the different temperatures do have specific effects on the nuclear activity as Sopina (1975) has shown by the use in intrastrain transplants of amoebae strains established at different temperatures, that the nucleus has an important role in the control of multiplication rates.

The present study showed that in conjunction with the changes at the cellular level and at the level of several organelles in cells grown under adverse culture conditions, there was also a concurrent decline in the relative proportions of Type I mitochondria and an increase in the numbers of Type II and Light Int forms, indicating that mitochondrial functioning had also been affected. In the Light Int profiles the change observed upon prolonged starvation in the density of the matrix is reminiscent of the earlier E.M. work performed on A. proteus (Cohen, 1957). The Type II profiles also displayed fine alterations in the cristae present these with an occurrence of vacuolation.

In Chaos it has been suggested that carbohydrate metabolism was much reduced by growth at 10°C where a decreased respiratory quotient was recorded (Pace and Kimura, 1946). The preservation of the Type II structural form in higher proportions when there is an apparent decrease in cell functioning in amoebae maintained at reduced temperatures or without food, is thus consistent with the proposal that this structural form reflects a reduction in the metabolic functional efficiency of the organelle. Similarly the
observed increases in Type II mitochondria in cells grown at 26-28°C may also result from a disturbance of the normal metabolic processes of the organelle.

Daniels and Breyer (1968) attributed the changes they noted in the mitochondria of Chaos, when normal food intake had been halted, to a switch to the use of lipids as the predominant metabolic fuel during starvation. They found that the percentage of altered forms increased after the fifth day of food deprivation which corresponds to the time interval in the present work when the proportions of Type I mitochondria began to decrease and when the amounts of lipid droplets were still relatively abundant in the cytoplasm. After this stage of starvation in the present investigations a gradual decrease in the droplets was noted in agreement with the earlier workers (Mast and Hahnert, 1935; Wilber, 1942; Andresen, 1945; Andresen and Holter, 1945; Heller and Kopac, 1955).

Heller and Kopac proposed that at least three key metabolic phases occurred during starvation in amoebae and that the cells entered the second phase after approximately five days. The appearance of the vacuoles that included an electron dense material (Fig. 4.15) in cells starved for 7 days and longer indicates that some form of metabolic changes have been effected by this culture treatment. The earlier light microscope studies on starved amoebae (Mast and Prosser, 1932; Zeuthen, 1948) had suggested a possible increase in dense granular spheroid bodies as the time interval without food increased. It was considered that these might possibly represent inclusions for the storage of waste materials or for the maintenance of ionic regulation within the starving cell. Although Chapman-Andreson (1976) found no difference in the numbers of Heavy spheroid bodies (HSB's) from fed or unfed cells contrary to these earlier reports, the structures she describes (which are seemingly identical to that illustrated in Fig. 4.6) cannot be likened to the ones reported here (Fig. 4.15). A more detailed study of these structures would be of interest.

An altered metabolic phase after five days of culturing without food would account for the presence of the lipid membranous inclusions that increased in numbers between days 6-13 (Figs. 4.11 and
4.14). If this is the case the effect might be to increase the free fatty acid content within the mitochondria which may be responsible for the observed structural changes. Wilber (1942) proposed that in starved cells the neutral fat stores of the lipid droplets are broken down into free fatty acids. Increases in free fatty acids have been reported to reduce the efficiency of ATP synthesis (Torchetto, 1974). The production of a 'semi-uncoupled state' affecting oxidative phosphorylation itself or by possible competitively binding to the nucleotide translocases in the mitochondrial membranes by fatty acids have been considered, (Wojtczak, 1976). It is therefore conceivable that the structural alterations in mitochondrial types reported here occur because of a fall in the electron transport energy production concomitant with a reduction of the normal substrates upon starvation or because of an interference of the nucleotide translocases in the organelle.

The activity of mitochondrial ketogenic thiolase is reported to be stimulated by starvation in rat liver cells (Reed et al., 1977) which further suggests changes in the regulation of lipid metabolism in starved cells. Other enzyme activities as well as respiratory rates are known to be lowered in organelles isolated from the liver, kidney and heart of starved rats (Gold and Costello, 1975), and the enzymes indirectly associated with lipogenesis are reported to be less stable than those of glycolysis and the TCA cycle in young rats (Freeland, 1967). In the nephric cells of frog kidneys, cytochrome oxidase activity is markedly changed during starvation periods of 9-12 days, suggesting a reduction in cell respiration (Karnovsky and Himmeloch, 1961).

Changes in the lipid composition of mitochondria have also been observed in animals maintained at temperatures lower than the optimal growth range for the organism (Caldwell and Vernberg, 1970; Hazel, 1973). In prokaryotes some of the membrane processes that are critical to changes in membrane fluidity are affected by varying the incubation temperature (Maeda et al., 1976; Miller and Koshland, 1977).

Caldwell and Vernberg demonstrated that in goldfish and bullheads reared at lower temperatures the mitochondria isolated from the gills contained increases in unsaturated lipids with a higher
percentage of phosphatidyl ethanolamine and cardiolipin. This is presumed to preserve a specific liquid-crystalline phase in the mitochondrial membranes. Such subtle changes in the lipid composition with cold-acclimation is known to adversely affect oxidative phosphorylation efficiency (Wodtke, 1973) and may also influence the permeability and barrier properties of the mitochondrial membranes having an affect upon the translocation of ADP and ATP. (Kemp et al., 1969; Spencer et al., 1976). As well as affecting the enzyme activities of the electron transport chain, reduced temperatures have also been reported to uncouple oxidative phosphorylation (Hannon, 1960).

The data collected from the cell samples maintained at different temperatures and then returned to room temperature prior to fixation indicate that the observed changes in the proportions of the mitochondrial types were of a temporary nature and as such could be correlated with the changes in overall cell functional activity. Thus when processes which normally require energy production are inhibited or arrested Type II mitochondria or a similar form predominate whereas if the cells are restored to a more normal environment the proportions revert to levels similar to those of control cells. Cells maintained at high growth temperatures (above 26°C) also possess lower proportions of Type I possibly because of a reduction in the efficiency of the organelles due to degradative events, or because of the depletion of certain factors from the mitochondria due to increased demands for metabolites throughout the cell at these temperatures.

A similar conclusion is possible from the observations on re-feeding starved cells although here the results were more variable possibly due to the diverse ability of the cells sampled in the ingestion of food organisms.

From these results several proposals may be offered to account for the increase in Type II profiles when cell activity is lowered either by starvation or lowered temperatures:

(i) This form is favoured in fixation after a switch to the utilisation of lipids as the predominant metabolic fuel in the cells.

(ii) Starvation might deplete the precursor substances necessary for the production or maintenance of Type I, while temperature
alterations might affect their synthesis or passage into the mitochondria.

(iii) The reduction in cell activity is a direct consequence of the lowering of intracellular energy levels and this is detected by an alteration in the mitochondrial type present.

The following sections of the work were conducted to investigate further these alterations with special reference to the latter proposition.
Chapter Five

The action of substances which uncouple oxidative phosphorylation.

5.1. Introduction

The production of ATP by the process of oxidative phosphorylation is one of the prime functions of a cell's mitochondria. If the mitochondrial structural forms present in A. proteus result from a functional difference related to respiration or ATP synthesis, it might be presumed that factors which directly affect the process of phosphorylation may also alter the mitochondrial configurations. The intentions of this present section of the work were therefore to investigate the effects of three substances: dinitrophenol (DNP) pentachlorophenol (PCP) and m-chloro-carbonyl cyanide phenylhydrazone (CCCP), which block oxidative phosphorylation; to determine whether at concentrations that had a detectable effect upon the behaviour and functioning of the whole cell, any alteration in mitochondrial structure was occurring.

Many such substances have long been known to produce dramatic physiological effects on cells and organisms, generally stimulating cell metabolism. DNP has been employed as a herbicide and insecticide. During the 1930's, it was used extensively in an attempt to clinically reduce obesity in man and also in many self-medication products until its potential danger was realised (Parascandola, 1974).

PCP has also been used as a herbicide, molluscicide, insecticide and in the preservation of wood, because of its potent effects on micro-organisms and lower invertebrates (Bevenue and Beckman, 1967). Stimulated respiration with a disruption of oxidative phosphorylation was demonstrated to be the major action when PCP was used against Australorbis granatus, the snail intermediate host of the human schistosome (Weinbach and Nolan, 1956). However, as with DNP, fatalities to humans (Gordon, 1956) and to other higher mammals (Blevins, 1965) have been recorded for PCP.

Uncoupling agents such as these substituted phenols are considered to prevent ATP formation while allowing respiration to continue which results in an increase in heat dissipation (Lardy and Elvehjem, 1945).
The actual molecular basis of the uncoupling phenomenon is still not fully understood, although Parker (1958; 1965) concluded that the various classes of substances which are chemically unrelated, probably have a common action as their effects on mitochondrial function are qualitatively similar to those of DNP.

Slater and Lewis (1954) suggested they act by causing a breakdown of the high energy intermediates between the electron transport chain and the ATP molecule: an explanation consistent with the chemical hypothesis of energy transduction. In terms of Mitchell's chemiosmotic hypothesis, uncouplers are considered to affect membrane permeability in the mitochondria by their ability to carry protons across the membrane, thus abolishing the proton gradient and membrane potential necessary for ATP formation (Mitchell, 1966).

Using thin lipid bilayers, several independent reports have given support to Mitchell's postulate, as different classes of uncouplers have been demonstrated to decrease the electrical resistance of the bilayer increasing membrane conductance (Bielawski et al., 1966; Finkelstein, 1970; McLaughlin, 1972). Alterations to H⁺ ion permeability were also seen using mitochondrial preparations (Carafoli et al., 1969). In isolated mitochondria in general the more lipophilic an uncoupler is, and hence its greater adsorption into the membrane, then the lower the concentration necessary to affect the mitochondrial systems. This physical property has been proposed to explain the strong inhibition observed with the ring-substituted derivatives of the carbonylcyanide phenylhydrazones in 'in vitro' systems (Heytler and Prichard, 1962; Goldsby and Heytler, 1963).

Although most of the experimental data suggests a common action by uncoupling agents, certain anomalies have been reported. Katyare et al. (1971) demonstrated that DNP has a differential effect on the different phosphorylation sites of the electron transport chain. They showed that sites I and III were most sensitive, implying a multiphasic action of the phenol. Other mitochondrial reactions are also affected by uncouplers. Stimulation of ATPase activity (Myers and Slator, 1957) and of the adenine nucleotide translocases (Spencer et al., 1976) have also been reported.

Some of these effects of the uncoupling reaction have been
detected as structural changes in isolated organelles (Packer, 1960; Blair and Munn, 1972). Weinbach and Garbus (1968) presented evidence that such conformational changes were a result of direct binding of the PCP or DNP to the isolated mitochondria. They proposed that uncoupler binding disorganised the protein components of the mitochondrial membranes producing the gross structural changes (Weinbach and Garbus, 1969).

That phenol uncouplers do interact with specific protein sites in addition to the interaction with the phospholipids in the biomembranes has been demonstrated by the use of photoaffinity-labelling uncoupler, 2-azido-4-nitrophenol (Hanstein and Hatefi, 1974; Kurup and Sandai, 1977). Using such techniques a polypeptide located on the inside of the inner mitochondrial membrane and of molecular weight 30,000 has been implicated in the binding reaction (Hatefi, 1975). Binding of different uncouplers to such binding sites appears to be of a competitive nature (Hanstein and Hatefi, 1974). The effectiveness of binding to the mitochondria varies depending on which uncoupler is under consideration, being over 80% for PCP, 40% for the CCP's but less than 5% for DNP by isolated rat liver mitochondria (Bakker et al., 1974).

Despite a recent claim that net conformational changes need not be induced by uncoupler binding (Hanstein, 1976), alterations have frequently been observed at the ultrastructural level.

Muscatello et al. (1975) indicated that isolated mitochondria underwent a contraction on the addition of DNP; and that this condensation was indistinguishable from the one produced upon the addition of ADP to the suspension. A significant increase in condensed types was also noted in chick heart cells 'in situ' incubated with several uncouplers including DNP and PCP (Buffa et al., 1970) Such an increase could not however be found on the incubation of rat jejunum tissue with higher concentration of DNP (Jasper and Bronk, 1968); nor in a study of PCP upon sections of rat liver (Weinbach, Garbus and Sheffield, 1967). In the latter case however altered mitochondrial forms were produced upon fractionating the tissue. When shrew heart tissue from animals which had previously received a high dose of DNP by injection were studied, the mitochondria appeared
swollen and showed a tendency towards cristae loss (Didio et al., 1975). The vast differences in dose might be one factor explaining these apparent discrepancies in action.

In a study on *Euglena gracilis*, Kahn (1973; 1974) showed that some cells could become reversibly adapted to growth in the presence of DNP at concentrations of $10^{-5}$ M. He suggested that perhaps the flagellate is capable of either more efficient utilisation of the available energy or undergoes an altered mode of energy coupling under such treatments.

As with *Euglena*, *A. proteus* offers a line of study on the action of uncouplers not possible when isolated organelles or tissue slices are observed. It was thus of interest to determine the concentration range and dose that was having a direct effect on the amoebae and then to investigate the mitochondria within cells fixed from similarly treated cells.

5.2. Effects of the uncoupling agents on cell morphology and cell viability

Exposure to treatments of DNP, PCP, and CCCP within the effective concentration range for each substance (Fig. 5.1, Table 5.1) produced qualitatively similar results on cell functioning. Once the basic concentration range had been determined for a fixed time interval, the dose regime was altered by varying the duration of the treatment from one to seven hours for a specific uncoupler concentration.

The effectiveness of each reagent was more evident when exposure was carried out at a pH near to the pK value for the substance, as it is likely that the passage across the membranes by the chemical was facilitated (Simon, 1953; Finkelstein, 1970; Khan, 1974). It is suggested that only undissociated uncoupler penetrates and inhibits the cell; once inside the cell the agent is completely ionised at the internal pH of approximately 7.0. Incubations for DNP, PCP and CCCP were therefore performed at pH 4.0, 4.5, and 6.0 respectively (Parker, 1965).

Control amoebae responded to a lowering of the pH to pH 4.0 by
Fig. 5.1: Effect of PCP (○○), DNP (■■), and mCCCP (□□) on cell survival in Amoeba proteus following one hour exposures.

Cell Survival (%)

Log_{10} Unocoupler Conc. (M)
Table 5.1: Effect of increased concentration of the uncoupler substances on cell survival following

<table>
<thead>
<tr>
<th>Uncoupler Concentration</th>
<th>% survival:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCP</td>
</tr>
<tr>
<td>$1 \times 10^{-6}$</td>
<td>96.7</td>
</tr>
<tr>
<td>$2.5 \times 10^{-6}$</td>
<td>95.3</td>
</tr>
<tr>
<td>$4 \times 10^{-6}$</td>
<td>94</td>
</tr>
<tr>
<td>$5 \times 10^{-6}$</td>
<td>81</td>
</tr>
<tr>
<td>$6 \times 10^{-6}$</td>
<td>56</td>
</tr>
<tr>
<td>$7.5 \times 10^{-6}$</td>
<td>42.7</td>
</tr>
<tr>
<td>$1 \times 10^{-5}$</td>
<td>11</td>
</tr>
<tr>
<td>$4 \times 10^{-5}$</td>
<td>-</td>
</tr>
<tr>
<td>$5 \times 10^{-5}$</td>
<td>-</td>
</tr>
<tr>
<td>$6 \times 10^{-5}$</td>
<td>-</td>
</tr>
<tr>
<td>$7.5 \times 10^{-5}$</td>
<td>-</td>
</tr>
<tr>
<td>$8 \times 10^{-5}$</td>
<td>-</td>
</tr>
<tr>
<td>$1 \times 10^{-4}$</td>
<td>-</td>
</tr>
<tr>
<td>$2 \times 10^{-4}$</td>
<td>-</td>
</tr>
</tbody>
</table>

Each point was estimated as the mean of three experiments, i.e. each point represents 50-150 cells.
an increased duration of the cell cycle. They retained elongated pseudopods and after an initial period of weak attachment to the substratum, demonstrated cytoplasmic streaming identical to that of cells maintained in more alkaline media. The lower pH occasionally produced binucleate cells upon division similar to an earlier finding of Ord (1968) where pH shocks on division spheres generated binucleates.

In the presence of any of the three uncouplers at the concentration considered more fully and at the pH near to the pK of each, attachment to the substratum was generally lost completely within the first ten minutes of exposure. Within 30 minutes the pseudopods had begun to be retracted although considerable variation in external form was still evident. An enlargement of the contractile vacuole was noted in most cells. By 1 hour of the treatment, the cells tended to assume a spherical rosette configuration from which very short pseudopods were extended. Cells surviving after 3 - 4 hours displayed little detectable cytoplasmic streaming and the outer membrane had become smooth in appearance. There was commonly a degree of cytoplasmic aggregation within the sphere with corresponding signs of swelling directly under the membrane. If treatment was continued, such forms proceeded to cytolysis.

Cell damage resulting in cytolysis in the case of all three uncouplers usually occurred during treatment itself or within the first 5 hours following treatment when the cells had been transferred back to normal Chalkley's medium. Cells in which permanent damage had not occurred reattached to the substratum first as a rosette form from which pseudopods were eventually extended. For shorter incubations cells usually recovered normal locomotion within 1 - 2 hours and proceeded to division without any noticeable delay. As the exposure was extended, the interval necessary for cell recovery to normal activity increased correspondingly for each uncoupler.

5.2.1. Effects of DNP

Stock solutions of $10^{-3}$ M DNP were prepared by dissolving 0.0184 gm up to 100 ml with distilled water containing 2 ml. of IM NaOH. Subsequent dilutions were made using Chalkley's medium and the pH adjusted prior to use. A number of different incubation conditions
were considered to demonstrate that entry of the uncoupler into the cells had been achieved.

5.2.1.1. Incubations at pH 4.0

A pH effect was noted for uncoupler action of DNP. Cells were found to survive much higher concentrations at pH 5.9, retaining a pseudopodal form when concentrations up to $10^{-3}$ M DNP were employed. Although as the exposure was increased cells at this concentration lost attachment and contractile vacuole enlargement was evident, cell survival after 2 - 3 hours treatment remained at the 100% level. On the other hand at pH 4.0 DNP concentrations in the range $5 \times 10^{-5}$ M to $10^{-4}$ M produced repeatable adverse effects on cell viability. The effect of pH upon one hour exposures of three concentrations of DNP are given in Fig. 5.2, Table 5.2.

After 2 - 3 hours of exposure to $10^{-4}$ M DNP at pH 4.0 subsequent recovery was reduced, falling to below 40% after 5 hours (Fig. 5.4, Table 5.4). Survival was significantly higher for cells that had been fed until immediately prior to the exposure of DNP than those cells which had been starved for 48 hours before treatment (Fig. 5.3, Table 5.3). Significance with $p<0.05$ was determined by the Student's 't' test; a 't' value of 2.529 resulting from the analysis with 7 degrees of freedom. This difference was considered of importance and for all comparisons of the effects of the different uncoupler agents cells of a similar feeding state were exposed to the chemicals. Overnight incubations with $1 \times 10^{-4}$ M DNP were lethal for both fed and unfed cells.

With $1 \times 10^{-5}$ M DNP, cells survived extended periods of incubation at pH 4.0 although they did not undergo division. Gradual cell loss was observed so that after 20 days, by which time pH 4.0 cells had completed four division cycles, only 40% survival was observed. Cells maintained under these conditions showed very poor sign of attachment and as treatment continued the floating forms became very granular in appearance having irregular pseudopods.

Cell damage by DNP was shown to be mainly at the cytoplasmic level for concentrations of $1 \times 10^{-4}$ M. In two experiments where sixty operations were performed, the nuclei of cells which had been treated for 6 - 7 hours demonstrated normal cloning in 90% of the hybrids when transferred to untreated cytoplasm. Whole cells after such an incubation gave only 30-35% survival (Fig. 5.3).
Fig. 5.2: Effectiveness of DNP and PCP at different pH for 1 hour treatments

Each point represents a total of 75-150 cells collected from three separate experiments. Survival was confirmed by assessing cloning ability.
Fig 5.3: Different sensitivity of starved and fed cells to increasing the exposure to $1 \times 10^{-4}$ M DNP at pH 4.0.

Figures represent means and standard errors from at least 60-140 cells collected for each experimental treatment time for the fed and starved cells. The nuclear sensitivity is based on the survival of 60 micrurgically operated cells where the nucleus of a 7 hour DNP-treated cell was transfered to host untreated anucleate cytoplasm.

A significant difference exists between the fed and starved cell response, $p < 0.05$ (Student's t test $t = 2.529$ with 7 d.f).
Table 5.2: Effectiveness of PCP and DNP at varying pH

<table>
<thead>
<tr>
<th>pH</th>
<th>5 x 10^{-6} M PCP</th>
<th>5 x 10^{-5} M DNP</th>
<th>7.5 x 10^{-5} M DNP</th>
<th>1 x 10^{-4} M DNP</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2</td>
<td>21.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3.5</td>
<td>-</td>
<td>86.0</td>
<td>42.0</td>
<td>0</td>
</tr>
<tr>
<td>3.7</td>
<td>61.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4.0</td>
<td>-</td>
<td>96.0</td>
<td>81.0</td>
<td>79.0</td>
</tr>
<tr>
<td>4.1</td>
<td>81.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4.3</td>
<td>90.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4.5</td>
<td>81.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4.6</td>
<td>94.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5.1</td>
<td>96.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5.9</td>
<td>-</td>
<td>98.0</td>
<td>100</td>
<td>95.0</td>
</tr>
<tr>
<td>6.1</td>
<td>96.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

N.B. Figures are % survival and are the means of at least three experiments to give 75-175 cells for each experimental point.

Table 5.3: Relative sensitivity of fed or unfed amoebae to the action of DNP (10^{-4} M, pH 4) for increasing treatment times

<table>
<thead>
<tr>
<th>Time of cell exposure (hrs.)</th>
<th>% survival for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fed cells</td>
</tr>
<tr>
<td>½h</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>79 ± 8.5</td>
</tr>
<tr>
<td>2</td>
<td>82 ± 3</td>
</tr>
<tr>
<td>3</td>
<td>74 ± 4.3</td>
</tr>
<tr>
<td>4</td>
<td>61 ± 2.4</td>
</tr>
<tr>
<td>5</td>
<td>39 ± 7.1</td>
</tr>
<tr>
<td>6</td>
<td>35 ± 10</td>
</tr>
<tr>
<td>7</td>
<td>33 ± 10.4</td>
</tr>
</tbody>
</table>

N.B. Figures are the means of at least three experiments to give at least 60-140 cells for each treatment point.

Significant difference between fed and unfed cell response, p < 0.05 calculated by the Student's 't' Test (t=2.529 with 7 d.f.)
Fig. 5.4: Sensitivity of amoebae to increased exposures to the uncoupling agents: PCP, DNP, CCCP.

Each treatment point represents the mean of 75-175 cells collected from at least three separate experiments; survival determined by single cloning of the treated cells.
Table 5.4: Subsequent survival of amoebae after exposure to doses of PCP, DNP, CCCP

<table>
<thead>
<tr>
<th>Cell exposure time (hours)</th>
<th>% survival:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCP</td>
</tr>
<tr>
<td>1</td>
<td>81</td>
</tr>
<tr>
<td>2</td>
<td>39.4</td>
</tr>
<tr>
<td>3</td>
<td>31</td>
</tr>
<tr>
<td>4</td>
<td>28.3</td>
</tr>
<tr>
<td>5</td>
<td>16.8</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
</tr>
</tbody>
</table>

PCP = $5 \times 10^{-6}$ M at pH 4.5
DNP = $1 \times 10^{-4}$ M at pH 4.0
CCCP = $8 \times 10^{-5}$ M at pH 6.0

Each sample point represents a mean calculated from at least three separate experiments so that between 75-175 amoebae were treated for each experimental point.
Reciprocal transfers could not be performed as the treated cytoplasm did not survive the operational manipulations involved in the attempts to introduce a donor untreated nucleus. Renucleated treated cytoplasm was incapable of repairing the wound resulting from the transfer operation. The membranes of cells incubated for this period with DNP show marked fragility. Due to the non-functional contractile vacuole there is evidence of water intake. Both of these factors would lead to cell rupture.

5.2.1.2. Incubations with 1% DMSO in the medium

Incubations had been conducted at pH 4.0 to enhance the uptake of DNP by the amoebae. Other means of administering the uncoupler were investigated which might aid cell penetration but permit a more normal growth pH. The addition to the culture medium of DMSO which is known to increase membrane permeability was considered.

Control cell viability was not affected by concentrations of DMSO up to 1%, the only noticeable difference being a slight lengthening of the cycle duration by about 10 hours. Higher concentrations resulted in cell lysis in incubations extending up to 24 hours and so were only employed for periods of less than an hour in the ultrastructural study.

Whereas cell division had been arrested at a DNP concentration of $1 \times 10^{-5}$ M at pH 4.0, cell cycling was observed at this concentration at pH 5.9 with 1% DMSO present. Although the cycle duration was extended by such a treatment, cells could be cloned to the completion of a fifth cycle.

At a concentration of $1 \times 10^{-4}$ M DNP with 1% DMSO the cells did not proceed through division events. The cells were generally present as a loosely attached or floating rosette form. Progressive cell death occurred but survival was higher than at this concentration at pH 4.0, so that after sixteen days a level of 25% was recorded.

It would appear that this means of treatment with DMSO levels as low as 1% was not producing cell penetration by the uncoupler to the extent seen near to the pK value. The use of DMSO was therefore not pursued for the investigation of the lethal effects of the other two uncouplers.
5.2.2. **Effects of PCP**

The effective concentration range for PCP was found to be of an order of magnitude lower than that for DNP (Fig. 5.1). At the concentration chosen to investigate the survival ability with increasing exposure times, PCP was also seen to be more potent, thus after 2 hours incubation at pH 4.5 with $5 \times 10^{-6}$ M PCP, subsequent cell survival was reduced to 40% (Fig. 5.4).

Initially a $1 \times 10^{-3}$ M stock of PCP was prepared by making 0.013318 gm up to 50 ml with distilled water in the presence of 1M NaOH, as PCP has a low solubility in water. The stock was maintained at pH 10.8, correction not being made until the subsequent dilutions were performed. However variations in the effects of the PCP were noted under such experimental conditions, which appeared to be correlated with the age of the stock solution. Hiatt et al (1960) suggested that photochemical degradation rapidly occurred when in solution reducing efficiency which would account for the result of the present study. PCP therefore was always prepared freshly from the crystalline solid which gave more reproducible lethality curves.

Incubation pH again proved a critical consideration (Fig. 5.2). At values above pH 4.5 most cells remained attached as pseudopodal forms throughout the incubations with concentrations of $1 \times 10^{-5}$ M for exposure times which would prove lethal at lower pH's. At pH 4.5 cytolysis usually occurred during the treatment itself or shortly after the treatment had ended. Those cells surviving treatments of more than two hours with $5 \times 10^{-6}$ M PCP usually took 2 - 3 hours before the swollen spheres began to reattach and flatten out into pseudopodal forms again. When cultured singly no significant division delays were recorded for those cells which recovered normal locomotory behaviour.

As with DNP exposure, cells of a mixed age were used in the study.

5.2.3. **Effects of CCCP**

With isolated mammalian mitochondria the carbonyl cyanide phenylhydrazines have proved to be a very active class of uncouplers (Goldsby and Heytler, 1963; Parker, 1965).
Incubations in the present study of the effects on intact amoebae illustrated that its potency on whole cell survival was similar to that of DNP but less than that of PCP (Fig. 5.1). At a concentration of $8 \times 10^{-5}$ M at pH 6.0 the cells became detached throughout the incubations, but for the first 2 hours the majority of cells retained short pseudopods. Only after 4 hour incubations under these conditions did a smooth-surface swollen spherical form, which was prone to lysis during treatment predominate. The pattern of recovery of cells surviving CCCP exposure was of a similar nature as for the other two uncoupler substances.

5.3 Ultrastructural changes associated with uncoupling agents

Alterations to the mitochondrial configurations within treated cells resulted from exposure of amoeba to all three agents considered in the present study. In all cases abnormal intermediate forms were generated which often involved gross alterations in profile shape accompanied by changes in the cristal membranes and in the matrix density. The appearance of these changes occurred rapidly upon incubation and were detectable before whole cell morphology had significantly altered and while the cells were still 100% viable should treatment be terminated. At higher concentrations of the reagents there was evidence of organelle rupture in some cells. A summary of the alterations produced upon exposure to the three uncouplers by the methods discussed below is shown in Table 5.7.

5.3.1. DNP treatments

Initial observations were carried out on cells which had been exposed to $1 \times 10^{-4}$ M DNP at pH 5.9. Exposures of 45 minutes resulted in a Type Int. profile predominating. This form has a L:W index approaching a value between those normally associated with Type II and Type Int. profiles in control cells (Table 5.5). Distinct Type I profiles had been reduced in number but were still present in some cells as were distinct Type II forms. Matrical filaments were observed in some of the Type Int forms.

As with the incubations to determine the lethal effects of DNP on whole cells, a number of different culture conditions were considered to study the effects at the ultrastructural level.
Table 5.5.
Variations in the length to width index of mitochondrial profiles following treatment with the uncoupling agents.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mitochondrial Profile Form</th>
<th>Profile Number</th>
<th>Index (± S.E.)</th>
<th>Graphical Representation</th>
</tr>
</thead>
<tbody>
<tr>
<td>None, pH 4.0</td>
<td>Type II</td>
<td>124</td>
<td>1.43 ± 0.04</td>
<td>[ ]</td>
</tr>
<tr>
<td>None, pH 5.9</td>
<td>Type Int</td>
<td>370</td>
<td>1.95 ± 0.03</td>
<td>[ ]</td>
</tr>
<tr>
<td>None, pH 4.0</td>
<td>Type I</td>
<td>216</td>
<td>2.62 ± 0.05</td>
<td>[ ]</td>
</tr>
<tr>
<td>DNP</td>
<td>Type Int</td>
<td>214</td>
<td>2.32 ± 0.08</td>
<td>[ ]</td>
</tr>
<tr>
<td>15 min 10^{-4} M pH 4</td>
<td>Type Int</td>
<td>231</td>
<td>2.45 ± 0.06</td>
<td>[ ]</td>
</tr>
<tr>
<td>30 min 10^{-4} M pH 4</td>
<td>Type Int</td>
<td>130</td>
<td>1.99 ± 0.08</td>
<td>[ ]</td>
</tr>
<tr>
<td>30 min 10^{-4} M pH 5.9 + 5% DMSO</td>
<td>Type Int</td>
<td>125</td>
<td>1.78 ± 0.05</td>
<td>[ ]</td>
</tr>
<tr>
<td>45 min 10^{-4} M pH 5.9</td>
<td>Type Int</td>
<td>247</td>
<td>2.37 ± 0.09</td>
<td>[ ]</td>
</tr>
<tr>
<td>PDP</td>
<td>Type Int</td>
<td>179</td>
<td>2.37 ± 0.09</td>
<td>[ ]</td>
</tr>
<tr>
<td>30 min 5x 10^{-6} M pH 4.5</td>
<td>Type Int</td>
<td>217</td>
<td>2.65 ± 0.06</td>
<td>[ ]</td>
</tr>
<tr>
<td>1 hour 5x 10^{-6} M</td>
<td>Type Int</td>
<td>269</td>
<td>2.23 ± 0.06</td>
<td>[ ]</td>
</tr>
<tr>
<td>CCCP</td>
<td>Type Int</td>
<td>247</td>
<td>3.04 ± 0.05</td>
<td>[ ]</td>
</tr>
<tr>
<td>1 hour 4x 10^{-5} M pH 6.0</td>
<td>Type Int</td>
<td>231</td>
<td>3.12 ± 0.10</td>
<td>[ ]</td>
</tr>
<tr>
<td>15 min 8x 10^{-5} M</td>
<td>Type Int</td>
<td>203</td>
<td>3.34 ± 0.12</td>
<td>[ ]</td>
</tr>
</tbody>
</table>

Profiles taken at random from between 8-10 cells for each sample point.
5.3.1.1. **Treatments at pH 4.0**

When incubations were conducted with $1 \times 10^{-4}$ DNP at pH 4.0, mitochondria of a condensed intermediate form predominated (Fig. 5.5) and represented 75-85% of the profiles within the cell. These changes were not simply due to a pH shock as both Type I and II mitochondria were clearly retained at this lower pH (Figs. 5.6 and 5.23).

Elongated types, with a long axis reaching $5\mu$ (Fig. 5.7), were often encountered after only 15 minutes exposure representing up to 10-20% of the profiles, and including alterations in overall shape producing L-shaped profiles (Fig. 5.8). Such profiles prevailed after 30-45 minutes also (Fig. 5.24); exposure times at which whole cells had begun to float and pseudopodal retraction had commenced. The L:W index for profile samples from cells exposed to this treatment gave values between those associated with Type I and Type Int. forms in control mitochondria (Table 5.5). The complexity and dimensions of the cristae were also suggestive of an intermediate form. Matrix density was of intermediate nature being denser than that of a Type II control organelle yet not as opaque as was normally encountered in Type I forms. Filamentous inclusions were noted occasionally within these profiles.

Similar configurations were produced in cells which were cultured at this pH for 10-20 days at the lowered concentration of $1 \times 10^{-5}$ M DNP. However, more normal Types I and II were present in these cells.

5.3.1.2. **Treatments with 5% DMSO present at pH 5.9**

A higher concentration of 5% DMSO was permissible without the concurrent lethality seen on extended incubations for the shorter DNP exposures performed in the present ultrastructural investigations. This level of DMSO did not eliminate the distinction between Type I and II mitochondria in control cells although occasional vacuolated regions were apparent in the cristal membranes of Type II mitochondria.

With $1 \times 10^{-4}$ M DNP treatment under such conditions, similar mitochondrial configurations to those observed with $1 \times 10^{-4}$ at pH 4.0 were seen. Filaments were evident in the intermediate-dense matrices of some of these profiles (Fig. 5.9 and 5.10). The L:W index again approached a value between that of Type Int and Type I
**Fig. 5.5.** : Type Int forms generated by a 15 minute incubation with $1 \times 10^{-4}$ M DNP at pH 4.0. x 20,000

**Fig. 5.6.** : Control Type I and Type II mitochondria within a cell cultured at pH 4.0 for 24 hours. x 20,000
Fig. 5.7. An elongated Type Int mitochondrion from a cell exposed to $1 \times 10^{-4}$ M DNP at pH 4.0 for 15 minutes.

$\times 24,000$

Fig. 5.8. An elongated Type Int profile of a different cell exposed to a $1 \times 10^{-4}$ M DNP at pH 4.0 for 15 minutes.

$\times 24,000$
**Fig. 5.9.** Profiles typical of a cell exposed to $1 \times 10^{-4}$ M DNP with 5% DMSO in the incubation medium. Cells fixed immediately following a 30 minute treatment. x 20,000

**Fig. 5.10.** Mitochondrial form following a 45 minute incubation in $1 \times 10^{-4}$ M DNP at pH 5.9 with 5% DMSO in the medium. x 30,000

**Fig. 5.11.** Mitochondria within a cell pretreated with $1 \times 10^{-3}$ M sodium periodate for 1 hour prior to treatment with $1 \times 10^{-4}$ M DNP for 1 hour. x 20,000

**Fig. 5.12.** Typical Type Int form generated within 5 minutes of injecting $1 \times 10^{-4}$ M DNP into a cell. x 30,000
profiles (Table 5.5). Diverse profile shape was evident as with this concentration of the uncoupler at pH 4. The only apparent difference under these two sets of incubation conditions was the presence of vacuolated regions in the cristal membranes similar to those seen occasionally in the DMSO control cells. It is possible therefore that these vacuolations resulted from the DMSO and not the DNP.

Cells after 4 days exposure to 1x $10^{-4}$ M DNP with 1% DMSO present possessed mitochondria of an elongated nature occasionally with a long axis of up to 5.5\mu. The matrix of these tended to be less dense than is normally found in Type Int.

5.3.1.3. Treatments following periodate pretreatment

It was hoped that a periodate pretreatment prior to incubation with DNP might result in a more rapid entry of the uncoupler into the cell. Previous studies with amoebae had shown that the resistance to a number of agents was reduced by a three hour pretreatment with $10^{-3}$ M sodium periodate (Sanders, 1969). Periodation removes the amoebae cell coat; the $\mathrm{IO}_4^-$ ions splitting bonds between adjacent C atoms carrying $\mathrm{OH}^-$ groups.

In the present study cells were exposed to periodate for one hour only, so that they did not take on a rosette form prior to the addition of the uncoupler. Before incubation with the DNP at pH 4, the amoebae were rinsed for thirty minutes with normal Chalkley's. With DNP treatments up to one hour a light Type Int profile was generated. Matrical filaments were evident in some of these, (Fig. 5.11) although occasionally these were also noted in periodate treated controls. However as the distinction between Types I and II profiles was less marked in these controls in addition to the presence of filaments in periodate pretreated controls this means of facilitating entry by the uncoupler into the cell was not pursued.

5.3.1.4. Administration of DNP by cell injection

The use of DMSO in the medium and periodate pretreatment has been considered in an attempt to confirm that the alterations in mitochondrial morphology encountered when cells were exposed to DNP at pH 4.0 was actually due to the action of the uncoupler upon entry into the cells. As noted, however, the DMSO and the periodate themselves
affect mitochondrial structures (e.g. the vacuolation of the cristal membranes with DMSO) even though the distinction between Types I and II might be retained.

To overcome the need for chemical treatments to facilitate entry across the cell coat and membrane, $1 \times 10^{-4}$M DNP was injected into a set of 20 amoebae. The pH of the injected fluid was adjusted to pH 6.8 to minimise an internal pH shock by the treatment. Cells were fixed 5 to 15 minutes after the DNP injection had been performed. As with the previous DNP treatments a condensed Type Int mitochondria predominated in between 80-90% of a cells' profiles. The cristae and matrix density typical of other DNP treated mitochondria were again observed (Fig. 5.12). Controls where phosphate buffer was injected into cells did not produce these changes. The effects of injecting amoebae with several substances including DNP will be considered in greater depth in Chapter 7.

5.3.2. PCP treatments

At the ultrastructural level PCP incubations produced rather more varied effects on mitochondrial morphology. At pH 4.5 with $5 \times 10^{-6}$M PCP Types I and II profiles persisted as minority groups while the majority of profiles adopted a series of Type Int forms. The Type Ints. were in slightly lowered proportions than on DNP treatments (Table 5.6).

Table 5.6 Percentage of Altered Type Int. profiles after exposure to the uncoupling agents

<table>
<thead>
<tr>
<th>Uncoupler</th>
<th>Conc. (M)</th>
<th>Profiles Counted</th>
<th>% as altered Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNP</td>
<td>$1 \times 10^{-4}$</td>
<td>1343</td>
<td>78.3</td>
</tr>
<tr>
<td>PCP</td>
<td>$5 \times 10^{-6}$</td>
<td>1409</td>
<td>74.7</td>
</tr>
<tr>
<td>CCCP</td>
<td>$8 \times 10^{-5}$</td>
<td>1371</td>
<td>98.3</td>
</tr>
</tbody>
</table>

30 minute incubations with PCP resulted in a condensed Type Int profile reminiscent of those generated by DNP (Fig. 5.13). The matrix density was of an intermediate opacity and the cristal
membranes were somewhat distended. The L:W index for overall shape resulted in a value lying between that of Type Int and Type I forms (Table 5.5).

Treatments of increased durations resulted in similar proportions of altered forms with the L:W index nearer to that of Type I controls but with a matrix generally of a lighter density. The occurrence of matrical inclusions was evident after 1 hour treatments (Fig. 5.14) along with a reduction in cristal membranes. These forms prevailed after 2 hours exposures (Fig. 5.25), a dose exposure where whole cell viability had been reduced to the 40% level.

Raising the concentration of PCP to $1 \times 10^{-5}$M also resulted in further alterations in the mitochondrial profiles preserved upon fixation. After a 30 minute exposure, elongated Type Int profiles were observed although these differed from those of $5 \times 10^{-6}$M treatments in possessing more tubular cristae of a less distended nature (Fig. 5.15). Matrical inclusions were again noted. With longer exposures at this concentration which were lethal to the whole cell, the mitochondria became disrupted with a breakdown of the membrane systems of the organelle.

5.3.3. CCCP treatments

5.3.3.1. Morphological changes

The most striking alterations in mitochondrial morphology resulted from treatment with the uncoupler CCCP. The changes involved a gross alteration in profile shape, a matrix of intermediate density, and a change in the cristal membranes; all characters associated with the other two uncouplers. Between 95-100% of the profiles of such cells underwent an alteration (Table 5.6) with an increase in the L:W index to well over that of normal Type Int and Type I forms. (Table 5.5).

Even with $4 \times 10^{-5}$M CCCP at pH 6.0 after exposures of 30 minutes, when amoebae are still attached to the substratum and displaying locomotory behaviour, Type Int forms predominate (Fig. 5.16). This form became more pronounced after 1 hour exposures (Fig. 5.17).

At $8 \times 10^{-5}$M, the concentration used in the lethality studies, the Type Int form was present after only 15 minutes of treatment and
Fig. 5.14. : Matrical Filamentous inclusions within the Type Int profiles generated in a cell exposed to $5 \times 10^{-6}$ M PCP for 1 hour at pH 4.5.

$x \times 20,000$

Fig. 5.13. : Type Int profiles present in a cell after exposure to $5 \times 10^{-6}$ M PCP for 30 minutes at pH 4.5

$x \times 25000$

Fig. 5.15. : Elongated Type Int forms within cell exposed to $1 \times 10^{-5}$ M PCP for 30 minutes.

$x \times 25,000$
**Fig. 5.16.** Mitochondria generated by a 30 minute exposure to $4 \times 10^{-5}$ M CCCP at pH 6.0. x 25,000

**Fig. 5.17.** Aberrant forms produced by a 1 hour exposure to $4 \times 10^{-5}$ M CCCP. x 25,000

**Fig. 5.18.** Aberrant types generated by a 1 hour exposure to $8 \times 10^{-5}$ M CCCP. x 14,000

**Fig. 5.19.** Disruption produced by a 2 hour exposure to $1 \times 10^{-4}$ M CCCP. x 14,000
CCCP Recovery

Fig. 5.20. : Type Int profiles from a cell fixed after a 15 minute exposure to $8 \times 10^{-5}$M CCCP.

$\times 30,000$

Fig. 5.21. : Type Int profiles persisting in a cell given a 1 hour 'recovery' period in Chalkley's medium after exposure to $8 \times 10^{-5}$M CCCP for 1 hour.

$\times 30,000$

Fig. 5.22. : Return of two distinct mitochondrial forms in a cell exposed to $8 \times 10^{-5}$M CCCP for 1 hour but given a 'recovery' period of 4 hours in normal Chalkley's before fixation.

$\times 30,000$
persisted after 30 minutes and 1 hour exposures (Figs. 5.26, 5.18) by which time the disruption of the cristal membranes was becoming obvious. This disruption was more noticeable at higher concentrations and doses which were lethal to the cells (Fig. 5.19).

5.3.3.2. Reversibility of structural changes

With the generation of such extreme forms it was of interest to investigate the mitochondrial types present in cells where the CCCP treatment had been halted and the cells returned to normal media to permit recovery. Amoebae exposed to $8 \times 10^{-5}$ M CCCP for 1 hour followed by 1, 4 and 24 hour recovery periods were considered. Mitochondrial alterations occurred within a short time after $8 \times 10^{-5}$ M exposures (Fig. 5.20).

1 hour after the removal of the CCCP the cells had reattached to the substratum and were exhibiting normal cytoplasmic streaming. The mitochondria in these however, remained as a Type Int form although the cristae appeared less damaged (Fig. 5.21). 4 hours after treatment had ceased profiles had again begun to take on the characteristics of Type I and II mitochondria although the matrix of the Type I form was not as dense as control Type I. Approximately 80% of the profiles were as Type II with 20% scored as Type I forms (Fig. 5.22).

With a 24 hour recovery period, the proportions of Type I mitochondria had increased to 38% with 62% of a Type II nature, indicating that cells in which the mitochondrial content had been drastically altered could still be reverted to a more normal type once the uncoupler treatment had been removed.

5.4 Discussion

The present study demonstrated that the exposure of amoebae to the three uncoupling agents DNP, PCP, CCCP at concentrations which had a detectable effect on cell activity, produced changes in mitochondrial morphology (See Figs. 5.23 - 5.26 for comparison). These alterations, at least when CCCP was employed, were of a reversible nature. This effect of the uncouplers could be produced whether the reagent was introduced on the outside or the inside of the cell.

DNP and PCP were found to be effective in concentration ranges
COMPARISON OF FORMS GENERATED BY THE DIFFERENT UNCOUPLER TREATMENTS

Fig. 5.23. : Type I and II profiles near the contractile vacuole of pH 4.0 cultured control cell.

Fig. 5.24. : Type Int profiles in cell exposed to $1 \times 10^{-4}$ M DNP at pH 4.0 for 30 minutes.

Fig. 5.25. : Type Ints generated after a 2 hour exposure to $5 \times 10^{-6}$ M PCP at pH 4.5.

Fig. 5.26. : Mitochondria preserved in cell treated for 1 hour with $8 \times 10^{-5}$ CCCP.

All Magnifications $\times 14,000$
<table>
<thead>
<tr>
<th>Uncoupler and method of administration</th>
<th>Gross shape of profiles</th>
<th>Matrix Density</th>
<th>Cristal Form</th>
<th>Occurrence of Filaments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DNP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(i) $1 \times 10^{-4}$ M, pH 4.0</td>
<td>Diverse forms, elongated and often L-shaped</td>
<td>Intermediate density</td>
<td>Dilated</td>
<td>Occasional</td>
</tr>
<tr>
<td>(ii) $1 \times 10^{-4}$ M, pH 5.9 with 5% DMSO</td>
<td>Diverse elongated and spherical forms</td>
<td>Intermediate</td>
<td>Dilated</td>
<td>Occasional</td>
</tr>
<tr>
<td>(iii) $1 \times 10^{-4}$ M after $10^4$ preincubation</td>
<td>Mainly spherical</td>
<td>Light Intermediate</td>
<td>Tending to Dilate</td>
<td>Occasional</td>
</tr>
<tr>
<td>(iv) $1 \times 10^{-4}$ M injected</td>
<td>Diverse and elongated</td>
<td>Intermediate</td>
<td>Often dilated</td>
<td>Rare</td>
</tr>
<tr>
<td><strong>PCP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(i) $5 \times 10^{-6}$ M</td>
<td>Elongated and spherical</td>
<td>Mainly intermediate Type I and II retained in a few cells as minority species</td>
<td>Tending to dilate</td>
<td>Occasional</td>
</tr>
<tr>
<td>(ii) $1 \times 10^{-5}$ M</td>
<td>Elongated</td>
<td>Light intermediate</td>
<td>Disrupted</td>
<td>Occasional</td>
</tr>
<tr>
<td><strong>COCP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(i) $4 \times 10^{-5}$ M</td>
<td>Very elongated and swollen forms</td>
<td>Light intermediate</td>
<td>Dilated</td>
<td>Not seen</td>
</tr>
<tr>
<td>(ii) $8 \times 10^{-5}$ M</td>
<td>Distorted elongated and swollen forms</td>
<td>Intermediate</td>
<td>Generally Disrupted</td>
<td>Not seen</td>
</tr>
</tbody>
</table>
similar to those which are used in studies on mammalian systems. Although CCCP appeared less potent in amoebae than has been reported for mammalian tissue systems if its effectiveness was assessed using lethality as a criterion; examination at the ultrastructural level revealed that CCCP did produce alterations in mitochondrial morphology within dose ranges normally employed in other systems. Such structural changes occurred with rapidity at the non-lethal doses. It is therefore probable that its potency on specific mitochondrial functioning is in the same order of magnitude as in other systems, and that in the present study, the lower regions of the organelle sensitivity range have not been reached.

When studying the effects of these substances upon amoebae the importance of determining the optimal incubation conditions must be realised. As with other studies on amoebae, the pH of the inhibitor in the medium was shown to greatly affect its potency on cell functioning and upon the ultrastructure. Thus, as Chapman-Andresen (1967) had demonstrated, a concentration of $4 \times 10^{-5}$ M DNP lies close to the limits of toxicity for A. proteus when applied at pH 3.96, but is ineffective at higher incubation pH's. It is not surprising therefore that other workers could find little effect on cell survival for amoebae after the administration of $5 \times 10^{-4}$ M solutions at pH 6.2-6.8 for exposures of up to two days (Shumaker, 1958; Flickinger, 1972; 1976). The results presented here for the three uncoupler substances accentuate the necessity of considering the ionisation of a chemical at different pH levels when using it in whole cell exposures and that caution must be taken when interpreting the results of any such chemical studies.

The pH effect discovered in the present work is consistent with the hypothesis that for inhibitors which are weak acids, only the undissociated species penetrates into the cell. Thus for each reagent concentrations will exist which have no effect on respiration and other cell functions at one pH, yet will cause complete inhibition at a lower pH near to the pK value for the compound (Beevers and Simon, 1949; Simon, 1953). An extension of this theory was proposed to explain the accumulation of DNP within Euglena, which was considered to occur because of the pH gradient present across the cell membrane at the lowered culture pH and not due to a carrier mechanism (Kahn, 1974).
Lower concentrations of DNP were required to arrest protoplasmic streaming in Physarum as the pH was lowered (Allen and Price, 1950). Similar increases in toxicity with lowered pH approaching the compound's pK have been established for the effects of PCP upon fish (Crandall and Goodnight, 1959).

The retraction of the pseudopods and the enlargement of the contractile vacuole were the first observable effects of the uncoupling action in the present work. These are energy-consuming processes which can only continue as long as a supply of available energy is maintained. The arrest of these functions by the uncoupler treatments therefore provides evidence that a depletion of the cell's energy content has occurred. The significantly greater effect of DNP when administered to starved rather than fed cells further demonstrates that energy depletion arises upon treatment with the uncouplers, as starved cells possess lower stores of metabolites than those maintained with food and are therefore more sensitive. Energy depletion was also suggested when mitosis was arrested by a 1 x 10^{-5} M DNP concentration at pH 4.0. Mitotic arrest by inhibitors in sea urchin eggs has been shown to result from lowered ATP levels (Epel, 1963).

The results of the nuclear transfer experiments showed that no irreversible damage occurred in the nucleus at a concentration lethal to the whole cell. It may be concluded that the effects of the uncouplers were upon the cytoplasm or membrane. Recently ATP-depletion has been shown to alter the conformation of cell membranes (Gazitt et al., 1976; Juliano and Gagalang, 1977). Damage by the uncouplers was generally of a reversible nature providing cells had not reached a dose causing cytolysis.

That a proportion of the cells recovered to normal cycling and activity after extended incubations of up to 7 hours with the three uncouplers suggests the energy levels within such cells are either sufficient throughout the exposure to maintain vital basal metabolic activities within the cell; or that possibly low levels of energy are being produced by other pathways. It was noted that, on removing the cells from the uncoupler solutions, they initially reattached and then only after a further period of 1 to 2 hours began to display normal locomotory behaviour. This indicates that a reversal to normal functioning requires the removal of the inhibitor from the
cell prior to a return to energy-consuming activities.

Other cell systems have shown a level of reversibility i.e. with the slime mould Physarum the inhibition of protoplasmic streaming by DNP is reversed if exposure to the compound is not too prolonged (Allen and Price, 1950). With isolated rat liver mitochondria there is evidence that the inhibition by uncouplers is of a reversible nature (Higgins and Rogers, 1976).

As mitotic events persisted when the DNP was administered at pH 5.9 at a concentration of $1 \times 10^{-5} \text{M}$ and with 1% DMSO included in the medium, it was concluded that such a low concentration of DMSO was not sufficient to significantly increase the penetration by the uncoupler into the cell at levels detectable by simple alteration in growth criteria. Similarly the higher survival with $1 \times 10^{-4} \text{M}$ DNP than exposure at pH 4.0 when these conditions of DMSO were used would indicate a reduced penetration by the uncoupler.

Whether DNP and the other uncouplers actually bind to the mitochondria upon interaction (Weinbach and Garbus, 1968; Hanstein, 1976), or whether they remain in a soluble unbound form within the cell (Kahn, 1974) remains unsettled. The present work demonstrated that whatever the interaction of the agents is within the cell, gross mitochondrial ultrastructural alterations result as a consequence of its entry into the amoebae.

With the addition of DNP, PCP, CCCP to amoebae, a condensed configuration was generated in the majority of profiles after a relatively short exposure time. This form has been designated a Type Int form, as the parameters of the L:W index, the cristal dimensions and complexity and the matrix density all appear to range between Type I and Type II mitochondrial configurations (see Tables 5.5 and 5.7). With CCCP, and to a lesser extent with DNP, the alterations in gross shape, with a concurrent change in the internal organisation of the organelles, were most obvious. Both elongated and diverse forms were commonly produced.

Such morphological changes upon uncoupling are consistent with the findings of other workers. Thus Buffa et al., (1970), who investigated the affects of several uncouplers on cultured chick embryo heart myoblasts showed an increase in denser, elongated forms.
Increased matrical densities and dilated cristae were reported by Morisset (1974) in cells from tomato root tips incubated with $5 \times 10^{-5}$ M DNP.

However, other published results from 'in vivo' work produced conflicting observations when other mammalian tissues were studied (Jasper and Bronk, 1966; Weinbach, Garbus and Sheffield, 1967; Didio et al., 1975). In none of these was an increase in condensed forms observed; however, in all these high concentrations of the uncouplers were employed, which produced degrees of rupture to the mitochondrial membranes. Such structures would appear to be the result of pathological alterations indicating mitochondrial disorganisation rather than due to actual functional differences. In the present work at higher concentrations of PCP and CCCP a proportion of the profiles had also begun to rupture and lose structural integrity.

The work with isolated mitochondria is also disputed. A contracted form was produced by the addition of $2.5 \times 10^{-6}$ M DNP and $5 \times 10^{-6}$ M PCP to rat liver mitochondria in respiratory state IV (Muscatello et al., 1975) and with the higher concentration of $5 \times 10^{-4}$ M DNP in a separate study (Weinbach, Garbus and Sheffield, 1968). A similar result could not be found by others with the addition of $2 \times 10^{-5}$ M - $1 \times 10^{-4}$ M DNP to state IV mitochondria and, as the exposure was extended, an increase in expanded types was reported (Hackenbrock, 1968).

The presence of matrical inclusions upon exposure to the uncoupler agents would further suggest altered metabolic processes in the mitochondria. Similar structures were generated by other treatments both in the present study (Chapter 6) and in the work of Ord (1976). Matrical inclusions in other cell types are thought to be indicative of a disturbance of normal functioning.

The significance of these structural alterations upon uncoupling may be considered. Uncoupling action involves both a blocking of ATP synthesis as well as a stimulation of ATPase activity (Myers and Slater, 1957), which is presumed to increase the levels of ADP within the organelle. Current theory as to what the condensed state represents at the molecular level when generated in normal state III coupled respiration (Hackenbrock et al., 1971) proposes that the binding of ADP to the mitochondrial membrane when both ADP and
substrate levels are high is of importance in the condensation phenomenon (Weber, 1972). That relative nucleotide levels may be of significance in determining configurational forms has also been advanced by Innis et al., (1976) whose elegant labelling experiment demonstrated that higher levels of ADP were present in sea urchin oocytes when the mitochondria were in a contracted form, while an expanded form was preserved after fertilisation when the ADP levels had been reduced. Muscatello (1975) considered there was little difference between the contracted form due to uncoupling and that associated in isolated organelles when ADP had been added to the preparation. If ADP levels are relevant in regulating mitochondrial configurations then certain similarities between uncoupled and coupled state III forms where levels are higher might be anticipated.

In the present study it can be concluded that all three uncouplers affect mitochondrial structure in amoebae by producing a condensed Type Int form with an L:W index and cristae approaching those common in Type I mitochondria but where the highly dense matrix of the Type I form has been eliminated. The subtle difference between the Type Ints produced by the three uncouplers are probably due to specific interactions between the individual reagents and the mitochondria which are superimposed upon the basic alteration in form resulting from the uncoupling process itself.

The reversion to more normal conformations with increasing recovery periods studied with CCCP treatments demonstrated that with sublethal doses the mitochondrial alterations were not of a permanent nature. The general increase to proportions similar to those of control cells of profiles resembling Types I and II as the recovery period increased, showed that when the cell's mitochondrial content has approached normality, control whole cell behaviour was again apparent.

The presence of the structural alterations upon uncoupling demonstrates that mitochondrial conformation can be related to changes in specific organelle functioning concerned with energy production. The results are consistent with the proposal that the mitochondrial types found in normal cells may also be representing functional differences and as such the next sections of the work were designed to investigate this further.
Chapter Six

The effects of factors acting upon the electron transport chain.

6.1. Introduction

In addition to inhibitors specifically affecting the phosphorylative processes within the inner mitochondrial membrane complexes, inhibitors acting directly on the electron transport steps of respiration and hence indirectly on phosphorylation were considered. Several treatments aimed specifically at mitochondrial functioning were used in an attempt to gain more information about the mitochondrial types of amoebae. Interruption of the passage of electrons along the respiratory chain was initially studied by the removal of oxygen, which acts as the terminal electron acceptor in the chain. Chemical means of arresting respiration were achieved by exposure to antimycin A and potassium cyanide (KCN); two metabolic inhibitors which disrupt electron transport between cyt b to cyt c₁ and between the terminal cytochromes and the oxygen acceptor in the chain respectively. The effects of these treatments on whole cell morphology and then upon cell ultrastructure were carefully studied for any detectable correlation of effect.

6.1.1. Anaerobiosis

Anaerobic culture conditions would seem ideal for the investigation of the mitochondrial types in A. proteus, as mitochondrial conformation in many cell types has been demonstrated to be greatly influenced by a deprivation of oxygen. In the cells of animals which have evolved to exist in environments of very low oxygen availability mitochondria may still be numerous but are generally of a smaller size and display a reduction in cristal membranes and in the organisation of the inner membrane (Bjorkman and Thorsell, 1962; Harlow and Byram, 1971). In rat myoblasts, on the other hand, extended culturing in low oxygen atmospheres gave rise to mitochondria which showed signs of fragmentation with the presence of distorted cristae (Auclair et al., 1976).

An early report of the effects of anaerobiosis upon ultrastructure concluded that yeast cells were devoid of mitochondria in the absence of oxygen and that the organelles were only resynthesised 'de novo' on subsequent aeration (Wallace and Linnane, 1964). More
detailed studies however demonstrated that mitochondrial-like particles were present during anaerobiosis and that these could be isolated from the yeasts (Criddle and Schatz, 1969; Plattner and Schatz, 1969; Watson et al., 1970). As anaerobiosis was prolonged, these 'promitochondria' were present first as a condensation of the normal mitochondrial morphology and then as degraded structures in which cristal disorganisation similar to that produced by CCCP was observed (Luzikov, 1973; Luzikov et al., 1971). In *Neurospora crassa* alterations were found where mitochondrial loss as well as degeneration was evident (Howell et al., 1971).

Mitochondrial structural changes occur in different tissues of many plants upon anaerobiosis. Although species of rice are capable of germination in the absence of oxygen (Opik, 1973), prolonged anoxic conditions result in destructive mitochondrial changes (Ueda and Tsuji, 1971; Vartapetian et al., 1976). The morphological differences may be correlated to a reduction in the cytochrome content of the organelles (Vartapetian et al., 1975; Opik, 1975). It has recently been shown that the preservation of mitochondria in rice tissue under anaerobic conditions may be prolonged by the presence of exogenous glucose in the culture medium (Varapetian et al., 1977).

Similar changes in the appearance of the mitochondria during anaerobiosis have been demonstrated in the roots of tomato (Morisett, 1974), *Triticale*, (Oliveira, 1977), pumpkin (Vartapetian et al., 1977), and cucumber where the damaged mitochondria recovered a more normal appearance if the anoxic state was not too prolonged and was replaced by a period of re-aeration (Coulomb and Coulomb, 1972). All these workers conclude that the decrease in mitochondrial structural integrity is associated with a decrease in respiratory functioning.

The alterations in the mitochondrial membranes induced by anaerobiosis have been shown to protect yeast cells from the action of mutagens which specifically affect mit. DNA (Pinto et al., 1975), indicating that lack of oxygen has other effects on the organelle. This would seem to be a specific protection for mitochondria as damage to nuclear DNA increased in mouse L cells on nitrofuran exposure when oxygen concentrations were reduced to zero (Olive and McCalla, 1977).
The only previous study concerned with anaerobic culturing of *A. proteus* was that of Hulpieu (1930). At the time it was not possible to relate the gross cellular morphology changes and survival ability to events that arose at the ultracellular level. The present work was therefore of interest in considering the mitochondrial forms in amoebae which resulted from various durations of anaerobiosis and also to study cells undergoing recovery in aerated media to see if changes similar to those reported in other cell systems were produced.

### 6.1.2. Metabolic Inhibitors

In studies where the effects on ultrastructure have been compared for hypoxia and KCN exposures the metabolic inhibitor was seen to produce the greater disruption of mitochondrial conformation (Auclair et al., 1976). It was therefore desirable to study the effect of this at the ultrastructural level in amoebae.

In isolated rat liver mitochondria, antimycin A and KCN prevent the transformation from the condensed to the orthodox form in state IV respiration (Hackenbrock, 1968). KCN was also known to prevent the action of mitochondrial swelling agents on isolated organelles (Tapley, 1956). Structural alterations with antimycin A resulted in the production of odd cup-shaped profiles (Jamieson and Palade, 1968); while a number of aberrant mitochondrial forms arose in the gills of fish exposed to rotenone, another metabolic inhibitor (Oberg, 1967). Rotenone binds to the mitochondria preventing electron transport at an earlier stage of the respiratory chain between FAD and Coenz Q.

Of these agents which have been shown to interfere with mitochondrial ultrastructure, KCN has long been used as the classic inhibitor of respiration. Its effects on *A. proteus* were first studied by Brinley (1929) who prematurely concluded that the toxic effect of cyanide was upon the cell membrane and considered at that time that the internal protoplasm incurred no permanent injury. As with the early study of anaerobiosis upon *A. proteus*, Brinley was unable to study the effect of KCN upon the cell's ultrastructure. The present work therefore set out to investigate the action of KCN and to note any ultrastructural changes. A comparison between the action of these inhibitors with that of anaerobiosis was attempted.
6.2.1 The effects of anaerobic culturing on the whole cell

Hydrogen and nitrogen were both employed to produce an anaerobic environment in which to culture the amoebae. The observed morphological changes did not result from the experimental design (which included the catalytic substance when hydrogen was used and routinely had the redox indicator within it) since untreated aerated cells within the vessel underwent normal growth and activity. Nor did the changes arise from the pressure changes which occurred on the evacuation of the apparatus as the short period involved had no detectable effect on aerated cells.

6.2.1.1 Hydrogen atmospheres

The initial period following the replacement of air by hydrogen was characterised by cell reattachment and a persistence of cytoplasmic streaming not significantly different from that observed in control cells. As the incubations progressed, however, cells maintained in a hydrogen atmosphere underwent a series of changes. By three hours of exposure, the amoebae which remained attached to the substratum became elongated and monopodal and appeared to have lost polarity in movement. Approximately 40% of the cells had detached and during the next hour these floating forms assumed a blistered appearance in which the cell membrane, particularly at the uroid region, was comprised of small cytoplasmic blebs which were often lost from the cell. In many cells an enlarged contractile vacuole was evident. The dimorphism of an elongated, monopodal and an irregular floating form persisted after ten hours of such culturing. Cytoplasmic streaming in the cells was sporadic and mainly confined to the monopodal form, although in the floating form an occasional pseudopod was extended into which the cytoplasm was forced.

With exposures of 16-24 hours only 5-10% of the cells remained attached. Monopodal and floating forms were still observed, but a large proportion had begun to swell and about 30% had assumed a spherical form. As the incubations were extended the numbers of these spheres increased. Although very slow, intermittent cytoplasmic streaming continued in some cells, the capacity to arrest and ingest food organisms had been lost by the amoebae after 24 hour exposures and had most probably occurred before this time.
There was evidence of cytolysis if the treatment was continued, and by 48-52 hours virtually all the surviving cells were present as swollen spherical forms in which cytoplasmic streaming had ceased completely. Such cells did not recover a normal appearance or cycling behaviour if returned to aerated medium after this time and hence it was concluded that this duration of anaerobiosis employing hydrogen resulted in irreversible damage.

Cells subjected to only 18 hours of these culture conditions did however revert to a normal foraging pseudopodal form when transferred to well-aerated Chalkley's medium. With shorter anaerobic periods higher survival ability was recorded, recovery occurring with relative rapidity. (Fig. 6.1). By the end of an 18 hour anaerobic exposure up to 90% of the surviving cells had lost attachment and were either of a spherical or monopodal type, yet within 20 minutes after a return to aerated medium, an initial reattachment was noted. 60% of these surviving cells displayed attachment after 1 hour of reaeration and although these were largely cells which had been monopodal at the end of the anaerobic period, a few spherical forms had also reattached to the substratum.

Attachment was initially of a very spread out nature giving the cells a translucent appearance. From this 'squat' form pseudopods were gradually extended so that after a two hour return to air up to half of the cells surviving were as a pseudopodal form, about 40% were as the squat form and the remainder continued as floating spherical forms. Where lysis occurred during the recovery period, it usually resulted from the cells which had become very swollen and spherical during the anaerobic period. This spherical form could not however be assumed to be totally inviable for although subsequent survival was much reduced, recovery was noted following reaeration even if a longer period was required before normal behaviour was again acquired.

6.2.1.2. Nitrogen atmospheres

To demonstrate that the effects on cell morphology described in 6.2.1.1. resulted from a lack of oxygen and were not due to a specific effect of the hydrogen atmosphere, nitrogen was used as the replacement gas in some experiments. Cells maintained in nitrogen environments
Fig 6.1: Survival potential of amoebae exposed to anaerobic culturing and potassium cyanide treatments.

- H₂ anaerobic culturing.
- 10⁻² M KCN.

% Cell survival.

Treatment duration (hours).

Each point represents between 50-125 amoebae. Viability was confirmed by scoring two cell cycles following treatments.
became monopodal within the first hour of exposure and although cytoplasmic streaming was continued, attachment to the substratum was very poor. This monopodal form predominated during the first 10 hours of culturing. In contrast to anaerobiosis resulting from hydrogen incubations, no blistering of the outer membrane was noted when nitrogen was employed. 89% survival was recorded if cells were returned to aerated medium following incubations under these conditions for 8 hours.

By 18 hours, cytolysis was observed at levels approaching those for cultures maintained under hydrogen. The surviving cells were present as either a spherical form or as monopodal types with only 10-15% remaining attached. Incubations of longer durations than 18 hours were not performed with nitrogen atmospheres as it was evident that by this time the resulting cell behaviour was identical whether hydrogen or nitrogen were employed. In the early stages of anaerobiosis the differences between the two atmospheres, which resulted in the membrane blistering in hydrogen but not with nitrogen, would indicate an effect with the hydrogen atmosphere in addition to that resulting from the lack of oxygen. However after about 10 hours of culturing the remaining cells reacted similarly to both gases.

6.2.2. Effects of anaerobiosis on mitochondrial ultrastructure

6.2.2.1. Hydrogen atmosphere

For the investigation of the effects of anaerobiosis at the ultrastructural level samples of 12-25 amoebae were fixed on opening the culture vessel after 5, 10, 15, 18 and 48 hour exposures to H₂. Similar sized samples consisting of cells exposed for these durations followed by recovery periods of 1, 3 and 5 hours in aerated medium prior to fixation were also viewed. The data on the proportions of Type I and II mitochondria collected from these cells is recorded in Table 6.1 and graphically illustrated in Fig. 6.2.

It is clear that as exposure time was increased the proportions of Type I profiles were reduced so that by 15-18 hours in a hydrogen atmosphere only Type Ints and II mitochondria remained. Control cells in the anaerobic culture apparatus maintained normal proportions of distinct Type I and II forms, hence eliminating the possibility that the change had resulted as a consequence of any part of the experi-
Fig. 6.2.: Proportions of Type I mitochondria within A. proteus sampled at various times after removal from anaerobic culture periods of different durations.

Mitochondrial counts were made on 12-25 cells for each experimental group so that at least 2,000 profiles were scored for each sample point in general.
Table 6.1 Proportions of Type I profiles with exposure to anaerobic culturing and during subsequent periods of aeration

<table>
<thead>
<tr>
<th>Duration of anaerobic exposure (h)</th>
<th>Time from end of anaerobiosis to fixation</th>
<th>No. Cells/sample</th>
<th>No. profiles/sample</th>
<th>% profiles as Type I</th>
<th>S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 (H₂)</td>
<td>0</td>
<td>13</td>
<td>2413</td>
<td>31.7 ± 3.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>13</td>
<td>2219</td>
<td>44.6 ± 2.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>13</td>
<td>2558</td>
<td>48.5 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>10 (H₂)</td>
<td>0</td>
<td>17</td>
<td>2023</td>
<td>21.2 ± 4.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>16</td>
<td>2700</td>
<td>39.0 ± 2.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>15</td>
<td>2224</td>
<td>42.8 ± 3.1</td>
<td></td>
</tr>
<tr>
<td>15 (H₂)</td>
<td>0</td>
<td>15</td>
<td>2000</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>12</td>
<td>1834</td>
<td>29.8 ± 1.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>13</td>
<td>2003</td>
<td>44.5 ± 6.6</td>
<td></td>
</tr>
<tr>
<td>18 (H₂)</td>
<td>0</td>
<td>22</td>
<td>2000</td>
<td>No Type I. Disrupted</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>13</td>
<td>1500</td>
<td>Type II only</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>20</td>
<td>2435</td>
<td>Type Int only, No Type I</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>8</td>
<td>1413</td>
<td>35.9 ± 4.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>39.9 ± 7.7</td>
<td></td>
</tr>
<tr>
<td>8 (N₂)</td>
<td>0</td>
<td>17</td>
<td>2004</td>
<td>18.3 ± 1.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>13</td>
<td>2171</td>
<td>34.5 ± 1.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>14</td>
<td>2059</td>
<td>45.1 ± 2.3</td>
<td></td>
</tr>
</tbody>
</table>
mental set-up such as the redox indicator containing thioglycolic acid.

In addition to the reduction in the proportion of Type I mitochondrial structure were also observed. The cristal membranes of the Type II-Int profiles that were preserved had a dilated appearance and often included vesicular regions. These were evident after 5 hours anaerobic culturing (Figs. 6.3 and 6.4) and persisted in the mitochondria of cells subjected to longer periods of anaerobiosis. The Type II profiles in monopodal cells on the one hand displayed reasonable levels of structural integrity (Fig. 6.5), while signs of mitochondrial degeneration were noted in the cells which had assumed a spherical form after 18 hours anaerobiosis (Fig. 6.6).

Dilated cristae persisted in the mitochondria of the small numbers of cells which survived 48 hours of these culture conditions although there was a possible reduction in their number. In a high percentage of these profiles, and in those from cells of shorter incubations, bundles of filamentous inclusions were noted within the mitochondrial matrix and in some instances profiles with more than one set were observed (Fig. 6.7).

More pronounced matrical inclusions consisting of parallel arrays of filaments were generated in mitochondria of cells maintained in an oxygen-depleted rather than oxygen-free atmosphere. Such hypoxic conditions arose when total evacuation of the culture vessel, as judged by a positive reaction with the redox indicator, had not been successful. Amoebae survived such environments for periods of up to one week, being generally present as a non-mobile floating form. 85-95% of the mitochondria in these cells were as Type II profiles in which the cristae were peripherally located to accommodate the matrical microfilamentous inclusions (Fig. 6.8).

Other structural changes were observed in amoebae incubated for prolonged periods of hydrogen atmospheres. There appeared to be a reduction in the number of Golgi complexes although no quantitative study was conducted. In several cells the cytoplasmic filamentous structure formed dense aggregates (Fig. 6.9). Within the nucleus, although the nuclear membrane was well preserved, alterations were noted including a compacting of the electron-dense nucleoli so that
Fig. 6.3. : Vesicular or vacuolated mitochondrial cristal membranes following 5 hours exposure to anaerobic culturing with a hydrogen atmosphere.
   x 32,000

Fig. 6.4. : Cristal membrane alterations in a cell exposed to 5 hours of hydrogen anaerobic culturing.
   x 32,000

Fig. 6.5. : Mitochondria preserved within a monopodal cell that had been subjected to an 18 hour exposure to hydrogen anaerobic conditions.
   x 20,000

Fig. 6.6. : Mitochondrial degeneration within a cell that had assumed a spherical form following 18 hours of hydrogen anaerobic culturing.
   x 20,000
Fig. 6.7. : Filamentous matrical inclusions generated by a 48 hour exposure to hydrogen.
\[ \times 50,000 \]

Fig. 6.8. : Inclusions within matrix of a cell cultured in a low or depleted oxygen atmosphere for 1 week.
\[ \times 50,000 \]

Fig. 6.9. : Aggregation of cytoplasmic filaments in a cell cultured in a hydrogen atmosphere for 48 hours. (Cells do not generally recover after an anaerobic period of this duration).
\[ \times 8,000 \]
the outer perimeter appeared smooth in contrast to this more diffuse form in control nuclei.

From Fig. 6.2 it will be seen that the proportions of mitochondrial types in amoebae which had been returned to aerated Chalkley's in the investigation of recovery and survival ability, gradually reached levels normally encountered in control cells. The 5-10 hour exposed amoebae showed the most rapid return to normal levels with 40% of the profiles assuming a Type I form within an hour in those cells which had reattached to the substratum as squat forms. After 3 hour recovery periods, the mitochondrial proportions of the five hour exposed sample had risen to almost 50% Type I, very close to control levels. Those cells which had been subjected to a 15 hour hydrogen exposure required three hours before the proportions of Type I exceeded 40% while those given an 18 hour exposure had not reached this level again until a five hour recovery interval had been completed.

The presence of two distinct types of mitochondria after 18 hours of anaerobiosis was not discernable until after 3 hours of re-aeration (Fig. 6.10); as after one hour in air the profiles were either a Type II or Int form. This demonstrated that amoebae which took longer to return to a pseudopodal morphology similarly required a greater interval of time before recovering a normal mitochondrial content.

That the mitochondrial respiratory enzyme content had not been completely or irreversibly depleted during anaerobic culturing was demonstrated by cytochemical staining for cytochrome oxidase with DAB (See chapter 7 for a more detailed account). A positive reaction was achieved with a 3 hour DAB incubation in cells which had been exposed to 18 hours of anaerobiosis (Fig. 6.11).

6.2.2.2. Nitrogen atmospheres

Cells were prepared for the E.M. after subjection to nitrogen environments for eight and 18 hours exposures and were either fixed immediately or after 1 and 3 hours of recovery in air.

A nitrogen atmosphere for 8 hour exposures resulted in a decrease in the proportions of Type I mitochondria to below 20% of the total profile content (Table 6.1, Fig. 6.2). Profiles were
Fig. 6.10.: Distinct Type I and Type II forms within a cell given a 5 hour 'recovery' period in aerated medium following an 18 hour hydrogen exposure. x 40,000

Fig. 6.11.: Deposition of reaction product in the DAB reaction to localise cytochrome oxidase in profiles of a cell grown anaerobically for 18 hours. The DAB incubation was for 3 hours on an unfixed cell. x 40,000

Fig. 6.12.: Matrical inclusions in the Type II profiles which are present when a cell was exposed to a nitrogen anaerobic atmosphere for 18 hours. x 40,000
generally of a light Type Int or Type II form. On subsequent aeration the numbers of distinct Type I forms increased in a manner similar to when hydrogen was used (Fig. 6.2). Unlike the 5 and 10-hour hydrogen exposures, however, the dilated cristae showed little signs of vesiculation after an 8 hour treatment with nitrogen.

The elimination of Type I mitochondria had again occurred after 18 hour incubations and as with hydrogen atmospheres, matrical inclusions were evident in the profiles preserved (Fig. 6.12). Type II profiles in which these inclusions persisted were still present after 1 hour re-aeration although a Type Int form had also returned after this time, indicating that the mitochondrial types were beginning to return to more normal forms. Further recovery has not been followed in this group.

6.3. Exposure to KCN

Concentrations in the range of $1 \times 10^{-3}$ to $1 \times 10^{-2}$ were considered. As no significant differences were detected in survival potential when incubations were conducted at pH 5.9 or 10.0 (KCN has a pK of 9.8) the former pH was routinely used for KCN treatments. In each experiment, groups of 25 cells of mixed age were exposed to KCN for varying periods and the amoebae surviving treatment cultured singly to confirm viability.

6.3.1. Viability studies

All cells survived overnight exposures to $10^{-3}$ M KCN and generally maintained a pseudopodal form. As the KCN concentration was increased subsequently cell viability was reduced (Fig. 6.13).

Cell exposure to $10^{-2}$ M was studied in greater detail (Fig. 6.1). After an hour all cells had lost attachment although pseudopodal extension was continued. Contractile vacuole enlargement was noted in the majority of cells. During the next hour cytoplasmic streaming was less frequent and the cells began to assume a spherical appearance; in some experiments cytolysis was already evident. Prolonged exposure induced cytoplasmic aggregation within the spheres, and this form persisted in those amoebae surviving six hour exposures. After a 6 hour incubation period, in general, about 40% of the cells
Fig. 6.13: Effect of increasing doses of KCN on the survival of A. proteus.

<table>
<thead>
<tr>
<th>Conc. [M]</th>
<th>% Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>$1 \times 10^{-3}$</td>
<td>100</td>
</tr>
<tr>
<td>$5 \times 10^{-3}$</td>
<td>92</td>
</tr>
<tr>
<td>$7.5 \times 10^{-3}$</td>
<td>64</td>
</tr>
<tr>
<td>$1 \times 10^{-2}$</td>
<td>25</td>
</tr>
</tbody>
</table>

50-100 cells used for each concentration and given a six hour exposure.
had cytolysed and further lysis was evident on return to normal Chalkley's.

Cells which were to recover from cyanide treatments of this dose, initially reattached as a rosette form before reverting to a pseudopodal type. Amoebae which had been exposed to 1 to 3 hour treatments of $10^{-2}$ M KCN, showed no detectable division delay compared to control cells. With longer incubations division was delayed by up to 24 hours although subsequent cycling was unaffected, indicating that the cells had received no permanent damage.

6.3.2. Effects of KCN on mitochondrial ultrastructure

Cells were fixed after 1 and 18 hour exposures to $10^{-3}$ M KCN, and after 15 and 30 minutes and 1 and 2 hours with $10^{-2}$ M KCN. The mitochondrial profiles resulting from such treatments were characterised by a matrix of a light-intermediate electron density, while the cristae were dilated and commonly displayed vesiculation reminiscent of that generated by anaerobic culturing with hydrogen. This Type Int form was most pronounced with $10^{-2}$ M exposures being present after only fifteen minutes, with virtually all the profiles assuming the form after one hour treatments, (Fig. 6.14, 6.15). $10^{-3}$ M for one hour exposures induced this form in over 80% of the profiles (Fig. 6.16), although distinct Type I mitochondria were still present as a minority group. When exposures were extended with $10^{-3}$ M, a lighter Type Int form predominated (Fig. 6.17).

The overall profile shape included both elongated and swollen spherical forms which were both characterised by the intermediate matrix and the dilated, peripherally located cristae. Profiles sampled for the estimation of the L:W index gave values similar to those normally associated with Type Int mitochondria:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. Profiles in sample</th>
<th>Index ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Type Int</td>
<td>370</td>
<td>1.95 ± 0.03</td>
</tr>
<tr>
<td>18 h. $10^{-3}$ M KCN</td>
<td>251</td>
<td>2.03 ± 0.05</td>
</tr>
<tr>
<td>1 h. $10^{-2}$ M KCN</td>
<td>241</td>
<td>2.13 ± 0.05</td>
</tr>
</tbody>
</table>
Fig. 6.14: Cristal vesiculation following exposure to $1 \times 10^{-2}$ M KCN for 30 minutes. x 32,000

Fig. 6.15: Cristal dilation and vesiculation with the presence of matrical filaments following exposure to $1 \times 10^{-2}$ M KCN for 1 hour. x 32,000

Fig. 6.16: Cristal vesiculation produced by a 1 hour exposure to $1 \times 10^{-3}$ KCN. x 32,000
Fig. 6.17. : Profile form typically preserved after an 18 hour exposure to $1 \times 10^{-2}$ M KCN. x 32,000

Fig. 6.18. : Cristal vacuolization in profiles of a cell exposed to $1 \times 10^{-2}$ M KCN for 1 hour. Two associated Golgi bodies may also be seen. x 32,000

Fig. 6.19. : An association of six Golgi bodies following a 2 hour exposure to $1 \times 10^{-2}$ M KCN. x 45,000
Fig. 6.20. : Type Int profiles generated by a 2 hour exposure to $2 \times 10^{-4}$ M Antimycin A.

$16,000$

Fig. 6.21. : Aberrant profile generated by a 2 hour exposure to $2 \times 10^{-4}$ M Antimycin A.

$32,000$
In some profiles filamentous inclusions were noted in the matrix (Fig. 6.15), while in others granular structures were present.

Besides the mitochondrial changes, another prominent feature of the KCN treated cells was the association of Golgi complexes into groups of 2 or 3 (Fig. 6.18), or in some cases even larger groupings (Fig. 6.19). The significance of this observation in this study and in other treatments remains speculative (Ord, 1978).

6.4. Exposure to Antimycin A

With $2 \times 10^{-4}\text{M}$ Antimycin A at pH 5.9 the amoebae continued as pseudopodal forms for up to six hours without any detectable differences from normal cells. At the ultrastructural level however alterations in the mitochondrial configurations were observed in cells fixed after 1 to 1$\frac{1}{2}$ hour treatments. Between 75-85% of the total mitochondrial content were represented at this time as Type Int forms (Fig. 6.20) although Types I and II still persisted as minority forms. The Type Ints were often of an aberrant gross shape while the internal membranes possessed an irregular appearance. The profiles included forms not unlike those generated by the uncoupling agent DNP (Fig. 6.21).

6.5. Discussion

The strain of amoeba used in the present study survived longer periods of anaerobiosis than those of the earlier work of Hulpieu (1930) where the cells were incapable of recovering locomotion if deprived of an oxygen supply for 15 hours or over. Apart from this difference in time sequences, the results presented here are in agreement with those of the early work, e.g. the blistering of the cell membrane occurred when hydrogen was used as the anaerobic atmosphere but was not observed when nitrogen was employed. It is therefore proposed that this blistering represents a specific hydrogen effect while the development of a floating monopodal and swollen spherical forms are more probably a direct result of the lack of oxygen as these were noted when both hydrogen and nitrogen environments were employed.

The ultrastructural observations provide evidence that
mitochondrial structural changes result when the cells are subjected to treatments in which electron transport is blocked. Vesiculation of the dilated cristal membranes was produced of a similar nature to that which occurred in other cell types under anaerobiosis (Ueda and Tsuji, 1971; Morisett, 1974). These cristal vesicles were more prevalent in cells from hydrogen environments than those where nitrogen had replaced air. The presence of matrical inclusions is also consistent with findings in other cells after anaerobic culturing (Oliveira, 1977).

These characteristic changes of anaerobiosis also arose in the mitochondria of amoebae in which electron transport had been arrested by the chemical means of KCN and antimycin A treatment in the present investigation. It may be concluded therefore that both methods of disrupting electron transport induced similar metabolic disturbances in amoebae, detectable as alterations in mitochondrial structure.

The reduction of the numbers and eventual elimination of Type I mitochondria upon anaerobiosis and the loss of this type after relatively short exposures to $10^{-2}$ M KCN, indicate a need for the presence of, and the ability to utilise oxygen in either the production or the preservation of this condensed configuration on fixation. The reappearance of distinct Type I profiles within reaerated cells, is also consistent with this proposal. With hydrogen exposures of different lengths, the reappearance of Type I forms appeared to be correlated with the time interval necessary for a return to a more normal morphology and levels of cell locomotion.

The generation of filamentous inclusions within the mitochondrial matrix under conditions of oxygen lack and depletion, and with KCN treatments, lends further morphological support to the concept that mitochondrial ultrastructural differences in *A. proteus* result from changes in functional activity. The presence of such inclusions in the mitochondria of other cell types is synonymous with arrested or degenerative processes occurring within the organelle. Crystalloid filamentous inclusions have been reported in the mitochondria of many cell types during human diseases e.g. chronic alcoholism (Svoboda and Manning, 1964); diabetes (Laguens and Bianchi, 1963); carcinomas (Watanabe et al., 1976; Marinozzi et al., 1977); and neural
disorders (Dodson et al., 1976). They were also shown to exist in mitochondria from hibernating snakes (Ebe et al., 1965; Kurosumi, et al., 1966).

Many authors have considered that matrical inclusion formation may be related to organelle aging (Elliot and Bak, 1964) or other destructive changes in cell metabolism (Fujita and Machino, 1964; Suzuki and Mostofi, 1967; Ollerich, 1968; Davis, 1976). On the few occasions when inclusions have been reported in normal cells they are present in much lower numbers than in pathological cells (Muganaini, 1964; Willis, 1965).

Matrical filaments have previously been observed in A. proteus following exposure to several mutagenic carcinogenic chemicals (Ord 1976; 1978) in addition to those in the current work. The chemical nature of these filaments remains unknown, but their appearance is reminiscent of the form which extracted phospholipids assume upon fixation (Stoeckenius, 1959). Anaerobic culturing of yeasts induces changes in the mitochondrial lipid content (Paltauf and Schatz, 1969), and at least in one of the human diseases, the inclusions have been shown to be phospholipid in composition (Marinozzi et al., 1977), possibly resulting from the breakdown of the cristal membrane. Oliveira (1977) also considered that anaerobic culturing caused the production of the filamentous inclusions from a degradation of the cristal membranes in response to a temporary impairment in organelle functioning.

It has been proposed in yeast cells that the mitochondria must be functionally active in order to maintain structural integrity, otherwise during anaerobiosis the organelles are degraded to produce 'promitochondria' (Luzikov, 1973). The preservation of a number of cristae within the profiles of the few cells that survive up to 48 hours exposures in the present study suggests that despite the morphological changes described, the mitochondria of amoebae possess greater structural stability than those in yeast. The studies which use plant material also demonstrate that cristae may be maintained in mitochondria in vivo for at least 24 hours of anaerobiosis (Morisett, 1974; Opik, 1975; Oliveira, 1977; Vartapetian et al., 1977).

A positive DAB reaction observable after 18 hours of anaerobiosis provides evidence that at least this part of the respiratory chain remains intact within amoebae mitochondria, although as stain-
ing was only demonstrable with unfixed cells (See Chapter 7), its activity may have been reduced. With Triticale roots DAB staining was abolished after only two hours of anaerobic culturing (Oliveira 1977) yet mitochondria from rice coleoptiles (Opik, 1975) and tomato roots (Morissett, 1974) gave positive results for up to three days without oxygen.

Amoebae in the present study were found to be more sensitive to $10^{-2}$ M KCN than the cells studied by Flickinger (1972) who claimed 37% survival for amoebae exposed to this concentration for 24 hours. The survival ability with the lower concentration of $10^{-3}$ M, however, was in agreement with Flickinger's data. Pace and Belda, (1944) showed in Chaos that the inhibition of respiration was virtually the same for all concentrations of KCN in the range $10^{-5} - 10^{-2}$ M. This would indicate that mitochondrial functioning is affected before the overall appearance of the amoebae is altered. The present results with KCN are consistent with this finding as identical changes arise with a $10^{-3}$ M concentration as are generated by $10^{-2}$ M yet the amoebae retain normal pseudopod behaviour at the lower concentration. It is possible that if mitochondrial functioning has been disrupted at the lower concentration, then the increased mortality at $10^{-2}$ M may result from additional toxic effects not solely directed at the mitochondria. $10^{-3}$ M KCN has previously been shown to affect certain cell functions in amoebae e.g. pinocytotic activity is reduced by short exposures (Shumaker, 1958; Chapman-Andresen, 1967).

In cultured heart cells $1 \times 10^{-3}$ M concentrations have been demonstrated to induce mitochondrial morphological changes (Auclair, et al., 1976). To date lower KCN concentrations have not been tested on the mitochondria structure in many studies, although with Allium cells mitochondrial alterations were not induced by $1 \times 10^{-4}$ M concentrations (Kartusch, 1976).

As with anaerobic culturing and KCN treatments, antimycin A treatments produced aberrant mitochondrial forms of a Type Int nature. Some of these profiles were also reminiscent of those generated by uncouplers, suggesting possibly similar disruption of mitochondrial functioning by this inhibitor.

It may be concluded from the present study that when electron transport is halted, mitochondrial conformational changes result
which cause the removal of the Type I forms and the generation of abnormalities. These alterations occurred with relative rapidity, especially when metabolic inhibitors were employed. The elimination of Type I profiles by such treatments permits the proposition that in normal amoebae this form is associated with the processes of the respiratory chain or the reactions that result from it.

Other explanations could be considered. It is possible that the blockage of respiration has an indirect effect on some other event within the organelle; for example, the prevention of a stage of an organelle generation cycle could result in the removal of the Type I form. However, in view of the observations in other cell systems, and from the results on the whole amoeba functioning: the arrest of locomotion, the detachment from the substratum and the prevention of contractile vacuole activity - the results would seem to be more consistent with the idea that the change is due to energy depletion.

The work in this chapter, therefore, suggests that the alteration of mitochondrial conformation is a direct consequence of the presumed arrest of electron transport and subsequently the inhibition of oxidative phosphorylation.
Chapter Seven

An investigation of the use of cytochemistry, autoradiography, and micrurgy in studying the mitochondrial types of *A. proteus*.

Three separate experimental approaches were attempted to gain more specific information on a number of functional aspects of the mitochondria in amoebae.

(i) The first technique involved the cytochemical staining for cytochrome oxidase to demonstrate the localisation of this part of the electron transport chain within the mitochondria of *A. proteus* and to investigate its activity under a number of experimental conditions.

(ii) In considering organelle DNA synthesis, autoradiography was employed to show whether all mitochondrial types had the ability to incorporate thymidine or whether label was restricted to only one form, which would suggest that this form represented a specific event in organelle biogenesis.

(iii) Finally a range of substances, including succinate as substrate, nucleotides and DNP were injected into a limited number of cells to test further whether mitochondrial ultrastructure could be related to metabolic considerations.

As each technique differs from the others it is necessary to briefly mention the theoretical background of each in introducing the aims and results of each section of the present work.

7.1. DAB cytochemistry to demonstrate cytochrome oxidase activity

Cytochemical techniques have proved of importance in elucidating the localisation of many enzymes within cells and as such act as bridges between biochemical and cytological approaches to certain problems (Van Duijn, 1976). Such methods have great potential for studies on single cell systems like *A. proteus* where it is not easy to grow large numbers of cells for more biochemical assays. In the present study the diaminobenzidine (DAB) reaction was employed for the demonstration of cytochrome oxidase activity.

7.1.1. Introduction

Since its introduction in the localisation of exogenous horseradish peroxidase (Graham and Karnovsky, 1966), DAB has been
used in the ultrastructural determination of peroxidative activities within many tissues and cells. All haemoproteins contain an iron porphyrin prosthetic group displaying peroxidative activity and so are potentially demonstrable by DAB (Essner, 1974; Hanker, 1975), providing there is sufficient activity to accumulate reaction product at the site of oxidation.

For the localisation of peroxidases at the EM level DAB has many advantages over other reagents. The distribution of the reaction product formed on its oxidation by cellular systems is of an amorphous, non-droplet nature that is insoluble in the dehydrating and embedding agents normally used. From 'in vitro' studies on the reaction products of the DAB oxidation, deposition is considered to result from a cyclical polymerisation of the reagent (Seligman et al., 1968). To enhance contrast of these products a post-fixation step was proposed using osmium tetroxide, as the formation of an osmium black compound of high electron opacity was indicated.

The reaction has been used to demonstrate peroxidase and catalase activity in peroxisomes when hydrogen peroxide is included in the incubation medium. Peroxisomes from a wide range of organisms have been observed (Novikoff and Goldfisher, 1969; Childs, 1973; Berchtold, 1975; Fok and Allen, 1975; Stelly et al., 1975; Hanna et al., 1976).

Within mitochondria the deposition of the reaction product is considered to result from cytochrome c oxidase activity via cytochrome c which acts as the initial acceptor of the electrons donated by DAB upon oxidation (Seligman et al., 1968; 1973). This is shown schematically in Fig. 7.1.

**Fig. 7.1.** Proposed scheme for the DAB reaction. Localisation occurs at the site of cytochrome c providing the cytochrome oxidase complex is functionally active.
The peroxidative activity of cytochrome c had been observed in chemical studies (Flatmark, 1965; Cammer and Moore, 1973). However this activity would not be sufficient in the DAB reaction for ultra-structural visualisation without the reoxidisation of the reduced cytochrome c by an active cytochrome oxidase complex (Seligman et al., 1973; Roels, 1974). The reoxidised cytochrome c is then capable of oxidising more DAB, causing an accumulation of reaction product (Reith and Schuler, 1972), with oxygen being utilised in the process (Hirai and Yasuhire, 1972; Reith and Schuler, 1972; Cammer and Moore, 1973). The necessity for cytochrome oxidase in the reaction within the mitochondria has been shown in all the above studies both by the complete inhibition of the reaction by KCN, and by heat treatment, which inactivate the enzyme complex but do not affect the peroxidative activity of cytochrome c itself. The DAB reaction product is deposited therefore at the site of cytochrome c in the mitochondrial membranes, accumulation occurring due to the involvement of cytochrome oxidase activity.

Thus DAB staining occurs on the outer surface of the inner membrane and within the cristal membranes on the surfaces facing into the intracristal space. Such localisation has been reported in a wide number of tissue and cell types: rat liver (Seligman et al., 1968); mammalian cochlear cells (Spector, 1975); adipose tissue (Barnard et al., 1971); HeLa cells (Posakony et al., 1975); invertebrate sperm cells (Anderson, 1970; Pearson and Walker, 1975); protozoa (Balber and Ward, 1972; Childs, 1973); plant material (Morisset, 1974; Opik, 1975; Pellegrini and DeVecchi, 1976; Oliveira, 1977) and Neurospora (Sturani et al., 1977).

In mitochondria from Tetrahymena and Paramecium a positive reaction was considered to result from the action of a cytochrome c peroxidase as staining could not be achieved without the presence of hydrogen peroxide in the case of Tetrahymena; while in Paramecium a positive reaction persisted in mutants lacking cytochrome oxidase (Hirai, 1974; Stelly et al., 1975). In addition to the main cristal reaction in adipose tissue mitochondria, a much weaker reaction on the outer membrane possibly due to the activity of monamine oxidase or cytochrome b5 was observed (Barnard et al., 1971).

These anomalies however would appear to be exceptions rather
than the general case in mitochondrial DAB cytochemistry and they do not detract from its potential relevance for the present study. In previous work using crude homogenates of *A. proteus* Thomson and Daniels, (1961) have reported only negligible amounts of cytochrome e oxidase, although others suggest that at least some terminal oxidase is present: possibly cytochrome e oxidase rather than cytochrome c oxidase (Moller and Prescott, 1955). As amoeba have been shown to undergo cyanide-sensitive respiration (Pace and Belda, 1944), which was seen in this thesis to induce mitochondrial alterations (Chapter 6), a re-evaluation of this part of the electron transport chain using this more recent experimental approach is desirable. It was hoped that this reaction will be of use in the study of mitochondria in control amoebae and also in those of cells subjected to certain treatments known to alter mitochondrial morphology.

The preparation and application of the DAB incubation medium to the amoebae in the present work has been described elsewhere (Section 2.7).

7.1.2.1. DAB staining for cytochrome oxidase activity in untreated cells

Unfixed cells were found to give a slight positive reaction after as little as 20 minutes incubation if 5% DMSO was included in the incubation medium. The reaction product was deposited on the inner membrane. If the incubation time was increased to two hours with unfixed cells, intense staining was observed, with the cristae and inner membrane being overstained (Fig. 7.4). Under such conditions using live cells, cell preservation was poor and the majority of the mitochondria appeared swollen. Attempts were made to stabilise structure by raising the sucrose content of the medium from 5% (Spector, 1975) to over 15% (Posakony et al., 1975), in order to provide osmotic protection for the amoebae. However organelle preservation was only marginally improved by such modifications, while staining ability was slightly reduced. The reaction product remained associated with the cristae in these cells.

In order to overcome the problems of preserving structural integrity, the amoebae were given a prefixation step as had been recommended for the reaction by others. As well as improving cell
preservation, prefixation should also increase the reagent's penetration to the oxidising sites of the cell. To a large extent the intensity of staining was found to be dependent on the degree of aldehyde prefixation and on the incubation duration.

Initially Karnovsky's fixative, routinely used in this laboratory, was employed for prefixing. However no convincing staining was detectable with subsequent DAB incubations, even when prefixation was limited to a 15 minute period. Neither did the reduction of the glutaraldehyde concentration of Karnovsky's to 0.25% (Stelly et al., 1975) followed by DAB incubation extended to overnight produce positive staining. The use of ultrapure glutaraldehyde also gave negative results.

Prefixation with freshly prepared 4% formaldehyde however did not inhibit the reaction. Reaction product was observed on the cristal membranes following incubations in medium containing 5% DMSO. To produce reasonable structure integrity a prefixation of 1 hour was desirable as formaldehyde does not prove such an effective fixative as Karnovsky's. After a 2 hour incubation in DAB medium following this prefixation, the reaction was still weak. Longer incubations of 5 to 10 hours were necessary to achieve good staining potential with reaction product deposited in up to 85% of the mitochondrial profiles of the cell. Not all the cristae within an organelle displayed uniform staining ability (Figs. 7.2 and 7.3); the number of positively staining cristae per organelle ranging from one to 15.

In addition to the mitochondrial reaction under these conditions, DAB-reaction product was also deposited in the structures identified as DNA-containing bodies (Section 7.2.2.1.), suggesting these possess a peroxidase and may be similar to peroxisome-like structures of other organisms (Fig. 7.2).

If KCN was added to the medium during incubations, no staining was observed, even in extended overnight incubations when prefixed material was under consideration (Fig. 7.5). This finding indicates the necessity of cytochrome oxidase activity in the present work for the occurrence of positive staining.
Fig. 7.2. : Deposition of DAB reaction product in the mitochondria and DNA-containing bodies following a 5 hour incubation in complete DAB medium of a 1 hour 4% formaldehyde-prefixed cell. 
  x 25,000

Fig. 7.3. : DAB reaction following a 10 hour incubation of a 4% formaldehyde-prefixed cell showing heterogeneity of cristal staining. 
  x 55,000
Fig. 7.4. : Deposition of DAB reaction product following a 2 hour incubation of an unfixed cell with DAB medium.

Fig. 7.5. : Lack of reaction product in mitochondria from a cell incubated for 5 hours in complete DAB medium but with the addition of $10^{-2}$M KCN. The cell had received a 10 minute prefix with formaldehyde. No counterstaining with uranyl acetate applied.

Fig. 7.6. : Positive DAB-staining in unfixed 'anaerobic' cell following a 3 hour incubation.

Fig. 7.7. : Weakly stained cristae in an unfixed 'MNU-treated' cell following a 3 hour incubation.

All magnifications $\times 25,000$
7.1.2.2. 'Anaerobic cells'

Having demonstrated a positive reaction with DAB staining for untreated amoebae, it was of interest to study cells which had been subjected to a period of anaerobiosis. The results of this investigation have been briefly presented in Chapter Six.

Amoebae surviving 18 hours hydrogen culturing were transferred to the DAB medium in a dark room. The incubations were performed in the presence of air. Unfixed cells were given a 3 hour incubation while prefixed cells received 5 hours. Positive results were obtained in the swollen mitochondria of the unfixed cells (Fig. 7.6), indicating that an active cytochrome oxidase had been retained by the mitochondria throughout the anaerobic period, or that enzyme had been synthesised during the DAB incubation period. Mitochondrial DAB staining was not observed when cells were given a formaldehyde prefixaion, although reaction product continued to be deposited in the DNA-containing bodies. This observation suggested that the cytochrome oxidase activity of such cells was possibly reduced by the preceding anaerobic treatment whereas the peroxidase of the DNA-containing bodies was not.

7.1.2.3. MNU-treated cells

A 12 minute exposure to $1 \times 10^{-3}$ M MNU (N-methyl-N-nitroso urethane) has been shown to cause alterations in the mitochondrial morphology of A.proteus (Ord, 1976). In the present work, amoebae which had been exposed to this treatment and then cultured for 4 days in normal Chalkley's were studied by the DAB reaction. After a 3 hour incubation a weakly positive reaction was seen in the swollen profiles of unfixed cells (Fig. 7.7). Such a reaction was eliminated by a formaldehyde prefixation suggesting that the terminal components of the electron transport chain were reduced in the mitochondria of MNU-treated cells, although they had retained at least partial functional integrity following exposure to the carcinogen.

7.2. Studies involving mitochondrial DNA synthesis

7.2.1. Introduction

The presence of mit DNA has been reported in many organisms (see reviews of Nass, 1969; Ashwell and Work, 1970). This DNA is
known to differ in its physical properties from nuclear DNA from the same source (Suyama and Preer, 1965). In mitochondria from higher organisms the DNA has been demonstrated to occur as circular configurations with perimeter lengths of approximately 5\mu (Nass, 1966), although in lower organisms open-ended mit DNA filaments have also been reported (Sinclair et al., 1967; Arnberg, 1972). Mit DNA in Xenopus consists of populations of small molecules weighing from 10-11 x 10^{-6} daltons, suggesting that the potential information content is limited. Mit ribosomal and transfer RNA's including isoaccepting forms, are known to be coded for by the organelle's DNA (Dawid, 1970; Aloni and Attardi, 1971; Chiu et al., 1974). Although each mitochondrion contains several DNA molecules, the number of proteins coded for is probably very small as these DNA molecules are likely to be polycopies of a single genome (Andre, 1970). At least cytochromes a and b of the respiratory chain are transcribed from the mit DNA, while cytochrome c is probably of nuclear origin (Soslav and Nass, 1971).

DNA synthesis has been shown to proceed in isolated mitochondria prepared from Physarum (Brewer et al., 1967), casting doubt on earlier suggestions that DNA within the organelle is of nuclear origin (Wilkie, 1963; Bell and Muhlethaler, 1964). Hybridisation experiments between mit DNA and nuclear DNA in Tetrahymena have further demonstrated the unlikelihood of an integrated 'chromosomal' copy of mit DNA being present in each nuclear genome (Flavell and Trampe, 1973). Replication of mit DNA is considered to be semi-conservative (Reich and Luck, 1966), probably originating at displacement loops (Arnberg, 1972; Robberson and Kasamatsu, 1972) and being directed by a specific mitochondrial polymerase (Westergrad and Lindberg, 1972).

Unlike replication of nuclear DNA, DNA synthesis is not confined to a specific phase of the cell cycle. Labelling experiments using chick fibroblasts, Physarum and Saccharomyces demonstrate that mitochondria do not synthesise DNA in synchrony with the nucleus or with other mitochondria (Meyer and Ris, 1966; Guttes et al., 1967; Williamson and Moustacci, 1971). In Tetrahymena mitochondrial labelling continued through the cell cycle (Charrett and Andre, 1968) but incorporation of H^{3}-thymidine increased slightly when macronuclear
DNA synthesis was occurring (Parsons and Rustad, 1968), possibly due to a depletion of the thymidine precursor pools rather than an actual increase in DNA synthesis.

Synthesis in HeLa cell mitochondria was seen to proceed at a constant rate throughout the cell cycle if the cells were synchronised by double thymidine blocking, but restricted to S or G₂ if the population was synchronised by a selective detachment method (Pica-Mattoccia and Attardi, 1972). In general it is concluded that part of the mitochondrial population is synthesising DNA at all times in the cell cycle.

Using *A. proteus*, Minassian (1974) in this laboratory showed that in cells from four different points in the cell cycle approximately 20% of the mitochondrial profiles incorporated tritiated thymidine. At that time osmium tetroxide was used as the sole fixative in this laboratory, and so she was not aware of the existence of different mitochondrial types. The present work therefore set out to extend Minassian's findings by using Karnovsky's fixative, to determine whether all labelled profiles were of one conformational form or whether both types incorporated the label. The time of exposure to the labelled precursor was also reduced to determine more precisely the temporal aspects of organelle DNA synthesis.

7.2.2. Incorporation of labelled thymidine by *A. proteus*

The following experiments were designed to consider whether the range of mitochondrial configurations present in *A. proteus* were correlated to different phases of an organelle divisional cycle where one of the forms might be specifically associated with DNA synthesis. This was studied by observing the incorporation of tritiated CH₃-thymidine into cells. The labelled thymidine, obtained from Radiochemicals Ltd., Amersham, had a specific activity of 19 Ci/mMol and was used at a strength of 0.5mCi/ml. It was desirable to reduce the incubation time in order to test whether labelling ability was restricted to only one of the conformational forms. Thus the 10 hour exposures of Minassian (1974) were shortened to between 2 to 6 hours (4 hours giving the optimal levels of labelling), followed by three rinses in normal Chalkley's for 1 hour prior to fixation.
A cold thymidine chase, used previously, was not employed.

As Minassian (1974) had shown that approximately 20% of the mitochondrial profiles were labelled in each of four different 10 hour age groups, it was considered necessary to study only two regions of the cell cycle in the present work. 1-6 hours and 24-30 hours, representing S-phase and mid G2-phase of the whole cell cycle respectively, were chosen.

7.2.2.1. 3H-thymidine incorporation by S-phase cytoplasm

In order to have some means of assessing whether the somewhat elaborate techniques involved in EM autoradiography had been adequately perfected in the present study, the initial experiments were performed on early S-phase cells in which nuclear DNA is actively synthesised. Hence a high level of nuclear incorporation was anticipated and served as a control. Amoebae of strain P18 were selected as division spheres and transferred to a drop of thymidine at either 1 or 3 hours old. The cells remained attached throughout the labelling period. Depending on the length of exposure and the period of rinsing, the cells were between 5-9 hours old at the actual time of fixation. Preparation of the EM autoradiographs followed the methods set out in Chapter Two.

Examination of the thin sections after the completion of the desired exposure period showed that the distribution of the silver grains within the S-phase nucleus was associated with the extranucleolar chromatin (Fig. 7.8) as previously reported (Minassian and Bell, 1976b). Cells given a 2 hour thymidine pulse showed sparse cytoplasmic labelling, but with a 4 hour period sufficient organelles had taken up the label. Cytoplasmic grains were localised over the mitochondria, membrane bound DNA-containing bodies, vesicles and food vacuoles. Some of the label did not appear to be associated with any organised structure.

It was not possible to locate the exact source of radioactivity within each mitochondria due to the large size of the developed grain of the L4-emulsion. However the silver grains were assigned to a particular cytoplasmic organelle if the centre of each was found to be in the limits of the organelle's circumference (Peters et al., 1969).
In an attempt to quantify the distribution of the label within the cytoplasm the total grain count was scored for a standard area of cytoplasm. The number of labelled and unlabelled profiles for mitochondria, DNA-containing bodies and vesicles were also counted. Estimated proportions of labelled organelles were made from 16 cells.

The % of cytoplasmic label originating from mitochondria was estimated to be 28% while 17% of the total mitochondrial profiles counted had incorporated the labelled precursor (Table 7.1). This figure is in close agreement with Minassian (1974). Although the post-staining did not result in as distinct a separation of the mitochondrial types, Types I and II were discernible (Fig. 7.8 and 7.9). Further analysis of the distribution of the label over the two types revealed that the label was associated with both forms in approximately equal proportions (Table 7.1).

25% of the total cytoplasmic grain count was localised on, or in close proximity to, the DNA-containing bodies (Figs. 7.8 and 7.10). A higher proportion of the total profile population of this organelle had incorporated label than had been observed for the mitochondrial population (Table 7.1). Developed grains lying over the vesicular spaces accounted for 16% of the total cytoplasmic label with approximately 7% of such spaces in the cytoplasm being labelled.

7.2.2.2. $^3$H-thymidine labelling of G$_2$-phase cytoplasm.

Amoebae of strain P$_{T1}$ were used in the 24-30 hour sample because of the high percentage of the DNA-containing bodies which had incorporated the label in the cells of strain P$_{Da}$. P$_{T1}$ was found to be free of these and hence this eliminated the possibility that these DNA-bodies played some significant role in altering the thymidine precursor pools available for the mitochondria.

Despite this precaution, mitochondrial labelling again represented only 22% of the total grain count in the sample of 12 cells studied, and the actual proportion of labelled mitochondria had decreased slightly to 14% (Table 7.1). The important finding however was that silver grains were distributed in equal proportions again between Type I and II profiles of the mitochondria labelled (Fig. 7.11).

The grains which had been localised in the DNA-containing bodies
<table>
<thead>
<tr>
<th></th>
<th>% of cytoplasmic grains over the mitochondria.</th>
<th>% mitochondrial profiles labelled</th>
<th>% Type I labelled.</th>
<th>% Type II labelled.</th>
<th>% labelled DNA-containing bodies.</th>
<th>% labelled vesicles.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S-phase cells</strong>&lt;br&gt;(5-9 hrs. on fixation)</td>
<td>27.3</td>
<td>17.4</td>
<td>18.5 ± 1.7</td>
<td>16.6 ± 1.1</td>
<td>33.1 ± 2.8</td>
<td>7.8</td>
</tr>
<tr>
<td><strong>G2-phase cells</strong>&lt;br&gt;(27-31 hrs. on fixation)</td>
<td>21.8</td>
<td>16.4</td>
<td>13.7 ± 1.4</td>
<td>15.7 ± 1.7</td>
<td>-</td>
<td>25.1</td>
</tr>
</tbody>
</table>

Each figure was obtained by averaging the results from 15-20 amebae.
Fig. 7.8: Tritiated thymidine incorporation by an S-phase cell incubated at 3-7 hours old. Nuclear, mitochondrial and DNA-containing bodies are all labelled. 

x 6,400

Fig. 7.9: Silver grains associated with a Type II and a Type I -Int profile after a 4 hour incubation in H3-thymidine. 

x 25,000
Fig. 7.10. : Incorporation of $^3$H-thymidine by a Type II mitochondrion and a DNA-containing body in the cytoplasm of a cell labelled at 3-7 hours old.

$\times 25,000$

Fig. 7.11. : Labelling of both Type II and a Type I-Int profile in a P$_{TL}$ cell exposed to the precursor at 24-30 hours old.

$\times 25,000$
of strain $P_{Da}$ appeared to have been redistributed in the $P_{Tl}$ cells as an increase in non-specific labelling and also as an increase in the numbers of labelled vesicular spaces.

7.3. **Micrurgical Investigations**

7.3.1. **Introduction**

The conformational alterations observed in previous sections of the present work, when treatments likely to arrest metabolic control and cause a disruption of energy formation were used, all occurred with relative speed. It was therefore considered important to study the effects of rapidly changing the amoeba's intracellular nucleotide and substrate levels to investigate whether ultrastructural changes might be specifically correlated with any of these factors or the reactions associated with them.

From the 'in vitro' work on mammalian systems three 'metabolic functional' explanations have been proposed to account for the observed transformations in mitochondrial form. (i) The condensed to expanded transformation occurs as a direct consequence of a mechanoochemical system for oxidative phosphorylation (Hackenbrock, 1966; 1968; Andrews and Hackenbrock, 1975); (ii) Energy-consuming transport and accumulation of ions affects the inner membrane and form of the matrix (Packer et al., 1968; Hackenbrock and Caplan, 1972; Schmidt et al., 1977); (iii) ADP binding and translocation by a carrier molecule across the inner mitochondrial membrane results in the changes seen in the EM and from light scattering data (Weber, 1972; Scherer and Klingenberg, 1974). The possibility remains that these different explanations may be interrelated to some extent.

It is necessary to ascertain whether such alterations in conformation arise in the cell during life. An 'in vivo' study of the mitochondrial transformations in sea urchin eggs following fertilisation indicated that a decrease in the ADP:ATP ratio was necessary to progress from a condensed to an orthodox conformation (Innis et al., 1976). Other 'in vivo' studies are scarce. *A. proteus* however, in which the different morphological types arise simultaneously in normal control cells, also offers a means of investigating 'in situ' whether the mitochondrial conformational differences are a consequence of the metabolic state of the organelle. By using micrurgical methods
the potential of the amoeba may be fully realised in that it has been possible to inject aliquots of a range of chemicals into the cells to study the effect on mitochondrial form. Previous studies have also investigated the possibilities of microinjection with amoebae (Goldacre and Lorch, 1950; Burnstock and Philpot, 1959; Wright, 1971). In the present work, the substrates of respiration and phosphorylation, excess phosphate ions, and DNP were all used.

7.3.2. Observations on cells injected with nucleotides, phosphate and succinate

In these initial experiments 1 and 3mM ADP, 10mM succinate, a mixture of 3mM ADP/10mM succinate, 3mM ATP, 3mM phosphate buffer and 0.1mM DNP were injected into groups of amoebae and the cells fixed after a short interval. All reagents were prepared on the day of injection and adjusted to pH 6.8. For each substance two injection periods were employed to obtain data on the types of mitochondria present and to note any changes in the relative proportions which may result from these treatments with time. The injections were performed by Dr. M.J. Ord upon mixed aged cells of strain PDa which had been starved for 24 hours prior to the operation.

Although the precise amount of each reagent injected was not accurately determined (see section 2.5) attempts were made to administer approximately 60 μm³ to each cell. No attempt was made to inject into a specific region of the cell, unlike the study of Goldacre and Lorch, (1950).

The first sample of cells for each reagent was fixed where possible within 5 minutes of the injection. Later periods ranged from 10-25 minutes. During the period between the operation and the addition of the fixative, the cells recovered well, apart from the DNP-injected amoebae. The majority had recovered a pseudopodal form on transference from the oil chamber to normal Chalkley's. The injection of ATP caused a momentary wrinkling of the cell membrane, while DNP caused a tightening or stretching of the outer membrane.

At the ultrastructural level to date, the numbers of cells studied has been limited because of technical difficulties encountered. However the preliminary results are of interest and warrant further study. The data collected from 75 operations are given in
7.3.2.1. **Phosphate Buffer**

Phosphate was used as a control solution to inject into the amoebae. Two distinct mitochondrial types were retained in these cells, though the Type I profiles were less distinct and tended to verge towards a dark Type Int form (Fig. 7.12). Type I profiles accounted for only approximately 40% in the five cells scored which is slightly less than would generally be found in unoperated cells of a mixed age (see Chapter Three).

7.3.2.2. **Succinate**

When 10mM succinate was injected into the amoebae, a mean value of 55% of the profiles were of a Type I form during the first 2–12 minutes, although after 15–24 minutes following the operation this figure had begun to fall. In some of the 12 cells injected with succinate Type I profiles of an elongate nature represented up to 90% of the total mitochondrial population (Fig. 7.13) although the calculated sample means were lower than this (Table 7.2). Compared to the phosphate control sample succinate administration resulted in elevating the proportions of mitochondria approaching the Type I form.

7.3.2.3. **ADP**

The relative numbers of Type I - dense Type Int profiles were also raised when ADP was injected. With cells receiving 1mM ADP the mitochondrial types preserved on fixation showed great variation in form ranging from a disrupted Type II to some cells predominantly composed of dense Type Int and Type I forms (Fig. 7.14). Counts were not attempted on this sample, but more consistent results were collected from operations involving 3mM ADP. Within 1–4 minutes with this concentration, over 60% of the mitochondria were as Type I profiles, while extending the period following the operation prior to fixation increased the relative numbers of Type I forms to over 80% with some cells containing 95% of this type (Fig. 7.15).

7.3.2.4. **ADP/Succinate**

A solution of 3mM ADP/10mM succinate administered simultaneously
Table 7.2: Proportions of Type I mitochondria within cells which had received microinjections of ADP, ATP, succinate, phosphate buffer and DNP.

<table>
<thead>
<tr>
<th>Reagent Injected</th>
<th>Time interval between the injection and fixation</th>
<th>No. of cells injected</th>
<th>No. of profiles counted</th>
<th>Comments on the mitochondrial types preserved</th>
<th>Proportion as Type I (% ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3mM Phosphate buffer (CONTROL)</td>
<td>10 mins.</td>
<td>5</td>
<td>962</td>
<td>Mainly Type Int- I and Type II</td>
<td>37.7 ± 1.9</td>
</tr>
<tr>
<td>1mM ADP</td>
<td>5-10 mins</td>
<td>7</td>
<td>-</td>
<td>Great variation from cell to cell</td>
<td>Not estimated</td>
</tr>
<tr>
<td>3mM ADP</td>
<td>1-4 mins.</td>
<td>8</td>
<td>1446</td>
<td>Type I and Type Int predominate</td>
<td>61.3 ± 7.2</td>
</tr>
<tr>
<td>3mM ADP</td>
<td>10-25 mins</td>
<td>9</td>
<td>1953</td>
<td>Distinct Type I</td>
<td>83.3 ± 3.9</td>
</tr>
<tr>
<td>10mM Succinate, 10mM Succinate</td>
<td>2-12 mins</td>
<td>6</td>
<td>1229</td>
<td>Types I and II</td>
<td>54.5 ± 6.2</td>
</tr>
<tr>
<td></td>
<td>15-24 mins</td>
<td>6</td>
<td>1220</td>
<td>Types I and II</td>
<td>41.1 ± 8.7</td>
</tr>
<tr>
<td>3mM ADP/10mM Succinate, 3mM ADP/10mM Succinate</td>
<td>1-4 mins</td>
<td>9</td>
<td>1793</td>
<td>Distinct Type I and II</td>
<td>44.3 ± 4.5</td>
</tr>
<tr>
<td></td>
<td>7-14 mins</td>
<td>8</td>
<td>1568</td>
<td>All types</td>
<td>36.0 ± 4.7</td>
</tr>
<tr>
<td>3mM ATP</td>
<td>5-10 mins</td>
<td>5</td>
<td>-</td>
<td>All as Type II or a light Type Int.</td>
<td>None observed</td>
</tr>
<tr>
<td>3mM ATP</td>
<td>15 mins</td>
<td>4</td>
<td>702</td>
<td>Types I and II</td>
<td>23.6 ± 2.6</td>
</tr>
<tr>
<td>0.1 mM DNP</td>
<td>5-15 mins</td>
<td>9</td>
<td>-</td>
<td>Majority as Type Int; approx. 10% as a disrupted Type II</td>
<td>approx. 90% as Type Int/I</td>
</tr>
</tbody>
</table>

The injection of phosphate buffer was taken as the control level with which to compare the other reagents employed. Amoebae were grouped together in oil chambers and the 4-6 cells injected singly. Between the injection operation and cell fixation, the cells had been transferred to normal Chalkley's medium in which all but the DNP-injected cells displayed signs of recovery. The cells were grouped together into the time intervals given above in order to investigate whether temporal factors were of importance.
Fig. 7.12: Two conformational types present following injection of 3mM phosphate, mins after injection of 10mM succinate.

Fig. 7.13: Group of Type I profiles in cell fixed 15 mins after injection of 10mM succinate.

Fig. 7.14: Type I profiles in cell following 1mM ADP injection (Fixed after 1-4 mins.)

Fig. 7.15: Type I profiles present 1-4 min. after an injection of 3mM ADP.

Fig. 7.16: Type II profiles in cell 5 mins after an injection of 3mM ATP.

Fig. 7.17: Two types of mitochondria following 3mM ATP, 15 mins after operation.

Magnification 20,000 x.
did not produce the significant increase in Type I profiles which were observed when ADP was injected singularly. In this instance, although initially the proportions were slightly higher than in phosphate controls, by 7-14 minutes the numbers had fallen to below this control figure (Table 7.2). The distinction between Types I and II were not always clearcut in this sample.

7.3.2.5. ATP

Immediately following the injection of 3 mM ATP only Type II or an indistinct light Type Int form were preserved (Fig. 7.16). By 15 minutes however a Type I form had returned representing approximately 25% of the mitochondrial population (Fig. 7.17).

7.3.2.6. DNP

The results of this sample have been described previously (Section 5.3.1.4.). Despite some of the profiles being of a very disrupted nature, the majority were of a Type Int-Type I appearance normally associated with DNP-treated cells. The disrupted mitochondria were presumed to be those receiving the high concentration of DNP near to the site of entry of the pipette into the cell as more general cytoplasmic damage was evident in this area.

7.4 Discussion of the results of the three experimental approaches

From the combined results of the present section several conclusions may be drawn.

The positive staining with the DAB reaction demonstrates the activity of a cytochrome oxidase in *A. proteus*. That the reaction occurs in a medium free of hydrogen peroxide and one containing catalase to remove endogenous peroxide indicates the involvement of an oxidase and not a peroxidase as others have proposed in the mitochondria of *Tetrahymena* and *Paramecium* (Stelly et al., 1975). The inhibition by KG3Nf further substantiates that an oxidase is concerned. Earlier spectroscopic studies proposed that the terminal oxidase in *A. proteus* was cytochrome e oxidase (Moller and Prescott, 1955). Cytochrome e (later designated cytochrome b_{5}) is located on the outer mitochondrial membrane (Barnard et al., 1971) and as such would not appear to be responsible for the reaction observed in this work.
The localisation of the reaction product in the cristae and inner membrane is consistent with the reports from a wide range of organisms which have concluded cytochrome oxidase activity has been demonstrated. That not all cristae within profiles show positive staining was similar to the heterogeneity in staining observed in mouse kidney tissue (Hanker et al., 1975).

The need for relatively long incubations following prefixation of the cells suggests that cytochrome oxidase activity in *A. proteus* is lower than in other cell systems where DAB cytochemistry has been employed. Very low levels of activity have previously been proposed. (Thomson and Daniels, 1961).

The use of prefixatives and stabilisers have been reported to decrease activity (Anderson, 1970; Litwin, 1975). The finding that sucrose concentrations above 10% inhibited activity is in agreement with studies on mammalian tissue (Litwin, 1975). It was also not surprising that staining was not demonstrated with Karnovsky prefixes as glutaraldehyde destroys cytochrome oxidase activity (Sabatini et al., 1963; Anderson, 1970).

Although formaldehyde did not prove to be as good a fixative as a double aldehyde mixture, sufficient structural integrity resulted to permit detailed visualisation of the reaction products on the cristal membranes. In all cytochemical work a balance has to be acquired between cell preservation and staining intensity. It was not possible to discern whether the mitochondria were present as Type I or II forms when unfixed cells were incubated, due to organelle swelling. With prefixed cells however, positive staining was generally associated with profiles of a Type II nature. As DAB may be considered as a substrate for the electron chain this observation may indicate that extended electron transport results in a Type II conformation. In mammalian mitochondria a positive DAB reaction has also been shown to produce profiles of an expanded orthodox form (Reith and Schuler, 1972).

The reduced activity demonstrated by a decrease in staining potential when anaerobic or MNU-treated amoebae were investigated indicates that the technique will be of use in further studies from this laboratory of treatments known to produce altered mitochondrial
morphology over extended periods; cytochrome oxidase being used as a marker enzyme for functional integrity. At present it may be concluded that cytochrome oxidase is present within the mitochondria of *A. proteus* in locations similar to those of other organisms.

The results of thymidine labelling showed that 14-17% of the mitochondrial profiles had incorporated label at both of the two phases of the cell cycle studied. These findings are consistent with the belief that no synchronous mit DNA synthesis occurs, and that instead part of the mitochondrial population is continuously synthesising DNA at all times of the cell cycle (Meyer and Ris, 1966). As DNA synthesis occurs in mitochondria from both S and G₂ of the total cell cycle, it seems probable that the controls for mit DNA synthesis differ from those involved in nuclear DNA synthesis.

Mitochondrial morphological alternatives in *Euglena* have been associated with ageing and specific events of an organelle generation cycle. (Calvayrac et al., 1972; Osafune et al., 1973). The present study hoped to clarify whether the mitochondrial forms of *A. proteus* also represented different temporal stages of an organelle generation cycle where it might be postulated that only one of the types was capable of incorporating labelled precursor. The findings were that Type I and Type II labelled in roughly equal proportions suggesting this is an unlikely proposition and that both forms have the ability to synthesise DNA.

However as the shortest interval between commencing the labelling incubation and cell fixation was 3 hours, one cannot totally exclude the possibility of only one form having the ability to synthesise DNA and that the grains associated with the second form result as a consequence of a temporal progression from the earlier synthesising form to the latter non-synthesising form. Thus extended exposures to the labelled precursors would conceal such an event, and though the present incubations were much shorter than those of Minassian (1974), in any attempt to eliminate this eventuality labelling pulses and rinsing periods would need to be decreased to a minimum. Such steps do not seem feasible at the present time using the existing procedures.

As pointed out by Minassian (1974) the possibility further exists that the actual number of mitochondria replicating DNA could well be
higher than the present results would suggest. It cannot be assumed that the ultrathin sectioning of the organelle always passes through the replicating molecule of a replicating mitochondrion, despite there being up to six molecules of DNA per organelle (Nass, 1966). Until the form of the DNA within the mitochondria of amoebae has been discerned and whether it is localised or diffuse throughout the organelle is known, and the extent of any over-simplification in the present analysis of labelled profiles cannot be determined. By studying unsectioned, isolated mitochondria one might be able to overcome these difficulties but such an approach has not been considered profitable at this stage of the work.

The association of label with the DNA containing bodies and the vesicles remains unclear; non-specific cytoplasmic labelled DNA not associated with mitochondria has previously been demonstrated in other high resolution autoradiography studies (Muckenthaler and Mahowald, 1966) and by sedimentation procedures in A. discoides (Hawkins, 1972).

The speed with which alterations were produced in the relative proportions of the mitochondrial types when microinjection was employed, indicated that the structural differences between types are more likely to be associated with metabolic functions rather than some phase of organelle biogenesis, substantiating the conclusions of the autoradiography work.

The microinjection studies indicated that a rise in intracellular ADP caused a definite increase in the numbers of Type I profiles within 1–4 minutes of the operation. A slight increase was also produced by the application of 10 mM succinate. ADP and succinate injected simultaneously did not significantly alter the proportions of mitochondrial types from the levels observed in phosphate injected controls, suggesting that when the concentration of these is raised together the organelle functions in maintaining structural control. Increased ATP resulted in a decrease in the occurrence of Type I forms although with time this type was again represented.

In spite of possible limitations of the present microinjection results, due to the small number of amoebae investigated, the findings are consistent with some of the other proposals in the current literature. That raised ADP concentration were associated with increased
numbers of condensed types of mitochondria is similar to the observations on sea urchin oocytes (Innis et al., 1976), and with the concept that specific interaction of the nucleotide with the organelle results in membrane structural changes (Packer, 1960; Weber, 1972; Scherer and Klingeberg, 1974). It is considered such ADP binding may alter ion transport or affect the organelle's respiratory state (Hackenbrock et al., 1971).

With ATP the opposite result was observed in that the injection of the trinucleotide generated Type II initially and it was only after the time interval had increased that a Type I returned. A recent 'in vivo' study has demonstrated that when ATP levels were lowered in cultured myoblasts by fluoroacetate, a more electron-translucent matrix was present in the swollen mitochondrial profiles which would seem to be at variance with the present findings (Buffa et al., 1977). However these authors conclude that the structural modifications might be produced by the actual interaction of the fluoroacetate with the multienzyme systems of the Krebs cycle which might account for the difference. Some caution must be taken in interpreting the results of the present injections with ATP, however, as the mitochondria of mast cells have been reported to assume a contracted form when cultured in media containing ATP (Bloom et al., 1970).

The increase in proportions of Type I in succinate-injected cells corresponds to the finding of Haydon et al. (1967) where rat liver cells incubated with this substrate possessed a mitochondrial content of condensed profiles. In exploratory experiments with amoebae for the cytochemical staining of succinic dehydrogenase activity I have also observed similar increases in elongated types with succinate present in the medium.

Further experiments are necessary to confirm the interesting initial microinjection results presented here. Certain modifications to the technique are envisaged. At present it has not been possible to localise precisely the area where the injected reagent has entered the cells, and the assumption has to be made that the injected fluid diffuses throughout the cytoplasm becoming well distributed. Despite reports that 8 M phosphate buffer does indeed mix well with the cytoplasm of A. proteus on injection (Burnstock and Philpot, 1959), it
would be of value to try to determine the actual point of introduction. An electron opaque marker such as ferritin or thorium dioxide injected together with the test solution would facilitate localisation of the extent to which mixing occurred prior to fixation (Giulian and Diacumakos, 1977).

Some of the variations encountered within sample groups at present may result from the slight differences in the time interval between injection and fixation which result within the groups when more than one cell per group were used in the operations. To overcome this each cell would have to be injected separately in single oil chambers to enable more standard timings between the operations and fixation. Such a step would prove very laborious and time consuming but may be necessary if the alterations are as rapid as the results at present allow one to conclude.

In conclusion it is suggested that the morphological changes reported here are resulting from definite changes in the mitochondrial functional state: whether this reflects molecular respiratory reactions, energy-requiring ion transport or the translocation of nucleotides must await further study. The studies therefore (i) demonstrate the presence of a cytochrome c oxidase in the mitochondria of Aiproteus; (ii) indicate that both Type I and Type II organelles are capable of synthesising DNA; and (iii) suggest metabolic processes underlie the ultrastructural types within the cell.
Chapter Eight

General Discussion and Conclusions

The project set out to determine the significance of the morphologically distinct mitochondrial types preserved in *A. proteus* with aldehyde fixation. Since the initial reports of their existence, no further work has been performed to shed light on what these forms are representing within the amoeba. It was thus the intention of this study to clarify the fundamental basis underlying these mitochondrial differences as it was considered that information concerning mitochondrial form and functioning in Amoeba could be extrapolated to other cell types where, though different forms have been recognised, attempts to study them 'in vivo' have been limited by cell size, the interdependence of cells in tissues and/or penetration problems of the chemicals used to elucidate form and function.

*A. proteus* has previously proved a good single cell model with which to determine the sites of action of carcinogens upon cell functioning (Ord, 1976; Chatburn, 1977). It was proposed to extend the range of application of this model by considering aspects of mitochondrial ultrastructure. It is believed that if in the control situation ultrastructural changes in mitochondria can be linked with changes in the functional state, then the generation of abnormal organelle forms in toxicological studies may have a diagnostic relevance. A deeper understanding of the mitochondrial forms within healthy untreated cells will therefore assist our comprehension of the cytoplasmic damage to mitochondria caused by carcinogenic and toxic agents at present under investigation in this laboratory.

The ultrastructure of Types I, II and Int were studied with reference to the overall shape, matrix density and the organisation of the cristae, so that changes resulting from experimental treatments could be effectively monitored. Such changes, together with variations in the relative proportions of the two main types under certain test conditions could be correlated with altered metabolic processes of the cell. Whole cell activities such as cell division, locomotion and contractile vacuole functioning were considered.

In the course of the work the importance of clearly defining culture conditions during treatments aimed at disrupting cell activity was realised, particularly with respect to the uncoupling agents.
Uncouplers are weak acids which will only penetrate the cell as the undissociated species (Simon, 1953) and should therefore be applied at pH's near to the pK value. This precaution has not always been followed by amoeba workers (Schumaker, 1958; Flickinger, 1972; 1976).

The two main mitochondrial types persisted in cells maintained at different growth temperatures and throughout regimes of progressive starvation; the mitochondrial population being affected in general only by alterations in the relative proportions of the structural types to each other, except under extreme conditions. It was concluded that when cell activities such as locomotion and food capture were impaired, by subjecting the cells to environmental stress, the numbers of Type I forms decreased. Conversely when cell activity was accelerated as evidenced by shorter generation cycles and when the cells were displaying efficient locomotion, Type I forms predominated.

Actual mitochondrial structural changes were generated by incubation for prolonged periods at the adverse temperature of 6°C, and when the oxygen supply was depleted; while more rapid alterations resulted from treatment with the uncoupler substances, metabolic inhibitors and under complete anaerobiosis. Mitochondrial changes included alterations in gross organelle shape, matrix density and cristal form and also the production of filamentous inclusions. That the site of action of uncouplers was upon cytoplasmic constituents was further tested by nuclear transfer experiments. These findings must be included in any proposal put forward to account for the differences in the mitochondrial types.

The early reports of the coexistence of two types of mitochondria in *A. proteus* (Flickinger, 1968a) and *Euplotes minuta* (Jurand and Lipps, 1973) considered that the differences might result from one of the following:

a. The two types represented different phases of an organelle growth and duplication cycle; the contracted, electron-dense form being a possible division stage.

b. The two types represent different metabolic or physiological states.

c. Within these protozoa two independent mitochondrial populations have evolved and have been maintained.

d. The finding was an artefact of fixation.
To explain the differences as mere fixation artefacts is totally inadequate when one considers the close proximity with which the different types are intermingled within the cytoplasm. In other systems it is rare for different mitochondrial types to be present at the same time within the same cell type. Thus differences in these might possibly be due to cell membrane alterations resulting as the cell ages or changes activity state, so that fixative penetration is differentially affected. In such cases the altered mitochondrial forms may therefore be simply reflecting the speed with which the fixative reaches and reacts with the organelle; hence the mitochondrial difference may be a secondary consideration. Where the two types coexist within the same cell it is hard not to accept that the forms result as a direct consequence of some actual chemical or physiological difference between the two types which is preserved by aldehyde fixation and not due to these other factors. The debate then is rather what are the chemical or physiological differences which are involved.

The suggestion that two evolutionary independent mitochondrial populations are present within the cell would be difficult to prove or disprove. However such an explanation is not favoured. The hypothesis would demand acceptance of an endosymbiotic origin for mitochondria, presumably invoking that an amoeba with both types had an advantage during evolution over a cell containing only one form. Although the results of the present study could be fitted with reservations to such a proposal, it would necessitate certain assumptions. One would have to accept that both types respond in an identical manner to uncouplers and metabolic inhibitors to produce the Type Int forms described. It also needs the acceptance that at several points in the cell cycle the two forms peak and dip for some indeterminate reason in relation to each other and that during starvation and anaerobiosis, the Type II population was more resilient in withstanding adverse conditions than Type I. Similarly one would have to concede that one mitochondrial population could be associated in some cells with specific cell regions such as the contractile vacuole, while in different individuals the mitochondria of the other population were more prevalent at these localities. Such assumptions are not very acceptable when one considers the anaerobic culturing and uncoupler treatment work where Type I was completely eliminated.
but returned on subsequent aeration, suggesting that it may be generated from existing Type II or Type Int forms and was not a separate organelle population.

Proposals that a temporal or functional cycle underlie the structural differences are more appealing and demand more serious considerations. The presence of both types throughout the cell cycle in proportions normally ranging from 35–65% indicates that if the forms are reflecting an organelle growth and division cycle then this is not synchronous with nuclear cyclic activity. This conclusion is consistent with the findings of other systems (Meyer and Ris, 1966; Williams and Moustachi, 1971).

Within the present resolution of EM autoradiographic techniques tritiated thymidine incorporation was demonstrated by both mitochondrial types which indicates that mit DNA synthesising ability is possessed by Types I and II alike. Between 15–20% of the profiles were labelled in the two periods of the cycle investigated. Even if this underestimates the actual numbers of organelles labelled per cell throughout the phases chosen, it is unlikely that the proportions labelled would reach the 35–65% range observed for Type I and II mitochondria at all stages of the cell cycle, as would be necessary if one of the forms represented a mitochondrial synthetic form and the other a divisional form. Lack of correlation between a particular type and a duplicative phase of an organelle cycle lessens the plausibility of merely a temporal biogenetic explanation. Neither does the rapid effect on mitochondrial structure of the metabolic inhibitors or uncouplers completely fit a biogenetic explanation.

It is considered therefore that the experimental findings of the present work are most fully satisfied by relating them to the metabolic functioning of the organelle. The speed with which Type I and Type II control forms are altered to a Type Int form with the uncoupling agents gives support to the proposition that the fundamental distinction between the different forms reflects variations in the physiological state of the organelle and primarily with events associated with energy transduction.

The interesting results from the microinjection work further substantiate this. Thus when ADP or succinate were introduced into the cell the numbers of Type I profiles increased above the levels
present in phosphate control injected cells. ATP injections gave opposite results i.e. of raising the levels of Type II-Int forms. Under conditions of oxygen deprivation Type I forms are reduced and eventually eliminated which suggests a need for a functionally active electron transport chain, or processes associated with this, for the maintenance of Type I. Anaerobic culturing has also been used to demonstrate mitochondrial changes in a number of other cell types (Ueda and Tsuji, 1971; Oliveira, 1977).

The production of matrix filamentous inclusions within the organelles of cells in which treatments affecting mitochondrial function were employed further indicated that form and function are closely linked. The presence of such inclusions in other cell types has likely been suggestive of degenerative processes occurring within the mitochondria (Svobda and Manning, 1964; Fujita and Machino, 1964; Oliveira, 1977); possibly reflecting a degeneration of the cristae induced by temporary impairment of organelle functioning.

Similarly the results from the stress culture conditions of starvation and changed growth temperatures may be explained by invoking functional considerations. During starvation many mitochondrial enzyme activities show decreased activity (Freeland, 1967) and the respiratory events are also reduced (Karnovsky and Himmelhoch, 1961; Gold and Costello, 1975). At lower temperatures oxidative phosphorylation is reduced (Hannon, 1960; Wodtke, 1973) with a decrease in oxygen consumption (Newell and Pye, 1971). Such factors might explain the drop in Type I profiles in amoebae grown under feeding and low temperature stress; while higher temperatures (above 24°C) affect metabolic processes in general (Schmidt-Nielsen, 1975) and possibly depleted the organelle of factors necessary for the maintenance of Type I forms.

'In vitro' work, using mainly isolated rat liver mitochondria, has demonstrated that alterations in structure were associated with changes in the organelle functional state (Hackenbrock, 1966; Packer et al, 1968). Whether these result as a consequence of reversible respiratory cycles (Hackenbrock, 1972); ionic accumulation (Schmidt et al., 1977); or nucleotide binding and translocation (Weber, 1972; Scherer and Klingenberg, 1974) is still open to debate.

The chemiosmotic hypothesis has gained general acceptance as the
framework for energy capture and the transmission stages of oxidative phosphorylation; the involvement of chemical intermediates (Griffiths 1976) and/or the occurrence of a protein conformational change are however also again finding wider credibility to account for the final stages when ATP formation is achieved (See the contributions of Boyer, Slater and Ernster in Boyer et al., 1977). Although this does not necessarily imply concurrent gross organelle changes during these stages a re-appraisal of the earlier claims for observable structural variation with functional change seems relevant (Hackenbrock, 1966; Penniston et al., 1968).

It may be unwise to attempt too rigid a comparison between 'in vitro' work and 'in vivo' conditions, although reports of mitochondrial morphology alterations with variations in cell function are widespread. Thus many studies have been attempted to relate structural 'in situ' changes with functional explanations (Hackenbrock et al., 1971; Weakley, 1975; Innis et al., 1976; Rosano and Jones, 1976). A cell system such as A. proteus where different morphologies are normally represented simultaneously, meets many requirements for functionally considering variation not inherent in these other systems where only one form or the other is preserved at a time depending on the level of cellular activity and where other factors related to the fixation process may be involved.

One of the most significant of the recent reports of mitochondrial change has been described for the transformation following fertilisation in the sea urchin oocyte (Innis et al., 1976). For the mitochondrial population to proceed from a condensed to an extended conformation in this instance, there had to be a decrease in the relative concentration of ADP. It was concluded that ADP levels had a direct effect on mitochondrial configuration. Oxygen consumption was up to four times higher after fertilization, although the rates of oxidative phosphorylation were not determined.

Similar conclusions are favoured for the present findings in A. proteus. Injections of ADP rapidly raised the proportions of Type I forms in the cell by either a simple binding and translocation of the nucleotide by the organelle, or by generating a higher number of organelles with the capacity to undergo oxidative phosphorylation. Similarly uncoupling oxidative phosphorylation and the disruption of
normal organelle structure by KCN and Antimycin A treatments may also indirectly alter nucleotide concentrations within the organelle by arresting respiration and induce Type Int. formation.

It is thus proposed that mitochondrial forms in amoebae are generated due to the metabolic state of the organelle. The following scheme is presented to summarise the different forms:

Type I organelles result from a high ADP concentration and have the potential for, or are carrying out, oxidative phosphorylation.

Type II is considered to arise when the ADP levels have been depleted once phosphorylation has been completed and may represent a 'switched-off' organelle state. Its presence in high numbers during starvation, temperature stress and anaerobiosis would thus be explained by the cell having a decreased efficiency for respiratory potential. Further the lower proportions of Type I profiles when cells were starved or kept at low temperatures may arise because of membrane changes or interference with associated enzymes or carrier molecules affecting function (Reed, 1977; Spencer et al., 1977).

Type Int in untreated cells would be accommodated by a functional scheme as representing transitional forms between the two more common extreme types. This form is possibly a less stable one as in normal cells it is found to account for only a small number of the profiles. It is suggested that the membranes preferentially enter either the Type I or II configuration for the greater part of the time. The generation of aberrant intermediate forms with uncoupler action and metabolic inhibitors are considered to result as a stabilising of the Type Int form due to the affect on ADP levels or possibly because of specific binding of the agents themselves. DNP and other uncouplers do undergo reversible binding with the organelles, though whether gross conformational alterations always result remains disputed (Weinbach and Garbus, 1968; Muscatello et al., 1975; Hanstein, 1976).

The matrix density changes are considered to be an integral part of the induced alterations to the membrane systems upon changed metabolic state, and possibly reflect alterations in the ionic and osmotic composition of this compartment. Development of filamentous inclusions is also envisaged as diagnostic of changed metabolic functioning.
Further lines of work may help demonstrate whether these conclusions are pertinent. It would be desirable to study the type of mitochondria preserved throughout mitosis and cytokinesis to see if the scheme proposed above is sufficient to account for any differences resulting at this period of intense cellular activity. (Qualitative evaluation suggests an increased number of Type Int forms at this time).

A 3-dimensional reconstruction of control and treated organelle types might provide a limited amount of information on the respective structures. If the Type Int profiles do represent transitional forms between the two major classes then a serial reconstruction may help demonstrate something of this transformation of Type I to II and vice versa. One could suppose the transformation might originate at one locality and sequentially pass to all parts of the organelle with time. Alternatively the transition could result in a progressive homogeneous change of the whole organelle. Heterogeneity in function has already been demonstrated in the cristae where some show positive DAB reactions for cytochrome oxidase activity while other regions of the same organelle do not. It is conceivable that whole organelle transformation is also a piecemeal event.

The possibility of making direct measurements of respiration and oxidative phosphorylation by the organelle may also yield useful information. To date such investigations have been unsuccessful and await a satisfactory isolating media and incubation conditions.

If the functional explanations presented in this thesis are valid, then a screening of mitochondrial ultrastructure will indeed be of use in assessing any cytoplasmic damage by toxic agents and thus in conjunction with the techniques of cloning and nuclear transfer manipulations will increase the scope of the present amoeba cell model (Ord, 1976). Any variance in the ratios of the control mitochondrial types may be taken as an indication that organelle function has been affected and this pointer will be ramified if structural changes such as inclusion generation, matrix density changes and gross organelle and cristae shapes are induced during treatment. Repeatable changes are also encountered for other cytoplasmic organelles, particularly ribosomes, after treatment with the carcinogen methyl nitrosourethane (MNU) (Ord, 1978).
It is therefore envisaged that mitochondrial structure will be of significance as an additional aspect of the Amoeba model system and in this respect the further development of cytochemical techniques as outlined in Chapter 7 will be desirable. The intentions of the present studies of attempting to relate the 'mitostructural' types of *A. proteus* with function, have been fulfilled, though in the course of elucidating the mitochondrial differences, by using alterations in cell activity and stress situations other avenues equally worthy of investigation have been opened.
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